

UNIVERSITY *of the*
WESTERN CAPE

Pollination and germination as limiting factors in the propagation of threatened cycads, *Encephalartos* (Zamiaceae)

Phakamani M'Afrika Xaba

A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae in the department of Biodiversity and Conservation Biology of the University of Western Cape.

Date of submission: 12 November 2014

SANBI
Biodiversity for Life

MILLENNIUM SEED BANK PARTNERSHIP
Kew

صندوق محمد بن زايد للمحافظة على الكائنات الحية
The Mohamed bin Zayed SPECIES CONSERVATION FUND

KEY WORDS

Encephalartos latifrons

Encephalartos altensteinii

Pollen viability

Pollen storage

Pollen germination

Artificial wet pollination

Artificial dry pollination

Seed storage

Seed pre-treatment

Seed germination

ABSTRACT

Cycads are the most threatened living organisms assessed to date (IUCN, 2010). *Encephalartos* is an African cycad genus which in South Africa has a high diversity but threatened status. This study focuses on the Critically Endangered cycad *Encephalartos latifrons*, which exhibits low seed viability (< 10) in *ex situ* living collections in the Kirstenbosch National Botanical Garden (KNBG). This is the largest known *ex situ* collection of *E. latifrons*, and the conservation programme of this species has been negatively affected by the unknown cause of low seed germination.

This study hypothesised that low seed germination in *E. latifrons* at KNBG was caused by: firstly, low pollen viability as a result of storage or environmental conditions; secondly by factors with the wet artificial pollination method and its synchronous application to the diurnal formation of pollination droplets in ovules; thirdly, seed storage factors (period and environmental conditions) and the lack of seed pre-treatment (scarification and hormone). This study compared *E. latifrons* to a more common cycad *E. altensteinii* with high seed viability (> 60%) at KNBG.

Encephalartos latifrons pollen viability results show that fresh and stored pollen of specimens cultivated at KNBG was high (>60%) and comparable to pollen from the wild, that was above the recommended standard (>40%) (Stanley & Linskens, 1974), and only started losing viability after three years. *In vivo* and *in vitro E. latifrons* pollen germination had a significant varied response to temperature, wetting and desiccation compared with *E. altensteinii* (*ANOVA* $p < 0.05$). Pollination droplets and thermogenesis occured in the afternoon to early evening in both species. Wet and dry artificial hand pollination methods resulted in significantly different pollen germination (χ^2 $p < 0.001$), embryo presence and seed germination in *E. latifrons* (*ANOVA* $p < 0.05$). Furthermore,

afternoon pollination when droplets overlapped with thermogenesis yielded significantly higher seed germination than in the morning period. Maximum seed germination after dry storage for six months in both species was significantly lower compared with seed stored for eight and 12 months (*ANOVA* $p < 0.05$). Dry seed storage for 12 months reduced time to seed germination period by 80% compared to eight month storage used at KNBG. However, environmental conditions under which seed was stored and pre-treatments, did not result in any significant change in the mean time to germination (MTG), maximum germination (*ANOVA* $p > 0.05$), or t_{G50} (time taken for 50% of seeds to germinate).

Wet pollination resulted in low pollen germination and in turn caused low seed germination in *E. latifrons* at KNBG. Therefore, *Encephalartos* pollen response to environmental conditions can vary according to species, given that *E. altensteinii* pollen was not negatively affected by wet pollination. Dry pollination in the afternoon is recommended for *E. latifrons* when pollination droplets are prevalent. Seed germination vigour can be improved by 12 months of dry seed storage. Seed pre-treatments (scarification and GA_3) does not influence seed germination in *E. latifrons* and *E. altensteinii*. Cycads seed undergo different types of morphophysiological dormancy. *Encephalartos latifrons* and *E. altensteinii* undergo deep simple morphophysiological dormancy.

November 2014

DECLARATION

I declare that "*Pollination and germination as limiting factors in the propagation of threatened cycads"* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.



Phakamani M' Afrika Xaba

November 2014

ACKNOWLEDGMENTS

I would like to thank my main supervisor Professor John Donaldson at South African National Biodiversity Institute (SANBI), for his patience, and guidance. Many thanks to my supporting supervisors Dr Jayanthi Najaradan, Kew Millennium Seed Bank Partnership Project, UK, and Mr Frans Weitz at University of the Western Cape (UWC).

I am grateful to Mr De Wet Bösenberg for much discussion of cycad reproduction biology and support with technical aspect of the project. Thank you to the now late Mr John Winter, and also to Mr Dickie Peterson, who first demonstrated the low seed viability problem in *Encephalartos latifrons* to me, which initially aroused my interest. Most of the work in this study was carried out at Kirstenbosch National Botanical Garden where thanks are due to cycad specialist groundsmen, Mr Thandile Mzukwa and Mr Mluleki Mbutse, for their dedicated support with experiments carried out with the living plants collection. Ms Maud Sebelebele and Ms Vuyisa Jwayi for their lab assistance.

Thanks to Mr Colin Fletcher for discussions on his experience with *E. latifrons* in the wild and to Mr William Tang and Dr Roy Osborne for discussions on aspects of reproductive biology. I am grateful to Mr Tlou Masehela for support with sourcing papers and Dr Ferozah Conrad for encouragement and editorial support. Dr Thabiso Mokotjomela, Professor Mark Gibbons and Dr Henning Winker provided statistical advice, Professor Hugh Prichard commented on the manuscript, Mr Les Powrie provided helping with formatting and Ms Alice Notten for help editing micrographs.

Thanks to SANBI's further studies committee and to Mr Philip le Roux, curator of Kirstenbosch, for allowing me to spend time on the project. Thanks to Ms Ada van Reenen and Ms Gail van Aswegen at SANBI and Ms Linda van Heerden at UWC for administrative support. Many thanks are also due to Dr Ernst van Jaarsveld, Professor Eugene Moll, and Professor Gavin Maneveldt for their advice and encouragement.

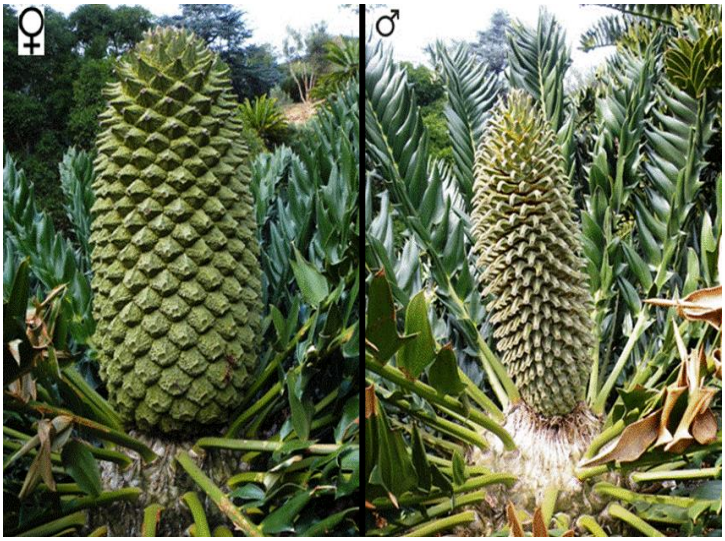
My sister Sizo Xaba and my mother, Dr Thembani Masilo, provided advice and encouragement.

This project was supported by the South African National Biodiversity Institute, the Kew Millennium Seed Bank partnership project, and the Mohamed bin Zayed Species Conservation Fund.

The work is dedicated to *iKhehla* (Peter Croeser)

who is the quintessential universal man.

PLANT PHOTOGRAPHS



Encephalartos latifrons cones at Kirstenbosch National Botanical Garden (KNBG).



Adult Specimen of *Encephalartos latifrons* at KNBG.



Encephalartos latifrons group planting at KNBG.

TABLE OF CONTENTS

KEY WORDS ..... **i**
ABSTRACT ..... **ii**
DECLARATION ..... **iv**
ACKNOWLEDGMENTS ..... **v**
PLANT PHOTOGRAPHS ..... **viii**
TABLE OF CONTENTS ..... **ix**
LIST OF FIGURES ..... **xi**
LIST OF TABLES ..... **xv**
LIST OF APPENDICES ..... **xv**
CHAPTER 1. INTRODUCTION **1**
CHAPTER 2. LITERATURE REVIEW AND THEORETICAL FRAMEWORK **7**
2.1. Reproductive Biology in Cycads **8**
2.1.1. Cycad Pollen 9
2.1.2. Cycad Pollination and Fertilisation 16
2.1.3. Seed Formation and Development in Cycads 22
CHAPTER 3. EFFECTS OF STORAGE, HYDRATION AND TEMPERATURE ON POLLEN GERMINATION IN *ENCEPHALARTOS*, AN AFRICAN CYCAD GENUS **31**
3.1. Abstract **31**
3.2. Introduction **33**
3.3. Materials and Methods **36**
3.3.1. In vitro testing of pollen viability and longevity 36
3.3.2. In vitro testing of pollen germination 37
3.3.3. In vitro testing of temperature effects on pollen germination 37
3.3.4. In vivo pollen germination in relation to ovulate cone temperature 38
3.4. Results **40**
3.4.1. In vitro testing of pollen viability and longevity 40
3.4.2. In vitro testing of pollen germination 42
3.4.3. In vitro testing of temperature effects on pollen germination 43
3.4.4. In vivo pollen germination in relation to ovulate cone temperature 45
3.5. Discussion and Conclusions **48**
CHAPTER 4. EFFECTIVENESS OF ARTIFICIAL POLLINATION IN A THREATENED AFRICAN CYCAD GENUS, *ENCEPHALARTOS* **51**
4.1. Abstract **51**
4.2. Introduction **53**
4.3. Materials and Methods **57**
4.3.1. Duration of sporophylls opening 57
4.3.2. Diurnal formation of pollination droplets 57
4.3.3. Pollen cone thermogenesis 58

4.3.4. Efficacy of pollination methods 58
4.4. Results 61
4.4.1. Duration of sporophylls opening 61
4.4.2. Diurnal cycle of pollination droplet formation 61
4.4.3. Pollen cone thermogenesis 62
4.4.4. Efficacy of pollination methods 64
4.5. Discussion and Conclusions 73
CHAPTER 5. EFFECT OF SEED STORAGE AND PRE-TREATMENT ON GERMINATION IN SPECIES OF THREATENED AFRICAN CYCADS (*ENCEPHALARTOS:* ZAMIACEAE) 77
5.1. Abstract 77
5.2. Introduction 79
5.3. Materials and Methods 83
5.3.1. Seed storage 83
5.3.2. Effect of seed pre-treatment on germination 86
5.4. Results 88
5.4.1. Seed storage 88
5.4.2. Effect of seed pre-treatment on germination 93
5.5. Discussion and Conclusions 96
5.5.1. Effect of seed storage on germination 96
5.5.2. Effect of seed pre-treatment on germination 98
CHAPTER 6. CONCLUSION AND RECOMMENDATIONS 100
6.1. Pollen viability, storage and responses to environmental conditions 102
6.2. Artificial pollination and poor germination 104
6.3. Seed storage and pre-treatments to release dormancy 107
6.3.1. Duration of seed storage 108
6.3.2. Environmental conditions of seed storage 109
6.3.3. Seed pre-treatment for germination 110
6.4. Practical outcomes and protocols arising from the structured analysis 111
REFERENCE LIST 113

LIST OF FIGURES

Figure 3.1 Example of a microscope photograph of *Encephalartos latifrons* pollen used to score pollen germination after 48h incubation. A = germinated pollen with pollen tube elongation, and B = non germinated pollen. 38

Figure 3. 2 Viability of pollen of *Encephalartos* that was tested when fresh (0 years) or stored for up to eight years at subzero temperature (-15°C). The solid line represents a regression analysis with Distance-Weighted Least Squares fit. The graph represents 17 *Encephalartos* species with (*n* = 32) samples. Squares represent *E. latifrons* from the wild, diamonds represent *E. latifrons* at KNBG and circles represent all other *Encephalartos* species from KNBG..... 40

Figure 3.3 Viability of pollen sampled from the KNBG. Data represent the means (± SE) for *Encephalartos latifrons* (black) from KBNG (*n = 2*), wild (*n* = 4) and *E. altensteinii (*grey) (*n = 6*) from KNBG. 41

Figure 3.4 Germination response in fresh *Encephalartos* pollen after hydration for five minutes, then drying for 24h or 15 d before incubation. Bars represent the mean (± SE) for *E. latifrons* (n = 4) and *E. altensteinii* (n = 7). 42

Figure 3.5 Pollen germination after incubation at different temperatures. *Encephalartos latifrons* (A) and *E. altensteinii* (B).The grey bar highlights the range in which pollen germination was > 40%. Lines represent the means (± SE) *(n =10).* 44

Figure 3.6 *In vitro* germination of *Encephalartos latifrons* pollen incubated at different temperatures. A = 10°C no germination, B = 20°C some germination, C = 30°C optimum germination and D = 40°C pollen sporoderm ruptured. 45

Figure 3.7 Germinating *Encephalartos latifrons* pollen in the pollen chamber of a sectioned nucellus 20 days after pollination. A = Entire image of the sectioned nucellus and B = magnified view of (A) pollen chamber area. PC = Pollen chamber and PG = Pollen germination into the nucellus..... 46

Figure 3.8 Ovulate cone temperature of two *Encephalartos* species during pollination receptivity. Solid lines represent mean cone temperatures for (A) *E. latifrons (n = 2)* and (B) *E. altensteinii (n = 3)* and dotted lines represent ambient temperatures for the corresponding period. Grey horizontal bands show the temperature zone for >40% germination derived from Fig. 3.5. 47

Figure 4.1 Diurnal pattern of pollination droplet formation monitored over 24h in *Encephalartos latifrons* (*n* = 2) and *E. altensteinii* (*n* = 9). 62

Figure 4.2 Temperature of pollen cones in *Encephalartos* species monitored in a controlled environment set at 20°C. A = *E. latifrons* (*n* = 7); B = *E. altensteinii* (*n* = 6). Ambient room temperature fluctuated by ±3°C due to delayed responses to heating and cooling. 63

Figure 4.3 Occurrence of aniline blue dyed pollen in the micropyle of *Encephalartos altensteinii* ovules after dry (A) and wet (B) pollination. Graph represents frequencies converted to means: 1= no dye, 2 = some dye, 3 = peak with dye and 4 = micropyle intensely blue. Each graph represents mean for 840 ovules. 65

Figure 4.4 Typical pollen accumulation in the micropyle of *Encephalartos latifrons* after pollination. PG = Pollen grain and M = Micropyle. 66

Figure 4.5 Effects of pollination time and pollination method on *Encephalartos altensteinii* seed germination. Bars represent the Mean (±SE) of seed germination at 30 weeks after sowing 120 seeds per treatment. 67

Figure 4.6 Accumulated pollen in the pollen chamber of sectioned nucellus of *Encephalartos latifrons,* 20 days after pollination. M= micropyle; PC = pollen chamber; NGP = non germinated pollen and GP = Germinated pollen. (A) Wet pollination method with pollen but no germination (B) Dry pollination method with germinating pollen. 69

Figure 4.7 Effects of the dry and wet artificial pollination method on seed viability in *Encephalartos latifrons* (LF = black) and *E. altensteinii* (AL = grey). Seed viability was measured through either the presence of an embryo or the proportion of seed that germinated after 30 weeks (sown after eight months warm stratification). EP = embryo presence and SG = seed germinated. Dry LF- EP (*n* = 180) and SG (*n* = 150), Dry AL- EP (*n* = 540) and SG (*n* = 150). Wet LF-EP (*n* = 180) and SG (*n* = 120), AL -EP (*n* = 480) and SG (*n* =180). (Means SE±). 71

Figure 4.8 Seed germination of *Encephalartos latifrons* pollinated with different artificial methods at 30 weeks. A = wet pollination and B = dry pollination. 72

Figure 5.1 X- rayed *Encephalarto*s seed for embryo presence. A = *E. latifrons* Pr = present embryo (full seed), Ab = absent embryo (empty embryo chamber). B = *E. altensteinii.* 86

Figure 5.2 *Encephalartos latifrons* seed. A = mechanically scarified and B = control with coronula unscarified. 87

Figure 5.3 Effects of storage time on seed germination in *Encephalartos*. Lines represent seed germination fitted into a binomial logistic model for A = *E. latifrons* (LF) and B = *E. altensteinii* (AL). Dashed straight lines denote time taken to reach 50% seed germination (t_{G50}). Data represent results for *Encephalartos altensteinii* stored for six months (*n* = 210); eight months (*n* = 180), or 12 months (*n* = 90) and *E. latifrons* for six months (*n* = 240) or eight month (*n* = 180). 89

Figure 5.4 Effect of storage duration on seed germination of *Encephalartos* seeds at 28°C. LF = *E. latifrons* and AL = *E. altensteinii*. Graph represents seed germination with mean SE ± at 30 weeks

for *E. altensteinii* stored for six months (*n* = 210); eight months (*n* = 180); 12 months (*n* = 90). In *E. latifrons* seed stored for six months (*n* = 240) and eight months (*n* = 180). 90

Figure 5.5 Effects of storage conditions on seed germination in *Encephalartos*. Lines represent seed germination fitted into a binomial logistic model for A = *E. latifrons* (LF) and B = *E. altensteinii* (AL). Dashed straight lines denote time taken to reach 50% seed germination (t_{G50}). Results represent seed stored for eight months from *E. latifrons* (n = 180) and *E. altensteinii* (n = 180). 91

Figure 5.6 Effects of storage conditions on *Encephalartos* seed embryo presence and seed germination, after eight months in storage at ambient room temperature and at 15°C with 15% Relative Humidity. Graph represents mean SE±. for *Encephalartos latifrons (*A) and *E. altensteinii* (B). EP = embryo presence in *E. latifrons (n =180)* and in *E. altensteinii (n = 540)*. SG = seed germination at 30 weeks for *E. latifrons (n =180)* and *E. altensteinii (n = 180)*. 92

Figure 5.7 Effects of pre-treatment on seed germination in *Encephalartos*. Lines represent seed germination fitted into a binomial logistic model. Dashed straight small lines denote time taken to reach 50% seed germination (t_{G50}). Data represents results from *E. latifrons* seeds subjected to (A) scarification, (B) scarification followed by 48h half-submerged in GA_3; (C) scarification followed by 96h half-submerged in GA_3. In *E. altensteinii* seeds subjected to (D) scarification; (E) scarification followed by 48h half-submerged in GA_3, and (F) scarification followed by 96h half-submerged in GA_3. *Encephalartos latifrons* data based on (*n* = 180) seeds per treatment and (*n = 150)* in *E. altensteinii*. 94

Figure 5.8 Effects of seed pre-treatment on germination of *Encephalartos* seed after eight months in storage. A = *Encephalartos latifrons* and B = *E. altensteinii*. Cntrl = control, Scar = scarification, Scar 48h half-submerged in GA3 = scarification followed by 48h half-submerged in GA3 and Scar 96h GA3 = scarification followed by 96h half-submerged in GA3. Graph represents germination with mean SE ± at 30 weeks. *Encephalartos latifrons* data based on (n = 180) seeds per treatment and (n = 150) in *E. altensteinii*. 95

LIST OF TABLES

Table 2.1 Eight cycad genera proven to be insect pollinated 29

Table 2.2 Number of free nuclei divisions during early embryogeny in gymnosperms (Schneckenburger, 1993; Chamberlain, 1935)..... 30

Table 2. 3 Number of cotyledons in gymnosperms (Schneckenburger, 1993; Chamberlain, 1935). 30

Table 5.1 Effects of *Encephalarto*s seed storage period on mean time to germination (MTG). **Error! Bookmark not defined.**

Table 5.2 Effects of *Encephalarto*s seed pre-treatment on mean time to germination (MTG)..... 93

LIST OF APPENDICES

Apendix 3.1 Results of pollen germination assay (hand drop technique) of 17 *Encephalartos* species, 32 samples from 0 to eight years of freezer storage (-15°C)..... 137

CHAPTER 1. INTRODUCTION

Growth in human populations, especially since the early 20th century, has placed immense pressure on the world's biodiversity, including plant species, of which more than 12 % (34 000) are in danger of extinction (Walter & Gillet, 1998). This threat is due to habitat loss and degradation, introduction of alien species, over-exploitation, pollution, disease and global change (Convention on Biological Diversity, 2009). In response to these threats, plant conservation has typically focused on two complementary activities, *in situ* conservation (protection within natural habitat) and *ex situ* conservation (preservation in botanic gardens, seed banks and gene banks) although there is a need for a better understanding of their interrelation and integration (Cohen *et al.,* 1991) to improve long term conservation strategic interventions (Li & Prichard, 2009). Living plant collections in botanic gardens are an intrinsic component of *ex situ* plant conservation (Namoff *et al.,* 2010).

The promotion of living collections as *ex situ* gene banks has gained momentum since the 1990s, with an increased recognition and policy alignment to international agreements such as the Convention on Biological Diversity (CBD), Global Strategy for Plant Conservation (GSPC), and Millennium Development Goals (MDG) (Donaldson, 2009). Thus, living plant collections in botanic gardens can be used as an 'insurance policy' against plant extinctions through activities such as securing threatened germplasm, producing material for reintroduction, research, education and ecological/habitat restoration (BGCI, 2012). The most effective application of *ex situ* conservation is in the country where the threatened species occur, and when it is linked to habitat conservation (Maunder *et al.,* 2001).

According to the IUCN Red List (2010), cycads are one of the most threatened groups of living organisms, where more than 60% of the ca. 300 species are classified as threatened with extinction. Cycads are the oldest known seed bearing plants (Norstog & Nicholls, 1997) and although they were once considered to be relicts of Mesozoic diversity (Chamberlain 1935), recent molecular studies (Nagalingum *et al.,* 2011) suggest that extant cycads have diversified within the last 12 million years. Cycads are presently confined to circum-tropical areas of North, Central and South America, Africa, Asia and Australia (Anderson *et al.,* 2007; Osborne *et al.,* 2012), and are increasingly threatened by habitat loss and a thriving trade in live plants. There are, however, a few exceptions of widespread and abundant species, especially in Australia. Due to the high level of threat to wild populations, the IUCN Cycad Status Survey and Action Plan identified *ex situ* conservation as a priority activity for cycad conservation (Donaldson, 2003).

Africa is an important centre for cycad diversity with representatives of all the cycad families (Cycadaceae, Stangeriaceae and Zamiaceae) and ca. 67 species in three genera (*Encephalartos*- 65 species; *Cycas* – one species; *Stangeria* - one species). South Africa has the highest cycad diversity within Africa (38 species) (Osborne *et al.,* 2012), and also has the highest proportion of threatened species. South Africa also has the highest number of species classified as extinct in the wild, and is the only country with recent cycad extinctions (in the last decade) (Donaldson, 2012) and also the highest proportion (12 out 37) of critically endangered species (IUCN, 2014). There is clearly a major extinction crisis facing the cycads in South Africa that requires urgent action (Donaldson, 2003). The main threat to South African cycads has been their removal from their natural habitat for private plant collections (Donaldson & Bösenberg, 1999; Donaldson, 2003) and some

conservationists have argued that the only chance of survival is through *ex situ* collections in botanic gardens (Kluge, 1988).

Encephalartos latifrons Lehm is a critically endangered cycad occurring in the Eastern Cape Province of South Africa, a biodiversity hotspot region known as the Maputaland-Pondoland-Albany hotspot (Donaldson, 2003 & 2006). *Encephalartos latifrons* is slow growing and coning is infrequent (Grobbelaar, 2002; Whitelock, 2002). Although early records noted a limited number of plants in the wild, figures are not mentioned (Pearson, 1916; Chamberlain, 1919; Dyer, 1965). There are currently fewer than 100 plants that are known in the wild, all are in private game reserves and farms in the Bathurst district, Eastern Cape Province and none of these plants are naturally recruiting (da Silva *et al.*, 2012).

Kirstenbosch National Botanical Garden in Cape Town has a century-old living cycad collection established in 1913 (Pearson, 1916). *Encephalartos latifrons* is the flagship of the collection with the largest known *ex situ* collection comprising 32 adult plants and 58 seedlings. The collection is significant in that it has genotypes that are no longer present in the wild which means that it can make a unique contribution to the gene pool (da Silva *et al.*, 2012).

Given the importance of the *E. latifrons* gene-pool at Kirstenbosch, seeds derived from this collection have high value for restoration and collection management. However, seeds of *E. latifrons* propagated at Kirstenbosch have exhibited consistently low viability with germination of about 10% (Winter, 2006). Similar findings were noted by Whitelock (2002), who stated that "seed viability is still pitifully small" in *ex situ E. latifrons* cultivated in Eagle Rock, California. The

cause of the low seed viability/germination is unknown, but seed set is well below that recorded for artificially pollinated habitat plants of > 60% (Fletcher, pers. comm.). In addition to supporting restoration efforts, registered nurseries are currently unable to meet the demand for cultivated *E. latifrons* plants resulting in very high values (up to R1 500 per cm of stem diameter/height) that drive the illegal harvesting of wild plants. This means that the collection at Kirstenbosch can also help reduce demand for wild plants but this can only be achieved if the viability and germination of *E. latifrons* seeds can be improved.

This study investigated the cause of poor seed viability/ germination in *E. latifrons* at Kirstenbosch by examining key components of the reproductive process. Firstly, the viability of pollen used at Kirstenbosch was tested focusing on viability and germination within temperature ranges that are typical of *in situ* and *ex situ* populations. Secondly, the pollination process was studied, comparing the effectiveness of wet and dry artificial pollination methods for pollen delivery to the ovules as well as the possible effects of pollination droplet formation on pollen capture. Finally, the effects of seed storage and seed treatment on germination were tested.

Studying highly-threatened species is a challenge because sample size is a limiting factor. With cycads the challenge is further compounded by two aspects of their biology.

All cycads are dioecious (Chamberlain, 1935) so that sample size for either male or female plants is more constrained than might occur in monoecious species. In the case of *E. latifrons,* the sex ratio in the wild is 4 males: 1 female resulting in extremely small potential sample sizes for female cones.

Cycads typically cone infrequently and, in the case of *ex situ Encephalartos* species, female plants may only cone once every 3-5 years (Robbertse & Claassen, 1995). As a result, the effective population size of coning individuals is even smaller than the overall population size. In order to overcome some of these challenges, this study used a comparative approach with *Encephalartos altensteinii*, a much more common species with relatively high levels of seed germination (> 60%) that has also been cultivated at Kirstenbosch for a century. This species has similar morphological characteristics and a natural distribution range that partially overlaps with *E. latifrons* (Dyer, 1965).

Several hypotheses were tested in this study.

The first hypothesis is that low seed germination is due to problems with pollen viability or germination. The sub hypotheses are that:

- ***Pollen viability in** E. latifrons **is lower than in other species with higher levels of seed germination and therefore accounts for low seed viability.***

- ***Viable pollen does not germinate due to a lack of favourable environmental conditions for germination, thus resulting in low seed viability.***

The second hypothesis is that low seed viability is limited by pollen delivery and entry into the ovule (pollination). The sub hypotheses are that:

- ***Poor seed set is due to the wet artificial hand pollination method in which pollen is mixed with water and is injected into the cone.***

- ***Low seed viability is due to limited entry of pollen into the micropylar canal, resulting from the lack of synchrony between artificial pollination and pollen droplet formation.***

The third and final hypothesis is that low seed germination is due to problems with seed storage, and seed germination problems related to seed dormancy. The sub hypotheses are that:

- ***Duration of seed storage affects seed germination***

- ***Poor seed handling and storage conditions result in embryo fatalities and cause low seed germination.***

- ***Low germination is caused by morphophysiological dormancy.***

The remainder of this thesis is set out in five chapters, with three core chapters in a format for later publication in journals. Chapter Two provides a literature review that places the questions and hypotheses in a broader context of what is already known about cycad reproduction and the treatment and storage of pollen and seeds, especially for cycads and gymnosperms. Chapter Three explores pollen viability and germination with a comparison of *E. latifrons* and *E. altensteinii*. Chapter Four explores artificial pollination (wet and dry) and the implications for *E. latifrons* and *E. altensteinii*. Chapter Five explores seed storage (duration and conditions) and pre-treatments to release dormancy. Finally, Chapter Six presents a discussion of all the results and synthesises the outcomes in order to identify the critical factors affecting poor seed set in *E. latifrons* and makes recommendations regarding the optimum conditions for propagation of *E. latifrons*.

CHAPTER 2. LITERATURE REVIEW AND THEORETICAL FRAMEWORK

This thesis focuses on the factors that may contribute to poor seed viability and low germination in the cycad *Encephalartos latifrons.* In order to identify and analyse the possible factors that may influence seed viability and germination, it is important to provide a context, based on existing literature, that identifies the unique features of cycad reproductive biology that may explain the problem, as well as features they share with other gymnosperms and even angiosperms and where there may be more general explanations for reproductive failure.

Cycads are gymnosperms, which include extant groups of Pinales (conifers, largest number of living gymnosperms), Ginkgoales (the maiden-hair with only one living representative, *Ginkgo biloba*), Gnetales (the horse-hair, Equisetales, and *Welwitschia*) and Cycadales (Anderson *et al.,* 2007). The cycads are considered to be one of the most ancient branches of the gymnosperms and represent the oldest extant group of seed plants, with a fossil record dating to the late Palaeozoic (300 MYA) (Wang *et al.,* 2007). Fossil evidence suggests that gymnosperms were dominant during the Mesozoic era but declined in more recent eras due to a variety of factors (White, 2002). Fossil evidence also suggests that numerous ancient cycad lineages are now extinct e.g. *Delemaya spinulosa* (Klavins *et al.,* 2003), and that modern cycad genera emerged within the last 65 MY. Recent molecular evidence supports the diversification of modern genera from 10-70 MYA (Salas-Leiva *et al.,* 2013) and indicates that species diversification took place in the late Cenozoic era

(10MYA) (Nagalingum *et al.,* 2011; Salas-Leiva *et al.,* 2013) giving rise to the 10 genera and ~326 species currently recognized (Osborne *et al.,* 2012). Molecular studies on *Encephalartos* indicate that the clade to which *E. latifrons* has been assigned diversified within the last 2 MY in relatively xeric habitats in South Africa (Yessoufou *et al.,* 2014). This raises questions about whether features of cycad reproductive biology have been conserved throughout the long evolution of cycads, or whether reproductive traits have diversified as part of the more recent speciation events. This will influence whether the questions being asked in relation to *E. latifrons* are deeply associated with cycad evolution or whether they represent specific problems in the reproductive biology and ecology of *E. latifrons.*

2.1. Reproductive Biology in Cycads

Cycads are all dioecious (Chamberlain, 1935), which requires the transfer of male gametes from the pollen cone to the ovulate cone at a time when the ovules are receptive. This feature of reproductive biology is ubiquitous in all cycads and in itself cannot explain poor seed set in *E. latifrons.* However, dioecy does introduce factors that may increase the risk in small populations either in the wild or in botanical gardens. An obvious factor is sex ratio, which often tends to be skewed towards male plants in small populations (Ornduff, 1987; Tang, 1990; Ornduff, 1991; Grobbelaar, 1999; J. Donaldson, unpublished data). A second risk factor is lack of reproductive synchrony between male and female plants, something that is often reported by cycad growers (Whitelock, 2002; Grobbelaar, 2002). In large populations it is assumed that throughout a coning event there will be some male plants shedding pollen and some receptive ovulate cones (Grobbelaar, 1996). This is not necessarily the case in small populations where pollen shed may occur when no female plants are receptive or

females may produce receptive cones when no pollen is being shed. In botanic gardens or intensively managed wild populations this lack of synchronicity can be resolved by storing pollen and artificially pollinating ovulate cones, however, this requires an understanding of the responses of cycad pollen to storage conditions as well as an understanding of the factors that affect pollination.

2.1.1. Cycad Pollen

According to Fernando *et al.,* (2010) there is considerable diversity in male gametophytes amongst the four orders of gymnosperms (Cycadales, Ginkgoales, Coniferales and Gnetales). The differences occur in pollen morphology (Dehgan & Dehgan, 1988; El-Ghazaly & Rowley, 1997; Owens *et al.,* 1998; Pacini & Franchi, 1999; Pacini *et al.,* 1999; Yatomi *et al.*, 2002; Norstog *et al.,* 2004; Tekleva *et al.,* 2007; Tekleva & Krassilov, 2009), growth pattern once in the ovule and nucellus (Chamberlain, 1935; Freidman, 1987; Friedman, 1993; Donaldson & Bösenberg, 1995; Owens, 2006), sperm delivery (zooidogamy and siphonogamy) (Ikeno, 1896; Ikeno & Hirase, 1897' Chamberlain, 1935; Friedman, 1993; Norstog, 1977; Owens, 2006), cell composition (Chamberlain, 1935; Pacini *et al.,* 1999; Norstog *et al.,* 2004; Owens, 2006), pollen tube morphology, development and composition (Fernando *et al.*, 2005; Williams, 2008), the sequence and pattern of cell division (Stanley & Linskens, 1974; Singh, 1978; Friedman & Gifford, 1988, 1997; Owens *et al.,* 1998; Owens, 2006) and the duration of pollen germination and fertilisation (Chamberlain, 1935; Donaldson & Bösenberg, 1995; Norstog & Nicholls, 1997).

Some of the differences in gymnosperm pollen may be due to pollination systems. Although previous analyses of gymnosperm pollen have assumed that anemophily is a shared trait (Stanley &

Linskens, 1974), insect pollination is now known to occur in several gymnosperm groups like Ephedraceae (Bino *et al.,* 1984a; b), Gnetaceae (Kato *et al.,* 1995), Welwitschiaceae (Wetschinig & Depisch, 1999), and has been experimentally proven in seven out of ten extant cycad genera including *Zamia* (Norstog *et al.,* 1986; Tang, 1987a), *Macrozamia* (Chadwick, 1990; Terry, 2001; Terry *et al.,* 2005), *Bowenia* (Wilson, 1993), *Encephalartos* (Donaldson *et al.,* 1995; Donaldson, 1997; Suinyuy *et al.,* 2009), *Lepidozamia* (Hall *et al.,* 2004), *Cycas* (Kono & Tobe, 2007) and *Stangeria* (Proches & Johnson, 2009). Despite these differences in pollination, cycad pollen still possesses similar traits to anemophilous gymnosperms in that a) most have orbicules (small a-cellular structures of sporopollenin) (Hesse, 1984; Pacini *et al.,* 1999) and b) they possess a multi-layered thick sporoderm (Gullvåg, 1966; Pacini *et al.,* 1999), which enables them to withstand desiccation, possibly as a result of a gymnosperm's prolonged pollination period. The similarities of entomophilous cycad pollen traits with that of anemophilous gymnosperms suggest that cycad pollen is robust and well adapted to potential detrimental conditions, since anemophilous pollen is more exposed to environmental conditions compared to pollen carried by insects.

2.1.1.1 Pollen morphology

Cycad pollen is diverse but generally has multiple layers (Dehgan & Dehgan, 1988) comprising a) the outer exine, b) a sexine with two layers (ectosexine and endosexine), and c) an inner nexine with three layers (nexine 1, 2 and 3). According to Dehgan & Dehgan (1988) cycad pollen is predominately monosulcate (boat shaped) and bilaterally symmetrical; it is variable from narrow to widely elliptic (sub-circulator), with an exine (main outer layer of the sporoderm) surface that can be psilate (smooth surfaced), foveolate (with rounded depressions 2 µm diameter), or fossulate (with

irregular surface grooves) (Punt *et al.,* 2014). Furthermore, cycad pollen size ranges from 11.07 µm in *Macrozamia miquelii* to 23.07 µm in *Ceratozamia kuesteriana* (Dehgan & Dehgan, 1988). This variability in cycad pollen morphology suggests that pollen will have variable response to similar environmental effects. This argument is further supported by the facts that the cycad pollen sporoderm varies in thickness amongst genera but is relatively uniform within them, and all have a laminated nexine, with a longitudinal furrow (sulcus) where the pollen tube grows through the intinine (Dehgan & Dehgan, 1988). *Encephalartos, Lepidozamia,* and *Macrozamia,* which are closely related genera, have similar characteristics including psilate exine surfaces and a thinner sexine layer suggesting some conservatism within this evolutionary lineage. In comparison, *Ceratozamia, Dioon, Microcycas, Stangeria,* and *Zamia* have a foveolate exine surface with thicker walls (Dehgan & Dehgan, 1988). *Encephalartos* pollen wall is reported to contain lipids and pollen also acidic polysaccharides (Mostert, 2000). It is unclear whether a combination of these components of pollen cells or the lack thereof, determines pollen sensitivity to environmental conditions.

2.1.1.2 Pollen development in cycads

The male gametophyte of cycads is formed within the protective compact cone (modified leaf), where the microsporangia occur on the under surface of the sporophylls (Chamberlain, 1935). Pollen cones can take several months from emergence to pollen shed e.g. four months in *Encephalartos altensteinii (*Xaba, unpublished data). During this time, pollen cells undergo meiosis giving rise to pollen mother cells (PMC) and four microspores (Pacini *et al.,* 1999). This process is common to all seed plants, but the subsequent development of the mature pollen grain differs between

gymnosperms (Fernando *et al.,* 2010) and angiosperms, with a much shorter sequence of development in angiosperms (Stanley & Linskens, 1974).

Within gymnosperms, the sequence of cell division that gives rise to mature pollen grains also varies among orders and families. The Araucariaceae and Podocarpaceae have the largest number of developmental steps (13 from the microspore) whereas Cupressaceae has the shortest with only five developmental steps (Fernando *et al.,* 2010). Cycad pollen goes through nine developmental steps leading eventually to two spermatozoids (Singh, 1978; Ouyang *et al.,* 2004), so it is intermediate within the gymnosperms.

2.1.1.3 Pollen tube development and spermatozoids

During pollen shedding in cycads, the sporophylls on the pollen cone loosen to expose pollen sacs, which eventually dry out to liberate pollen grains into the atmosphere (Chamberlain, 1935). During this period, the sporophylls on most seed cones, except *Dioon*, also loosen at the top for a number of days depending on the species, to allow insect vectors and the transfer of pollen directly into the vicinity of the micropyle (Donaldson, 1997). The second phase of pollen development begins after pollen is carried to the ovule and is drawn into the pollen chamber by resorption of a pollination droplet. This is typical of all gymnosperms (Chamberlain, 1935).

Once in the pollen chamber, pollen germinates and produces a pollen tube that is like a fungal haustorium that grows into the nucellus tissue from where it absorbs nutrients. Generally pollen tube growth in gymnosperms is relatively slow at < ~20 µm/h compared to the more rapid rate of ~80 - 600 µm/h even in primitive angiosperms such as *Amborella*, *Nuphar* and *Austrobaileya* (Williams

2008). All angiosperms and most gymnosperms are siphonogamous in which the pollen tube grows and discharges sperm cells into the egg cell (Fernando *et al.,* 2005, Pearson, 1909; Friedman, 1990). In contrast, *Gingko* and cycads produce pollen tubes that function primarily as vegetative structures for obtaining nutrition using a unique intracellular penetration that eventually exhausts the nucellus (Choi & Friedman, 1991). In cycads and *Ginkgo*, pollen tubes have multicentriolar bodies (blephoraplast cells), a unique feature that gives rise to motile spermatozoids (zooidogamy) (Norstog *et al.,* 2004). Normally, in cycads, two mature spermatozoids (diplospermy) are released from the pollen tube sac into the pollination chamber, following which one swims to fertilise the egg cell nucleus and the other fuses with the egg cells (single fertilisation) (Chamberlain, 1935). This contrasts with angiosperms which undergo double fertilisation. *Microcycas* is the only known cycad which develops 16 spermatozoids (Caldwell, 1907) and this is thought to be an ancestral characteristic (Norstog & Nicholls, 1997), although some authorities differ and suggest it is a derived trait (Norstog *et al.,* 2004).

The entire process from the deposition of pollen on the micropyle, followed by pollen tube growth and fertilisation of the egg, takes considerably longer in cycads and other gymnosperms than in angiosperms. In *E. altensteinii* it takes between 99 and 120 days from pollination to fertilisation (Donaldson & Bösenberg, 1995), as opposed to angiosperms which are reported to have extremely short fertilisation intervals of as little as 15 minutes (Williams, 2008).

2.1.1.4 Pollen storage and germination

In *ex situ* living cycad collections, numbers of plants are normally limited so coning by male and female plants may not occur at the same time or within the same year making it necessary to store

pollen. Besides physiological conditions, temperature and humidity are important influences on pollen viability during storage (Stanley & Linskens, 1974; Keller & Beda, 1984). Gymnosperm pollen can generally be stored for longer periods than angiosperm pollen (Stanley & Linskens, 1974). This is partly due to low metabolic activity in gymnosperm pollen at low temperature storage, but there are two additional factors that aid storage of gymnosperm pollen. One is the thick multilayer sporoderm, which can protect pollen against detrimental rapid desiccation. Second, moisture content of gymnosperm pollen is generally low, especially of conifers that are mostly 5 – 10% at dispersal (Owens *et al.,* 1998; Owens, 2006), although in Cupressaceae it can be as high as 30%. These factors are not necessarily applicable to all gymnosperm pollen, since it is now known that gymnosperm pollen is variable, but it does allow some understanding of what may be happening in cycads where pollen has not been extensively researched.

Conifer pollen is possibly the most studied of all gymnosperms, mainly because of the interest in commercialised species such as pines. Early *in vitro* pollen germination studies of *Pinus strobus* and *P. resinosa* showed that storage of pollen at 0°C to 4°C yielded the best germination results after a year of storage (Duffield & Snow, 1941). Pollen that was desiccated and stored at sub-zero temperatures gave the best results in *Pinus* (Ching & Ching, 1964) and in *Abies* pollen, although pollen reaction to storage can be species specific (Kormutak *et al.,* 2010). Post storage germination can be variable as shown by the results for five species of *Pinus* and *Picea abies* that were desiccated to ~10% moisture content (MC) and stored for two years at either -18°C or -198°C (Lanteri *et al.,* 1993). Siregar & Sweet (2000) recommended that for long term storage of *Pinus radiata,* pollen MC can be as low as 7%, and for short term storage (less than a year) pollen can be

desiccated to 30% MC. Lanteri, *et al* (1993) recommended drying to 10% MC for long term storage in five *Pinus* species.

Early experimental work on zoodigamous gymnosperm pollen storage was done by Newcomer (1939) with *Ginkgo.* This was later followed by Tulecke's (1954) devising a protocol for pollen storage. Chamberlain (1926) reported that cycad pollen could last about a month in ambient atmospheric conditions but since then our understanding of cycad pollen processing and storage has improved. Tang (1986) recommended a method of collecting, pre-treating and storing pollen, and showed that different cycad genera varied in their responses to similar pre-treatment and storage conditions. Furthermore, Mostert (2000) also showed that pollen within *Encephalartos* species differs in its ability to retain viability at sub-zero storage temperature. Osborne, *et al* (1992) reported an anomalous cyclic viability in *Cycas thouarsii* pollen, whereby the pollen showed a dormancy-like phenomenon when tested every six months over three years - viability was low after six months but high again when tested six months later. Again, these finding demonstrate how variable cycad pollen can be within genera and sometimes within species.

Osborne, *et al* (1991; 1992) also investigated pollen storage under different conditions and different germination media. The study concluded that desiccation of pollen prior to storage was beneficial as in conifers (Ching & Ching, 1964; Lanteri *et al.*, 1993; Siregar & Sweet, 2000; Kormutak *et al.*, 2010), but there was no benefit in storing pollen in liquid nitrogen (when compared to general atmosphere). They concluded that *Encephalartos* pollen could be stored for three to five years at -15°C. In addition, best results for germination were obtained using the hanging drop pollen germination assay with 15% sucrose solution with 0.005% boric acid at 28°C and incubated for 48h.

However Mostert's (2000) experimental work showed that 5% and 10% sucrose, with similar measures of boric acid, yielded a similar result. Pollen viability testing using stains has also been carried out with nitroblue tetrozolium stain (Tang, 1986a), acetocarmine and Alexander's stain (Mostert, 2000) while Kay, *et al* (2011) used aniline blue. Although stains offer a rapid testing method of cycad pollen, no seed fertility studies have been undertaken to further confirm accuracy of the results as carried out in *Encephalartos* by Osborne, *et al* (1992).

Temperature is also an important consideration for pollen germination. However, no investigation has thus far been carried out for cycads to determine the different temperature thresholds for pollen germination. Such knowledge would help distinguish whether specific temperatures are required for pollen germination, especially in the context of other studies showing that low temperature can increase plant sterility by restricting pollen development (Stanley & Linskens, 1974). In *Pistacia* species, pollen germinated between 5°C and 40°C and in *Gossypium*, germination occurred between 15°C and 45°C (Kakani *et al*, 2005). In *Pinus mugo*, pollen showed no germination below 20°C although germination peaked at 29°C (Nygaard, 1969). This implies that environmental conditions in both *ex situ* and *in situ* situations may affect pollen viability and may explain why pollination problems occur in some populations of *E. latifrons*.

2.1.2. Cycad Pollination and Fertilisation

For over 90 years cycads were thought to be wind pollinated. However, since the first study proving insect pollination (Norstog et. al., 1986), mutual relationships with pollinating insects have been established for seven of the ten recognized cycad genera (see Table 2. 1). The accumulated evidence on cycad pollination suggests some highly specific interactions that are comparable to fig

(*Ficus*) and fig-wasp (*Chalcidoidea*) interactions (Suinyuy *et al.*, 2013) and this raises questions whether these distinctive pollination features contribute to reproductive failure in *E. latifrons*. Key features of the cycad pollination syndrome are discussed to provide an understanding of potential problems in relation to the hypothesis addressed in this thesis. These include: dioecy, thermogenesis in pollen cones and its role in attracting insect vectors, insect movement and transfer of pollen to receptive seed cones, gaining access through loose sporophylls and delivering pollen to the apex of the ovule, and presence of pollination droplets at the time of pollination.

2.1.2.1 Dioecy

According to Givnish (1980), monecieous gymnosperms (Araucariaceae, Pinaceae, and Taxodiaceae) are predominantly wind pollinated, whereas dioecious gymnosperms (Cycadaceae, Stangeriaceae, Zamiaeceae, Cephalotaxaceae, Podocarpaceae, Ephedraceae and Gnetaceae) are predominantly animal pollinated. Dioecy coupled with preformed ovules, as in cycads, seems to represent a "high risk investment" compared to monoecious or hermaphrodite species that are more common in angiosperms. A classic example of the disadvantage of dioecy in cycads is the extinction in the wild of *Encephalartos woodii*, a species that is known from only one male plant, which can no longer reproduce sexually. Another disadvantage is if male and female plants are too far apart from each other for pollination to occur, and this can result in possible local pollinator extinction (Donaldson, 2003). In contrast, dioecy could reduce negative impacts of selfing and inbreeding depression (Freeman *et al.,* 1997; Ainsworth, 2000), especially in small isolated populations where inbreeding would be expected. Even so, a genetic study of *E. latifrons* using AFLP gene markers showed a high level of genetic diversity and a lack of population structure suggesting that the

species originated from a single population (da Silva *et al.,* 2012). Nonetheless, no close genetic uniformity was reported that could potentially explain pollination and reproductive failures in the *E. latifrons* at Kirstenbosch NBG.

2.1.2.2 Thermogenesis & pollination droplets

Poisson (1878) was the first to report on thermogenesis in cycad pollen cones. Since then much evidence has emerged to support the observation that cycad pollen cones generate heat during certain stages of development (Jacot-Guillarmod, 1958), with the most extensive evidence showing thermogenesis in 42 out of 43 species tested across ten genera (Tang, 1987b) as well as several detailed studies of thermogenesis in a few species (Terry *et al.,* 2004; Suinyuy *et al.,* 2010; Terry *et al.,* In press). Thermogenesis also occurs in basal angiosperms (Thien *et al.,* 2000), mostly in ancient families that have evolved in association with beetle pollinators. Many beetles require high body temperatures for activity and the warm environment inside thermo-regulated flowers may provide an energy reward during their visit (Seymour & Schultze-Motel, 1997). Additional reasons cited for heat production in flowering plants include protection from damage during cold spells, aiding development and early flowering, release of volatile odours and provision of desirable brood sites as reward for pollination (Minorsky, 2003). In such cases, volatile odours could indicate food, sex and heat resources (Thien *et al.,* 2000), which is a strong biological incentive for pollinators. Such reasons are also applicable in some cycads (Terry *et al.,* 2004; Suinyuy *et al.,* 2010; Tang, 1987b). Furthermore, in an Australian cycad *Macrozamia lucida,* the role of thermogenesis in combination with volatile cone emission is reported to have a "push-pull" pollination effect on *Cycadothrips chadwicki* (Terry *et al.,* 2007). Since thermogenesis can influence the timing of insect movement

between cones (Donaldson, 1997; Terry *et al.*, 2004; Suinyuy *et al.*, 2010), it may be expected that receptivity in female cones and especially the presence of pollination droplets, would be synchronised with periods of heating and insect dispersal from male cones. This has implications for wild pollinators as well as for artificial pollination in cultivated populations.

Daily patterns of thermogenesis in *E. villosus* (Donaldson, 1997) resulted in a temperature increase in the mid-morning, peaking (5°C above ambient) between 13:00h – 20:00h. Pollination droplets were present on >50% of ovules at any time during the day but were more prevalent (>80% of ovules) between 06:00h and12:00h. In *Zamia pumila*, the pollen cone reached peak temperature at ~ 20:40h tapering in the late evening (Tang, 1987b) whereas the pollination droplets were most frequent in the early morning (Tang, 1987a). These studies provide contradictory results and do not properly test the hypothesis of synchronicity between peak temperatures in male cones and receptivity in female cones so it is not clear whether this could be an issue affecting seed viability in *E. latifrons.*

2.1.2.3 Sporophylls opening and formation of pollination droplets

Prior to formation of pollination droplets in cycads, the sporophylls of ovulate cones separate to allow pollen or pollinators to enter the cone and then close again after a number of days. A number of cycad growers report that different species stay open for different periods. The only formal study undertaken into the number of days sporophylls remain open was in *E. inopinus*, where they were found to be receptive for only four days (Grobbelaar, 1996). Such knowledge is important in determining the optimal pollination period, especially if coupled with the knowledge of when, and how long, pollination droplets are present.

Pollination droplets are produced by most gymnosperms (including cycads) at the apex of the ovule when receptive (Chamberlain, 1935; Tang, 1987a; Donaldson, 1997). The function of pollination droplets is to capture, hydrate and resorb the pollen grain into the micropylar canal (Labandeira *et al.*, 2007), and similarities can be drawn to the stigma and style in angiosperms (Edlund *et al.*, 2004; Nepi *et al.*, 2009). In conifers, once the pollination droplet has resorbed pollen, the micropyle is sealed, possibly by a chemical mechanism (Owens *et al.*, 1980; Owens *et al.*, 1987), but this has not been confirmed in cycads.

2.1.2.4 Artificial pollination

Artificial pollination is necessary in cycad *ex situ* collections for viable seed set (Tang, 1986b), since *ex situ* collections typically have limited numbers of each plant species and also pollinators are not present. Artificial pollination may also be important in *in situ* conservation projects where plants are too far apart for pollination to occur as in a case of *E. latifrons* (Daly *et al.*, 2006) and *E. middelburgensis* (pers. obs.) or where no pollinators are present. There are two main methods for artificial pollination that are used for cycads: the dry and the wet method (Tang, 1986b; Grobbelaar, 2002; Whitelock, 2002). For *Encephalartos* the dry method involves pneumatically squirting dry pollen between the loose sporophylls (Grobbelaar, 2002). In the wet method, pollen is mixed with water and squirted with a syringe between the loose sporophylls (Grobbelaar, 2002). Since cycads have different types of cones (with different size, shape and structural components) depending on species, the most efficient pollination methods may vary between species (Tang, 1986b). According to Grobbelaar (2002), certain species yield better results when pollinated with a particular method. He recommended dry pollination for *Bowenia, Ceratozamia, Chigua, Cycas, Dioon, Microcycas,*

Stangeria and *Zamia* and wet pollination for *Encephalartos, Lepidozamia* and *Macrozamia.* The only comparative artificial pollination study currently known for cycads was for *Encephalartos* - Tang (1986b) reported that *E. ferox* had more viable seed (82 %) using a dry method where the top part of the cone was cut off for better access and pollen was blown inside the cone. The results suggest that the method of artificial pollination can make a significant difference to seed viability and supports the need for further research on this subject with reference to *E. latifrons*.

2.1.2.5 Fertilisation

Pollination in plants ends when the male cell fuses with the egg cell, thus marking the beginning of fertilisation. During the last stage of pollination in cycads, pollen tubes deposit motile spermatozoids into a fluid-filled archegonial chamber (fertilisation chamber) (Chamberlain, 1909). The spermatozoids swim into the archegonial chamber via two open neck cells to fertilise the haploid egg cell and only one spermatozoid fertilises the egg cell after shedding its ciliated band (Chamberlain, 1919). Even at this stage fertilisation may fail if spermatozoids are incompatible and are rejected by the egg cell (Steyn *et al.,* 1996). When fertilisation is successful, nuclear fusion occurs and a number of simultaneous nuclear divisions occur in the cytoplasm of the zygote to form a pro embryo that will eventually mature to a diploid (2n) embryo that is incubated by a 2n megagametophyte. Apart from a more rapid process of fertilisation in angiosperms than in gymnosperms, double fertilisation is another physiological difference, whereby a haploid (1n) egg produces a diploid (2n) zygote. The second male gamete fuses with a second haploid polar nucleus, to form a triploid (3n) primary endosperm nucleus (Weier *et al.,* 1982; Proctor *et al.,* 1996). There are also some gymnosperms that undergo double fertilisation, e.g. *Gnetum gnemon* and *Ephedra*

nevadensis. Double fertilisation in these gymnosperms results in only a 2n zygote without a triploid 3n endosperm as in angiosperms. Although, according to Kermicle & Alleman (1990), triploid endosperm results in vigorous "hybridity" from the unique combination of genes from the sperm and central cell. This suggests that diploid gymnosperm megagametophytes might lack the hybrid vigour of triploid angiosperms endosperm.

2.1.3. Seed Formation and Development in Cycads

According to Sporne (1974) the development of an integument by early vascular plants was one of the major evolutionary steps that enabled seed plants to become dominant. A seed primarily consists of a seed coat developed from the integument (functioning for protection, dormancy and germination regulation), the 3n endosperm in angiosperms or 2n megagametophyte in gymnosperms (providing nutrient storage) and finally the embryo (gene code and cotyledons). Seeds provide an effective survival strategy that has an adaptive advantage in diverse habitats. Some key seed characteristics include: protection of the developing embryo, an aid to dispersal, and provision of storage reserves to sustain the embryo post germination (Bewley & Black, 1994, 2001; Bonner, 2008). Roberts (1973) classified seed into two main groups according to storage ability; orthodox (desiccation tolerant) and recalcitrant (non desiccation tolerant) seeds. There is a general pattern that recalcitrant species are more common in tropical and subtropical areas (Tweddle *et al.,* 2003).

Gymnosperm seeds are diverse, varying in duration of seed formation, morphology, physiology and dispersal (Chamberlain, 1935). There is also diversity in cycad seed structure, seed size, seed coat thickness and duration of cones held by the mother plant (Tang, 1987; Osborne, 1988; Norstog & Nicholls, 1997; Grobbelaar, 2002; Jones, 2002; Whitelock, 2002). Preliminary work undertaken in

cycad seed kernel morphology supports the seed diversity found in cycads (Osborne, 1988). To better understand the background to the hypothesis that seed storage and seed dormancy are the factors causing poor germination in *E. latifrons,* this section reviews the following factors; seed development, germination, seed dormancy and storage and desiccation sensitivity.

2.1.3.1 Seed Development

Fertilisation and the development of a zygote mark the transformation from an ovule to a seed. This process in angiosperms is rapid but in gymnosperms and cycads in particular, it is slow (Norstog & Nicholls, 1997). Saxton (1910) was the first to study embryo development of the genus *Encephalartos* and divided it into four developmental stages. Chamberlain (1935) later streamlined the process in cycads into three currently recognized stages: a) a free nuclear stage; b) formation of the cell walls in the embryo; and c) differentiation into body regions. Directly after fertilisation, nuclear division occurs (Table 2.2) and somatic cells divide into two meristematic cells at the proximal and distal ends. The first organ to develop is the suspensor, an umbilical cord-like structure that is formed from the cell at the proximal end. The proper embryo and cotyledons develop from the cells at the distal end (Sedgwick, 1924), (Table 2.3). In cycads the proper embryo developmental stage coincides with cone disintegration and seed shed (Forsyth & Van Staden, 1983; Osborne, 1986; Bonner, 2008), although exact timing of embryo development may vary depending on the genus and species. In *E. gratus, E. natalensis* and *E. villosus* seed can take from four to six months to reach germination stage after cone shedding (Woodenberg *et al.,* 2007). This lengthy seed development process is common in other gymnosperms e.g. pines (Owens & Blakes, 1985) and *Ginkgo* (Lee, 1955).

2.1.3.2 Germination

Seed germination is triggered by water imbibition and ends with the elongation of the embryo axis (radicle). The germination process includes metabolic events such as protein hydration, sub-cellular structural changes, respiration, macromolecular synthesis and cell elongation (Bewley & Black, 1994; Bewley, 1997)**.** Seed germination typically occurs when external environmental conditions (light quality, nutrients, salinity, soil moisture and temperature) are favourable (Baskin & Baskin, 2001). In nine of the ten extant cycad genera, the seeds are radiospermic (radically symmetrical), and the radicle is exposed through a coronula (seed opening). *Cycas* is the only genus that is platyspermic (flattened), in which the seed splits in half (longitudinally) during germination to expose the radicle (Norstog & Nicholls, 1997). Seed germination in cycads is visible when the coleorhiza (root cap-like structure) protrudes through the coronula (Dyer, 1965). The coleorhiza pushes downwards and is penetrated by the enclosed primary root. This tap root goes deeper and begins forming secondary roots. The cotyledons remain embedded within the seed, where they continue absorbing food stored in the megagametophyte (Dyer, 1965). The leaf appears at the proximal end of the cotyledons (Chamberlain, 1935; Dyer, 1965; Norstog & Nicholls, 1997). Seed germination in **c**ycads and *Ginkgo* is similar with a retained trait of hypogeal germination, which contrasts with conifers, Gnetales and many angiosperms that experience epigeal germination (Doyle & Donoghue, 1992). Most cycad growers germinate seeds between 28°C - 35°C max on a heated bench (Forsyth & Van Staden, 1983; Grobbelaar, 2002; Jones, 2002; Whitelock, 2002).

2.1.3.3 Seed Dormancy and Storage

Seed dormancy is an important survival strategy in seed plants because it delays germination until conditions are favourable (Baskin & Baskin, 2001). According to Nikolaeva (1977), seed dormancy is divided into two types: endogenous and exogenous. Endogenous/morphological seed dormancy is a result of factors limiting embryo germination, e.g. development. Exogenous/physical dormancy is a result of structural factors arresting germination e.g. seed coat, endosperm or fruit. Cycads have a thick integument that forms three layers: the sarcotesta (external fruit-like tissue), sclerotesta (hard seed kernel or seed coat) and endostesta (papery internal layer) (Dehgan & Yuen, 1983). Cycad seed may experience morphophysiological dormancy (MPD) (Witte, 1977; Dehgan & Schutzman, 1983; Dehgan, 1996), which has also been reported in *Ginkgo biloba* (West *et al.*1970). Morphophysiological dormancy is a combination of morphological (e.g. hard seed coat) and physiological dormancy (undeveloped embryos) (Nikolaeva, 1977). Morphophysiological dormancy is divided into eight levels depending on required temperature, temperature sequence and hormone treatments to overcome dormancy (Baskin & Baskin, 2004). Dehgan (1996) showed that in *Cycas*, *Encephalartos* and *Zamia*, seeds are consistent with factors that can be characterised as non deep simple morphophysiological dormancy (ND-MPD), and require a lengthy warm stratification of up to eight months (Baskin & Baskin, 2001). Warm stratification involves the interrelationship of warm temperature and high moisture content (West *et al.*, 1970). Cycads have a high moisture content and are recalcitrant (Forsyth & Van Staden, 1983; Woodenberg *et al.*, 2007) and require different periods of warm stratification for embryos to mature depending on genera (Calonje *et al., 2011*).

After storage or warm stratification, even when the embryo is fully developed, some cycad species are still physical dormant as a result of the thick seed coat. Cycad species with these attributes include *Cycas revoluta, C. circinalis, Zamia floridana,* and *Z. furfuracea* (Dehgan, 1996). Breaking physiological dormancy of cycad species has been achieved by chemical scarification with sulphuric acid (H_2SO_4) (Dehgan & Schutzman, 1983; Frett, 1987; Dehgan, 1999; *Zarchini et al., 2011*). In *Encephalartos,* with a hard seed kernel, mechanical scarification (sanding down coronula at apex of the seed kernel) can improve germination (Donaldson, unpublished data). Scarification allows the rapid exchange of water and oxygen (amongst other factors) with the embryo, endosperm or megagametophyte (Salisbury & Ross, 1978), therefore aiding germination.

In order to overcome morphophysiological dormancy in *E. natalensis,* warm stratification at 20°C is recommended (Forsyth & Van Staden, 1983). Generally a six months period of warm stratification is recommended for *Encephalartos* seeds (Giddy, 1984; Whitelock, 2002). Similar recommendations have been made for *Cycas* (Dehgan, 1983) with longer periods of eight to nine months for most other genera and even up to two years for *Dioon* (Whitelock, 2002). In contrast, no warm stratification is necessary for *E. transvenosus* where seed cones can be held on the plant for prolonged periods after fertilisation (nine months), and germination may occur while seeds are still enclosed in the cone (Grobbelaar, 2002).This phenomenon has also been observed in *E. manikensis* (Vorster, 1995), *E. arenarius*, and *E. longifolius* (pers. obs.). This suggests that not all cycads experience morphophysiological dormancy, but most *Encephalartos* species may only experience physiological dormancy, since the embryo is undeveloped at seed shed but the seed coat is not thick enough to provide a mechanical barrier once the embryo is fully developed.

Studies by Forsyth & Van Staden (1983) established that the sarcotesta inhibits germination. As a result, Grobbelaar (2002) recommended that the sarcotesta should be removed prior to seed storage in all cycads except in the case of those with a difficult to remove (rubbery and sticky) sarcotesta such as *Zamia* and *Cycas*. In the wild, the sarcotesta is eaten and cleaned off by birds and small mammals (Goode, 1989), possibly minimising susceptibility to infections and priming the seed for germination.

In living plant collections, seed cleaning (removal of the sarcotesta) is followed by seed viability testing. When seeds are placed in water, fertile seeds sink and floaters are assumed to be infertile (Forsyth & Van Staden, 1983). Although there are no published accounts of the method's accuracy, it is used by most cycad growers. A representative sample can be dissected and checked for embryos to confirm seed viability (Grobbelaar, 2002).

According to (Kucera *et al.,* 2005) gene expression in seeds is regulated by signal transduction pathways (process by which a biological cell converts one kind of signal or stimulus) which is mediated by environmental and hormonal signals. External application of hormones has been proven to induce and promote germination. For example, gibberellic acid (GA) unlocks dormancy, promotes germination and counteracts the effects of abscisic acid (ABA). Since cycad seeds are reported to exhibit physiological dormancy, germination will occur when the seed is exposed to the optimum environmental conditions provided the embryo is fully developed (Witte, 1977; Dehgan & Schutzman, 1983; Dehgan, 1996). In *Encephalartos*, seed soaked in GA_3 for 24h and 48h showed rapid and uniform germination (Dehgan, 1999), although generally *Encephalartos* seed germinate with ease after the removal of the sarcotesta and warm stratification for 4 to 6 months (Dyer, 1965;

Forsyth & Van Staden, 1983; Giddy, 1984; Grobbelaar, 2002; Whitelock, 2002). However, scarification with H_2SO_4 in *Zamia floridana* and soaking for 24h in GA_3 (1000pm) resulted in a 100% germination within 2 to 4 weeks, and seed scarified had similar results (Dehgan & Johnson, 1983). Similar results were also obtained in *Zamia furfuracea* seed that was scarified for 20 minutes (maximum) in H_2SO_4 and soaked for 24 hours in GA_3 (Dehgan & Almira, 1993). Other cycad species that showed an improved germination with similar treatments include *Macrozamia communis* (Ellstrand *et al.,* 1990). Although *Cycas revoluta* showed improved germination of 63% with only 1 h H_2SO4 scarification, application of GA_3 did not have any marked effect compared to the control (Frett, 1987). In addition, the seeds showed 95% germination when treated with boiling H_20 (100°C) for 1h and with 25% H_2SO4 for 2h (Zarchini *et al., 2011*). Furthermore, mechanical scarification (sanding down the coronula at the apex of the seed kernel) of the hard seed kernel in *Dioon merolae* hastened germination but GA_3 negatively affected germination (Perez-Farrera *et al.,* 1999). Since scarification and growth regulator treatments are generally reported to improve germination in several cycad species, it was identified as a factor that needed investigation for *E. latifrons*.

Based on the complexity in reproductive biology highlighted above, the next three chapters will be exploring and testing hypotheses relating to the possible causes of low seed viability in *E. latifrons.*

Table 2.1 Eight cycad genera proven to be insect pollinated.

Species	**Pollinator**	**Reference**
Zamia furfuracea	*Rhopalotria mollis*	(Norstog *et al*., 1986)
Z. pumila	*Pharaxonotha zamiae* and *Rhopalotrida slossoni*	(Tang, 1987a)
Encephalartos cycadifolius	*languriid* beetle	(Donaldson *et al*., 1995)
Encephalartos villosus	*Porthetes* sp.	(Donaldson, 1997)
Macrozamia communis	*Cycadothrips chadwicki and Tranes lyterioides*	(Terry, 2001)
Bowenia serrulata	*Miltotranes subopacus*	(Wilson, 1993)
B. spectabilis	*M. prosternalis*	(Wilson, 1993)
Lepidozamia peroffskyana	*Tranes* sp.	(Hall *et al*., 2004)
Macrozamia machinii and *M. lucida*	*Cycadothrips chadwicki and Tranes* sp.	(Terry *et al*., 2005)
Cycas revoluta	*Carpophilus chalybenus*,	(Kono & Tobe, 2007)
Stangeria eriopus	*Urophorus picinus*, *Carpophilus famatus*, and *Carpophilus hemipterus*	(Proches & Johnson, 2009)
Encephalartos friderici-guilielmi	*Erotylidae* sp., *Metacucujus encepharlati and Porthetes hispidus*	(Suinyuy *et al*., 2009)

Table 2.2 Number of free nuclei divisions during early embryogeny in gymnosperms (Schneckenburger, 1993; Chamberlain, 1935)

Taxon	*Torreya*	*Bowenia*	*Ginkgo*	*Dioon*
Number of nuclei	4	64	256	1024
Number of divisions	2	6	8	10

Table 2. 3 Number of cotyledons in gymnosperms (Schneckenburger, 1993; Chamberlain, 1935)

Taxon	*Pinus*	*Cedrus*	*Araucaria*	*Taxus*, select *Eutacta*, select *Colymbea* and most cycads and *Ginkgo*	*Ceratozamia*
Number of cotyledons	**18**	**9**	**4**	**2**	**1**

CHAPTER 3. EFFECTS OF STORAGE, HYDRATION AND TEMPERATURE ON POLLEN GERMINATION IN *Encephalartos*, AN AFRICAN CYCAD GENUS

3.1. Abstract

Encephalartos latifrons, a critically endangered cycad propagated at Kirstenbosch National Botanical Garden (KNBG), experiences low seed germination. The cause of poor germination is unknown, but it has a negative impact on conservation programmes of this species. This paper investigates possible causes relating to i) viability of fresh and stored pollen, ii) effects of hydration then drying, and iii) the temperature under which pollen germinates.

The study compares responses of *E. latifrons* and *E. altensteinii*, a more common cycad that has a relatively high reproductive success of > 60% at KNBG. It also examines pollen viability for 17 species to establish whether *E. latifrons* has particularly low levels of pollen viability. Experiments were conducted to test pollen viability and pollen responses at different temperatures, as well as to test the effects of hydration and subsequent desiccation.

Pollen viability of 17 species was observed to be high and started dropping after three years. Fresh and stored *E. latifrons* pollen from KNBG was comparative to the wild and showed high viability. Pollen from *E. latifrons* germinated between 15°C and 35°C with optimum germination at 30°C, and *E. altensteinii* germinated from 10°C to 40°C, with optimum at 25°C. A χ^2 test inferred that pollen germination of *E. latifrons* was lower than in *E. altensteinii* when hydrated as part of wet pollination

methods. Results suggest that pollen drying out, after wetting in *E. latifrons* causes low pollen germination and results in poor seed set, although this contrasts with *E. altensteinii.* This means, the response of *Encephalartos* pollen to environmental conditions can vary according to species.

Key words: *Encephalartos latifrons; E. altensteinii; pollen viability, pollen desiccation, pollen germination temperature.*

3.2. Introduction

Ex situ plant collections have been identified as critical components of endangered plant conservation strategies (BGCI, 2012). However, collecting and maintaining plants in offsite collections has several challenges, which according to Walters (2003) include: selection of plants for phenotypes that satisfy horticultural, aesthetic and landscaping perspectives; loss of plants due to environmental conditions prevailing in gardens, and poor maintenance or change in collections policy. Further challenges also arise due to hybridisation and lack of genetic diversity (Maunder *et al.*, 2004), which can lead to inbreeding depression as a result of the small gene pool (Volis *et al.*, 2009). These challenges need to be overcome if *ex situ* collections are going to play a meaningful role in plant conservation efforts.

Cycads are the most threatened group of plants (IUCN Red List, 2010) and, for this reason, conservation is critical. One of the factors complicating *ex situ* conservation is that cycad seeds are reported to be recalcitrant (Forsyth & Van Staden, 1983). This means that *ex situ* conservation through conventional seed banking is currently not possible, making living plant collections the only viable *ex situ* conservation method. Several *ex situ* living collections have been established as a result, and Kirstenbosch National Botanical Garden (KNBG) is one such collection. KNBG has the largest *ex situ* collection of the critically endangered *Encephalartos latifrons* (da Silva *et al.*, 2012), but poor seed viability of < 10% (Winter, 2006) means that the collection yields only low numbers of propagules for conservation purposes. Similar problems have been experienced in other *ex situ* collections of this species (Whitelock, 2002). The cause of low seed viability is unknown and a structured analysis is required to identify the likely cause and propose solutions. One of the possible

causes could be low pollen viability or reduced viability due to either the storage or treatment of pollen preceding artificial pollination, or the conditions that prevail at the time of pollination (e.g. temperature). This chapter addresses these three factors.

In *ex situ* cycad collections, cones are often artificially pollinated using stored pollen because coning of different sexes may not occur synchronously in the same year and natural pollination systems are either absent or increase risks of hybridization. Effective pollen storage for artificial pollination is therefore crucial for successful propagation in *ex situ* collections. Previous investigations showed that cycad pollen can last between three and five years in sub-zero temperatures (-15°C) (Osborne *et al.*, 1992). Although all cycad pollen shares some characteristics such as a multi-layered sporoderm, being boat shaped, monosulcate and bilaterally symmetrical (Dehgan & Dehgan, 1988; Marshall *et al.*, 1989), other pollen characters vary between genera, such as thickness of the sporoderm layers and chromosome numbers (Dehgan & Dehgan, 1988). It is therefore not surprising that pollen from different cycad genera vary in their response to storage (Tang, 1986a; Osborne *et al.*, 1992). Despite aparent similarity in pollen within genera, pollen from different *Encephalartos* species have also been reported to respond variably to similar environmental conditions and storage (Mostert, 2000). This poses the question whether uniform pollen storage methods used for cycads in *ex situ* collections result in different viability for species such as *E. latifrons* (with low seed viability) *E. altensteinii* (with relatively high seed viability of > 60%), and other species cultivated at KNBG.

Pollen germination and pollen tube growth is influenced by several environmental factors, and temperature and moisture (relative humidity) are amongst the important ones (Stanley & Linskens, 1974; Acar & Kakani, 2010). Exposure to particular temperatures or levels of moisture at the wrong

time can negatively impact pollen germination if they lead to desiccation or exine rupture leading to eventual death (Duhoux, 1982). Given that temperature can affect moisture and in turn pollen desiccation, it is a crucial factor in regulating pollen germination in plants (Nygaard, 1969; Barrow, 1983). Temperature effects on cycad pollen germination have not been intensively studied and it is therefore not known how different temperatures affect pollen germination and seed viability. In the case of *E. latifrons,* conditions in *ex situ* collections at KNBG differ from natural conditions and could therefore affect pollen germination. This species' cones are receptive mainly in winter (in the month of August) when low temperatures prevail at KNBG (min 9°C and max 18°C mean) with high winter rainfall with a mean of 1 310mm per annum. Comparatively, temperatures in the wild are min 4°C and max 18°C, at the same time of the year with less than half the year round rainfall with a mean of 680mm. This raises further questions whether, prevailing environmental conditions in combination with the wet pollination method (mixing pollen with water) used at KNBG, is negatively impacting pollen germination, and thus results in low seed germination in *E. latifrons*.

This paper investigates three hypotheses: (i) that *E. latifrons* has low pollen viability compared to other species with better seed germination, especially in pollen that has been stored for a number of years; or (ii) low pollen viability as a result of pollen wetting and subsequent drying by the wet pollination method leading to low seed germination, and (iii) that cycad pollen germinates within a specific range of temperatures so that environmental conditions in cones may affect pollen germination. These hypotheses were tested on *E. latifrons* at KNBG. In addition the same tests were carried out on *E. altensteinii,* a more common species that does not experience low levels of seed germination. Overall pollen germination was tested in 17 species.

3.3. Materials and Methods

3.3.1. In vitro testing of pollen viability and longevity

The hang-drop pollen viability testing method was used (Osborne *et al.*, 1992). Pollen cones were harvested when initially shedding pollen and stored in a box covered with paper for seven days. Pollen was then sieved to remove excess cone tissue and insects, packaged in paper envelopes (5 g), and placed in a sealed plastic container with silica gel and stored at –15°C until use. *In vitro* pollen testing was performed under sterile conditions on a flow bench on a media solution comprising 10% (w/v) sucrose and 0.005% (w/v) boric acid (Osborne *et al.*, 1992; Mostert, 2000). Pollen (0.02g) was mixed with 200µl media solution and a 15µl droplet of solution was suspended upside down on a Petri dish with double distilled water at the bottom to prevent desiccation. Petri dishes were placed inside a sealed plastic container with moist paper towel and incubated for 48h at 28°C in the dark. There were five replicate Petri dishes for each pollen sample. After incubation, the droplet was diluted with 20µl double distilled water and a 15µl sample was mounted on a 76 X 26 mm slide, which was viewed at 100x magnification using a Laborlux K microscope. For each slide, 10 grid cells each with > 10 pollen grains were chosen at random and scored for the proportion of germinated pollen with visible elongation of the pollen tube (Fig 3.1).

Pollen germination experiments were repeated on 17 *Encephalartos* species from KNBG (*E. altensteinii, E. arenarius, E. caffer, E. friderici-guilielmi, E. ghellinckii, E. horridus, E. humilis, E. latifrons, E. lehmannii, E. longifolius, E. manikensis, E. ngoyanus, E. paucidentatus, E. princeps, E. transvenosus, E. trispinosus and E. villosus*) using pollen which had been stored for periods between zero and eight years. In addition, pollen collected from wild specimens of *E. latifrons* and then

stored for two to three years under the same conditions at KNBG were also tested. In total there were 35 pollen samples. The mean germination for each sample was calculated and analysed using a regression least square fit, since it was the best biological fit model and ANOVA (Statistica7®).

3.3.2. In vitro testing of pollen germination

The response of pollen to wetting and subsequent drying, as may occur during wet pollination, was tested. Pollen was collected as in the pollen viability tests (3.3.1) from *E. latifrons* and *E. altensteinii* at KNBG. Pollen from individual trees was first tested for viability and all found to be >70% viable. Pollen from individual trees was then combined, a control was tested, then 10g of pollen was mixed in distilled H_20, filtered to remove the water, and then left on a sterile flow bench to dry for 24h before testing, and also for 15 days then tested. Pollen counts of germinated pollen were carried out on a desktop computer screen using digital photographs (Fig 3.1). Four *E. latifrons* and seven *E. altensteinii* trees were used with 10 replicates of mixed pollen tested for each species. Results were tested using factorial ANOVA (Statistica7®).

3.3.3. In vitro testing of temperature effects on pollen germination

The effect of different temperatures on germination of fresh pollen was tested in *E. altensteinii* and *E. latifrons*. The same incubation method and number of trees were used as in the effects of pollen hydration and desiccation above. Germination was tested at nine temperatures from 5°C - 45°C with a 5°C increment. Results from ten replicates for each species and each temperature were processed in the same way as the previous experiment, and statistically analysed using factorial ANOVA (Statistica7®).

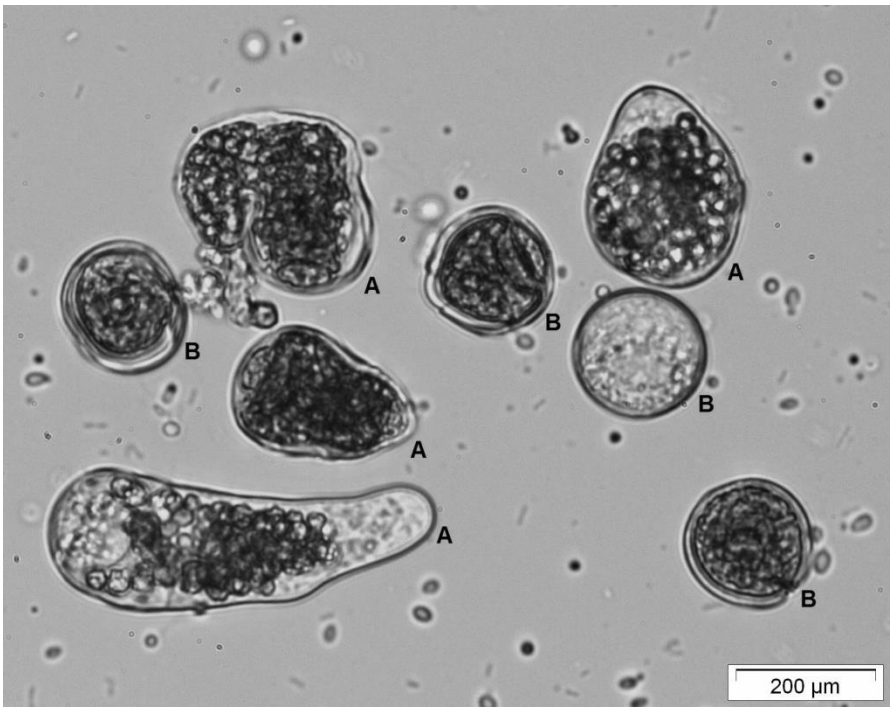


Figure 3.1 Example of a microscope photograph of *Encephalartos latifrons* pollen used to score pollen germination after 48h incubation. A = germinated pollen with pollen tube elongation, and B = non germinated pollen.

3.3.4. In vivo pollen germination in relation to ovulate cone temperature

The temperature of female cones was monitored on plants growing outdoors at KNBG using thermochron ibutton data loggers (Fairbridge Technologies®, South Africa) set to record temperature at 15 min intervals. The data were downloaded and analysed using "Cold Chain Thermodynamics®" (Fairbridge Technologies®, South Africa) software. One ibutton was inserted in the middle of the cone in between the sporophylls, to avoid direct sunlight. Two ibuttons were hung one meter away, also away from direct sunlight, to monitor ambient temperature. A mean of the two ambient temperatures was compared to the cone temperature. Ovulate cone temperature was monitored in two *E. latifrons* cones (2012) and three *E. altensteinii* cones (2011).

In vivo pollen germination was tested in ovulate cones that were pollinated three times every second day using the wet pollination method. Wet pollination meant that 5g of fresh pollen was mixed with 250ml of distilled water and injected between the open cone sporophylls. Ovules were extracted from the cones twenty days after the first day of pollination, then the sarcotesta was removed and soaked in a formalin propionic acid (FPA) fixative until dissection. The nucelli were extracted from the ovules to examine for pollen germination in the pollen chamber. The nucellus was hand sectioned with a thin blade under a microscope to obtain fine slices of the pollen chamber, then placed on a 76 x 26 mm slide and covered with a drop of aniline blue dye. The slides were examined using a Leitz Laborlux K® microscope with a fluorescent light source, and with a mounted Olympus SC® camera. In *E. latifrons* (n = 3 cones with 60 ovules) were extracted for viewing (2011, 2013 and 2014). This process was repeated in *E. altensteinii* using cones (n = 7 with 105 ovules) in (2010, 2011, 2012 and 2014). The relative frequency of germinated and ungerminated pollen grains was tested using a χ^2 goodness of fit test..

3.4. Results

3.4.1. In vitro testing of pollen viability and longevity

Data for 17 species in which pollen was stored for between zero and eight years (Fig 3. 2) showed an overall gradual decline in viability over the first three years followed by a more dramatic decline after four years. A Distance-Weighted Least Squares regression model fitted to the data provided the best statistical fit and makes biological sense if there is an overall loss of viability in later years.

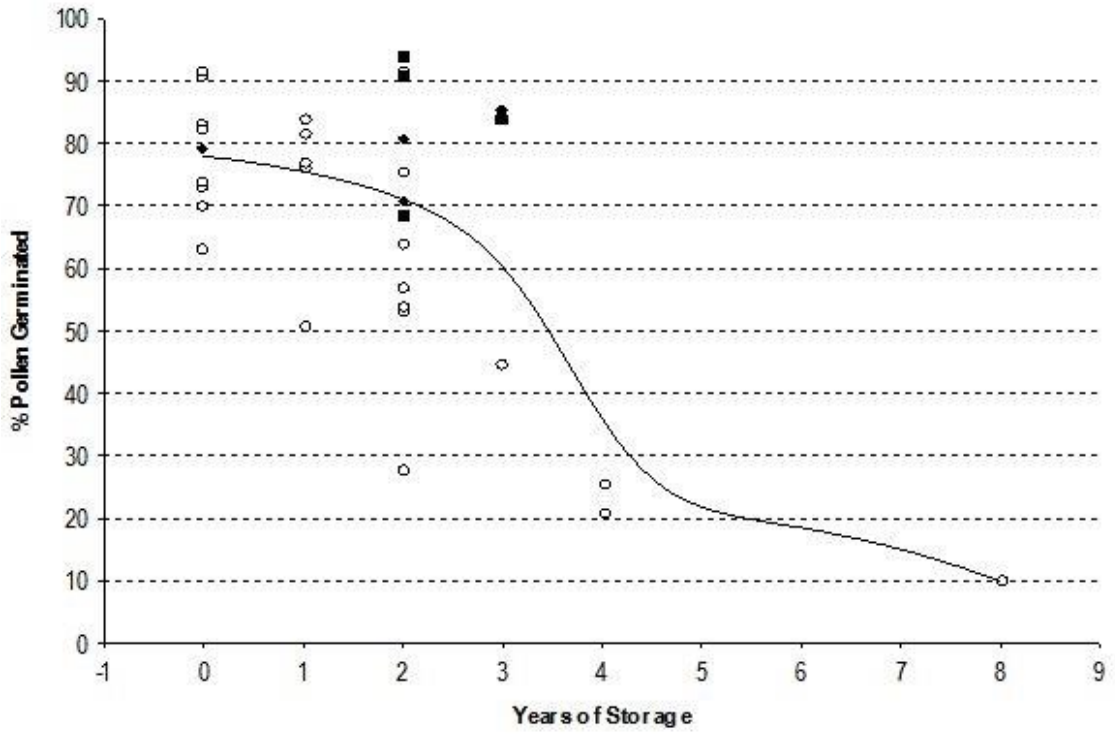


Figure 3. 2 Viability of pollen of *Encephalartos* that was tested when fresh (0 years) or stored for up to eight years at subzero temperature (-15°C). The solid line represents a regression analysis with Distance-Weighted Least Squares fit. The graph represents 17 *Encephalartos* species with (*n* = 32) samples. Squares represent *E. latifrons* from the wild, diamonds represent *E. latifrons* at KNBG and circles represent all other *Encephalartos* species from KNBG.

The viability of fresh *E. latifrons* pollen from KNBG ($\bar{x}$ = 79%) was within the same high range as *E. altensteinii* ($\bar{x}$ = 76%) (Fig 3.2). Similarly, after three years of storage, there was no significant difference (ANOVA $F_{1, 8} = 0.08861$ $p > 0.05$) between *E. altensteinii* from KNBG and *E. latifrons* from both the wild and KNBG (Fig 3.3). Furthermore, there was also no significant difference (ANOVA $F_{2, 11} = 1.1872$, $p > 0.05$) in pollen viability between species and years of storage within the first three years.

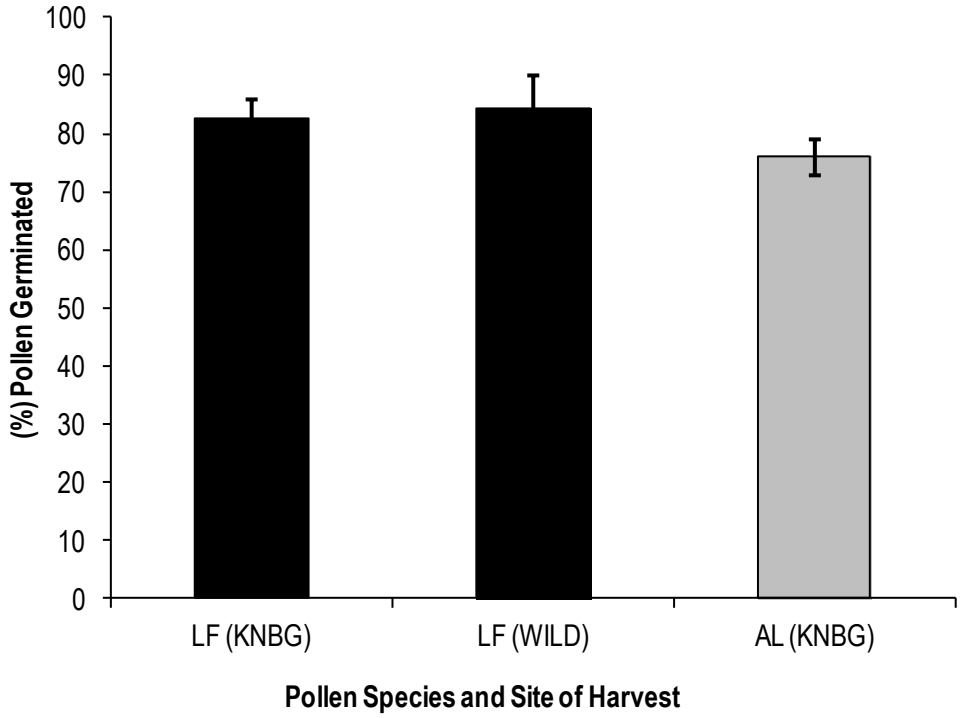


Figure 3.3 Viability of pollen sampled from the KNBG. Data represent the means (± SE) for *Encephalartos latifrons* (black) from KBNG (*n = 2*), wild (*n* = 4) and *E. altensteinii (*grey) (*n = 6*) from KNBG.

3.4.2. In vitro testing of pollen germination

Germination response of *E. latifrons* and *E. altensteinii* pollen that was wet and subsequently dried was varied (Fig 3.4). Germination of *E. latifrons* pollen was significantly lower than that of *E. altensteinii* (ANOVA $F_{1, 54}$=167.45, $p < 0.05$) (Fig 3.4). The Post Hoc Tukey HSD test showed that the *E. latifrons* control was similar to all the treatments in *E. altensteinii* ($p > 0.05$). However, *E. latifrons* pollen that was dried for 24h or 15d was significantly lower than the control, and the rest of *E. altensteinii* treatments including the control ($p < 0.05$) (Fig 3.4).

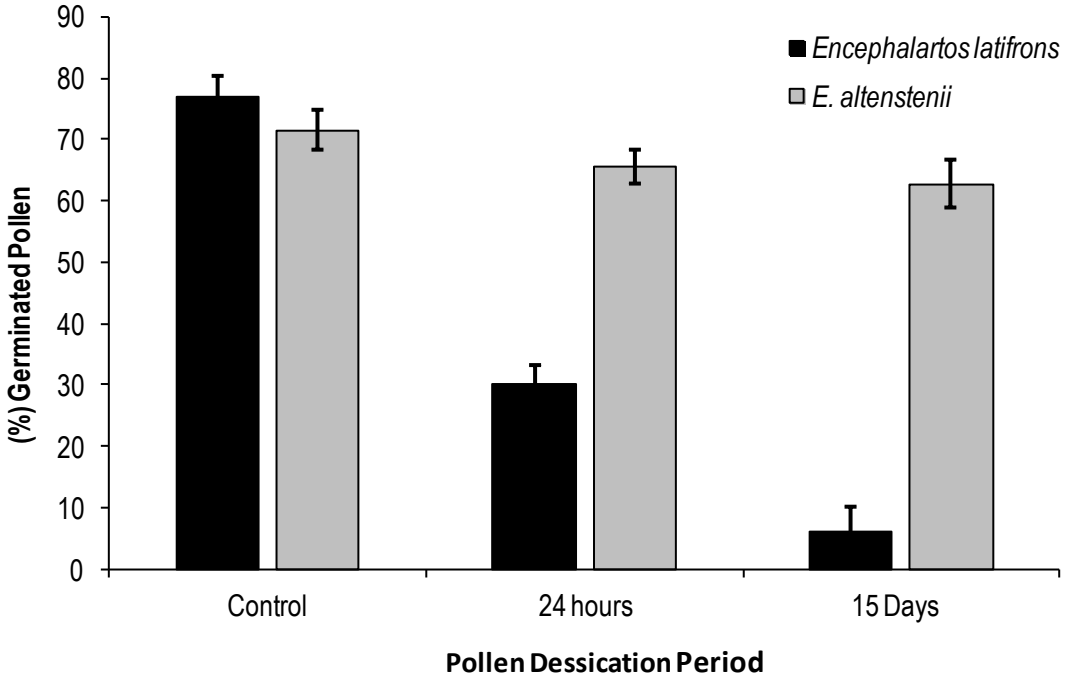


Figure 3.4 Germination response in fresh *Encephalartos* pollen after hydration for five minutes, then drying for 24h or 15 d before incubation. Bars represent the mean (± SE) for *E. latifrons* (n = 4) and *E. altensteinii* (n = 7).

3.4.3. In vitro testing of temperature effects on pollen germination

Pollen response to temperature was different in *E. latifrons* to that of *E. altensteinii* (Fig 3.5). *Encephalartos latifrons* responded within a narrower temperature band from 15°C - 35°C, compared to *E. altensteinii* which ranged from 10°C - 40°C (ANOVA $F_{8, 162} = 126.32$ $p < 0.05$). *Encephalartos latifrons* pollen did not germinate at temperatures < 15°C (Fig 3.5 A), whereas some *E. altensteinii* pollen germinated even at 10°C (Fig 3.5 B). The sporoderm in *E. latifrons* ruptured at a temperature of > 35°C (Fig 3.6 D) whereas sporoderm rupture only occurred at >40°C in *E. altensteinii*.. On the other hand, pollen sporoderm remained intact in both species at lower temperatures (< 10°C) (Fig 3.6 A).

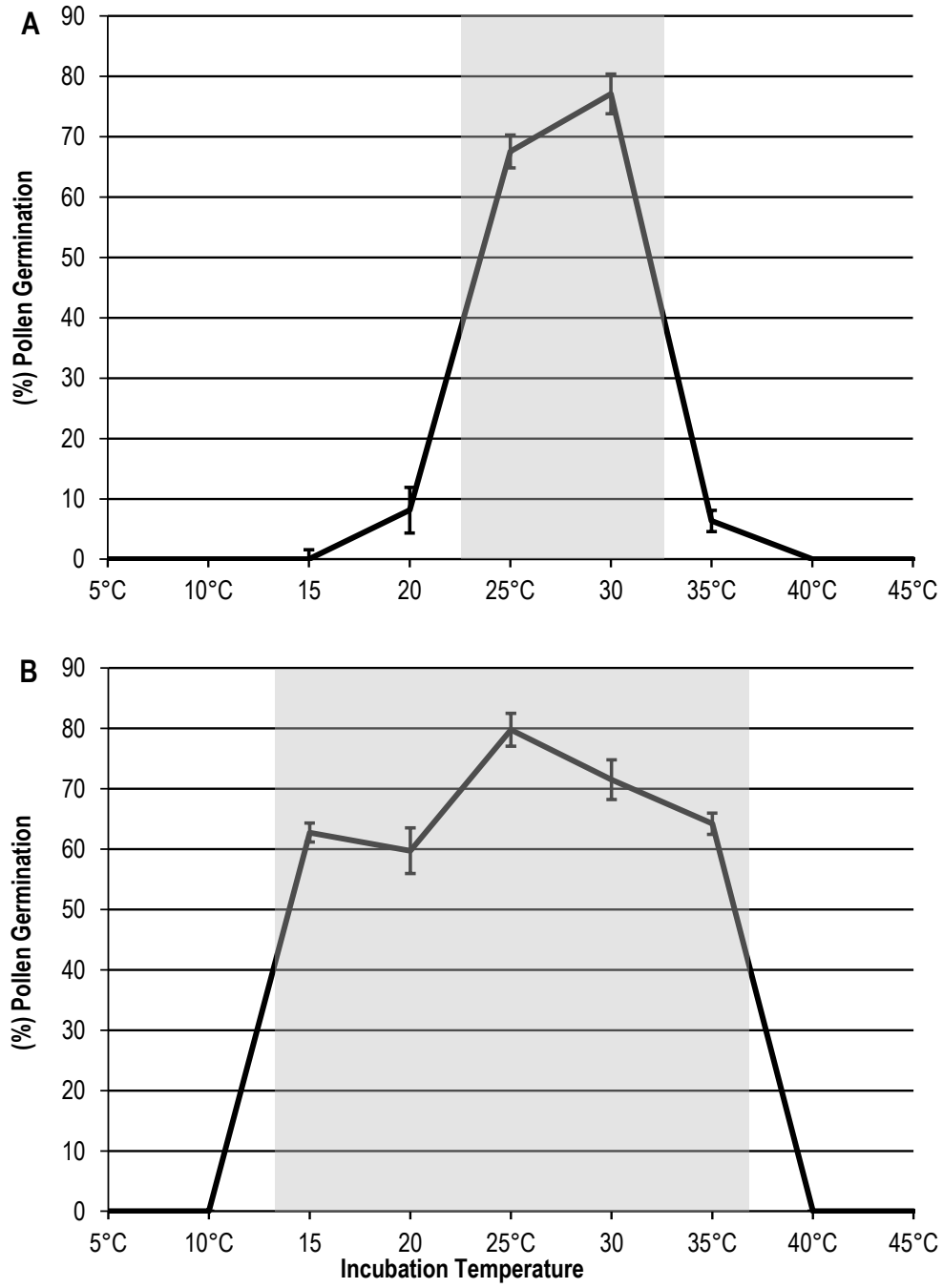


Figure 3.5 Pollen germination after incubation at different temperatures. *Encephalartos latifrons* (A) and *E. altensteinii* (B).The grey bar highlights the range in which pollen germination was > 40%. Lines represent the means (± SE) *(n =10).*

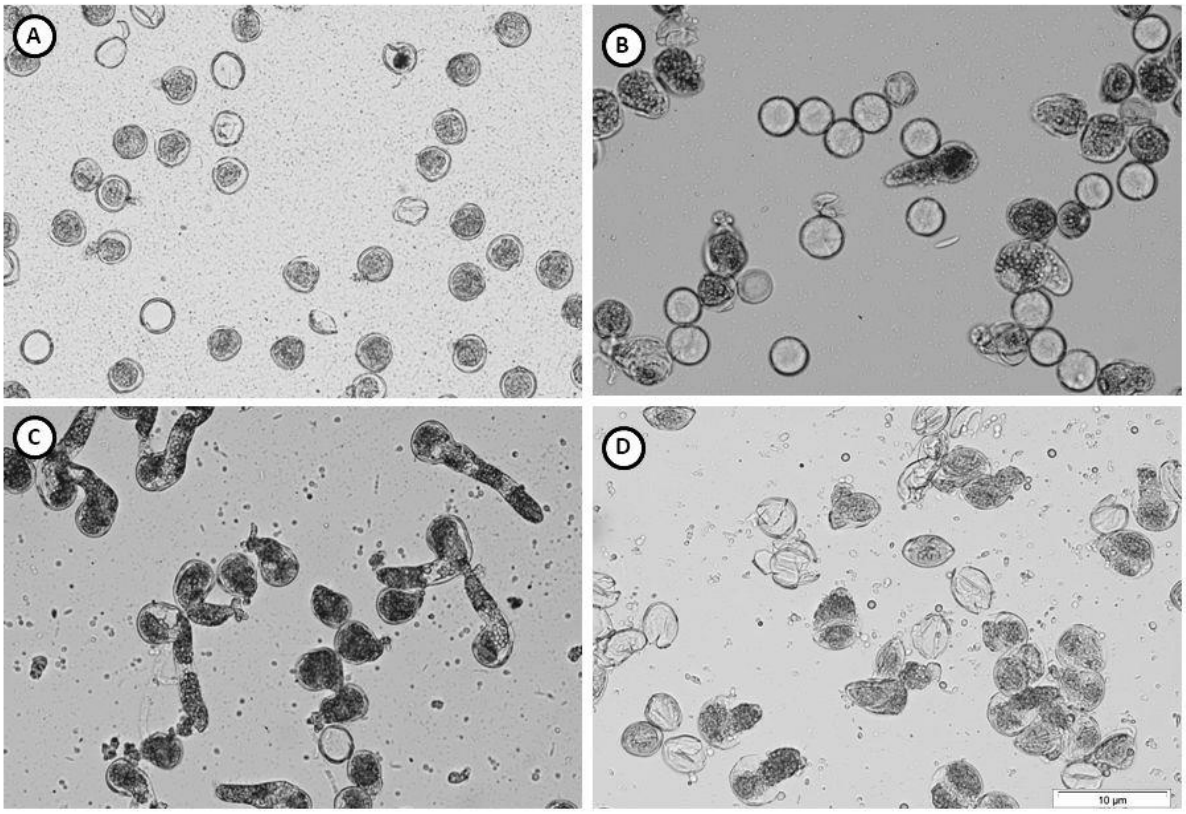


Figure 3.6 *In vitro* germination of *Encephalartos latifrons* pollen incubated at different temperatures. A = 10°C no germination, B = 20°C some germination, C = 30°C optimum germination and D = 40°C pollen sporoderm ruptured.

3.4.4. In vivo pollen germination in relation to ovulate cone temperature

In vivo pollen germination was observed in *E. latifrons* (Fig 3.7) and *E. altensteinii* by sectioning the nucellus. Pollen germination in *E. latifrons* ovules was significantly lower than in *E. altensteinii* (χ^2 n = 165) = 16.25, $p < 0.001$).

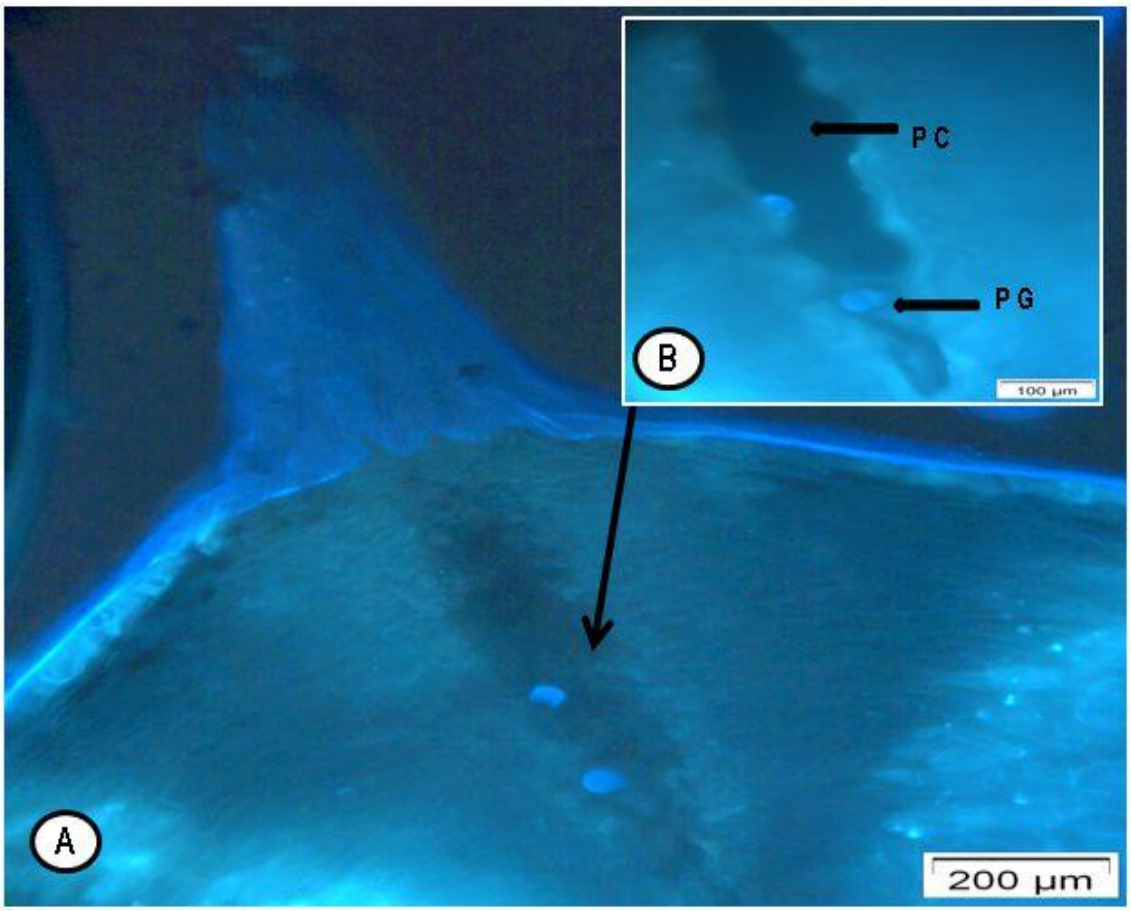


Figure 3.7 Germinating *Encephalartos latifrons* pollen in the pollen chamber of a sectioned nucellus 20 days after pollination. A = Entire image of the sectioned nucellus and B = magnified view of (A) pollen chamber area. PC = Pollen chamber and PG = Pollen germination into the nucellus.

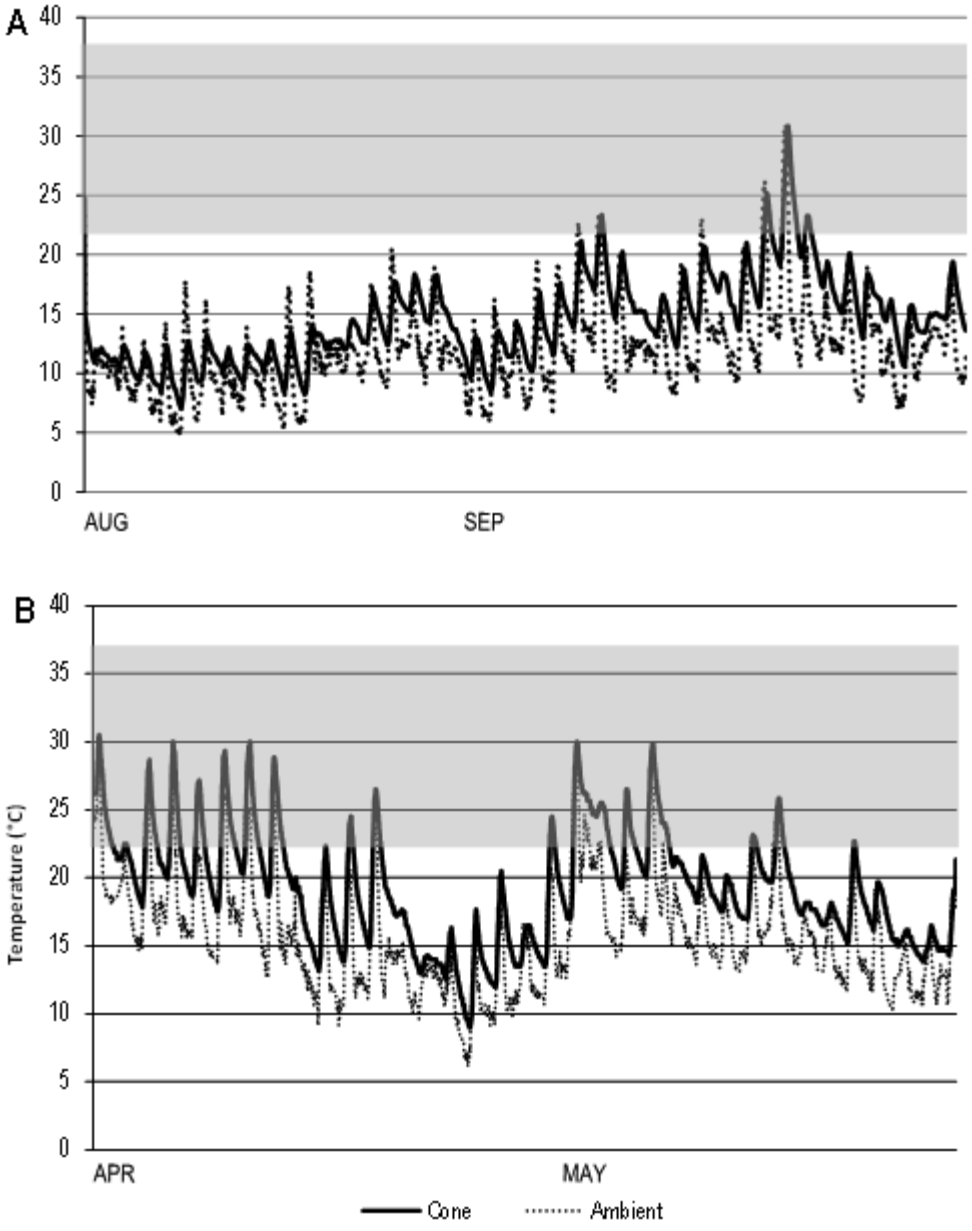


Figure 3.8 Ovulate cone temperature of two *Encephalartos* species during pollination receptivity. Solid lines represent mean cone temperatures for (A) *E. latifrons (n = 2)* and (B) *E. altensteinii (n = 3)* and dotted lines represent ambient temperatures for the corresponding period. Grey horizontal bands show the temperature zone for >40% germination derived from Fig. 3.5.

In both *E. altensteinii* and *E. latifrons*, female cone temperature lagged behind the ambient temperature (Fig 3.7 A & B). *Encephalartos latifrons* had an average cone temperature of 12.6°C, with a peak of 16.9°C and the lowest was 8.2°C. *Encephalartos altensteinii* had an average cone temperature of 14.6°C, with a peak of 22.7°C and the lowest was 9.1°C during the two months monitored after pollination. *Encephalartos latifrons* had four temperature peaks that reached the optimum pollen germination temperature (> 23°C) over two months of August and September (Fig 3.8 A), compared to *E. altensteinii* with 13 peaks over a similar duration from April to May (Fig 3.8 B).

3.5. Discussion and Conclusions

The objectives of this study were to detetermine whether low seed germination of the critically endangered *E. latifrons* was a result of poor pollen viability or germination as an inherent problem with *E. latifrons* pollen or as a result of (i) storage, (ii) pollen desiccation after hydration with artificial wet pollination, or (iii) environmental conditions prevailing at KNBG.

Environmental stresses and age are often cited as the primary causes of low pollen viability (Stone *et al.,* 1995). The findings in this study on cycad pollen generally support this notion. Seventeen *Encephalartos* species tested for pollen viability generally showed a decline in viability after three years (Fig 3.2). This trend following pollen storage supports similar findings by Osborne *et al.,* (1992), where it was reported that pollen retained viability at -15°C for three to five years. The viability of fresh *E. latifrons* pollen was high (97%) and comparable with fresh pollen of five other species that were sampled at KNBG (Fig 3.2 & Appendix 1). In addition, pollen viability of *E.*

latifrons from both the wild and KNBG, that was stored for two and three years, was not significantly different (ANOVA, $p > 0.05$). The results indicate that fresh and stored *E. latifrons* pollen from KNBG has relatively high viability and is higher than the 40% threshold identified by Stanley & Linskens (1974) as an indicator of sufficient viability for effective fertilisation to occur. The initial concern was that either the method or period of pollen storage was inappropriate for *E. latifrons* even though there was no obvious reason why *E. latifrons* should be different from other *Encephalartos* species. The results provide some evidence that pollen viability in *E. latifrons* is not very different to congeneric taxa. This means that either pollen is not the problem or that the problem is not the actual viability of the pollen but the response of pollen to particular treatments during pollination and to conditions that prevail at KNBG. The hypothesis that low seed germination is a result of poor pollen viability in *ex situ* cycad collections at KNBG is therefore not supported by the results.

The stark differences in the sensitivity of pollen to drying out between *E. latifrons* and *E. altensteinii* is surprising, given that there are only minor morphological differences in pollen size, shape and surface texture (Marshall *et al.*, 1989). Nevertheless, the difference in responses is consistent with differences detected in the germination of pollen at different temperatures, where *E. latifrons* responded within a narrow temperature band from 15°C to 35°C compared to *E. altensteinii* which geminated from 10°C – 40°C (ANOVA, $p < 0.05$) (Fig 3. 5). Furthermore, *in vivo* studies showed that *E. latifrons* pollen germination was significantly lower compared to *E. altensteinii* when pollen was mixed with water and injected into a receptive cone (χ^2 goodness of fit test, $p < 0.001$).

There are at least two variables at KNBG that could explain differences in pollen germination between species, i) the time of year when reproduction takes place, and ii) the method of pollination.

Pollination of *E.latifrons* occurs in winter and for *E. altenstenii* it is in autum at KNBG. As such, the species will experience different environmental conditions (temperature) during the pollination period. Measurements of ovulate cone temparature (Fig 3.8 A & B), show that temperature in *E. altensteinii* reach 20°C - 35°C for ca.19 days, and are within the range where pollen germination occurred. This is in contrast to *E. latifrons* cones, which reached optimum pollen germination temperatures for only six days during the reception period, suggesting that pollen germination may be compromised by cold ambient conditions at KNBG *(*Fig 3.8 A & B).

Furthermore, the lower germination of pollen using the wet pollination method in *E. latifrons* suggests that wetting pollen may result in desiccation if not resorbed into the ovule rapidly. These results give at least circumstantial support to the idea that ambient conditions prevailing at the time of pollination may influence the effectiveness of pollination. Despite the apparent similarities in cycad pollen, observations of different pollen germination responses between *Encephalartos* species are not unique to this study, but were also noted in a previous study by Mostert (2000). Perhaps the seemingly minor morphological and sporoderm differences play a greater role than suspected in cycads, in particular for *Encephalartos.* These questions need to be further investigated. Nevertheless, the lower pollen germination using the wet artificial pollination method in *E. latifrons* suggests that the pollination method used at KNBG may be the cause of low seed germination. For this reason, the effects of wet and dry pollination are more intensively investigated in Chapter Four.

CHAPTER 4. EFFECTIVENESS OF ARTIFICIAL POLLINATION IN A THREATENED AFRICAN CYCAD GENUS, *Encephalartos*

4.1. Abstract

The critically endangered cycad *Encephalartos latifrons* experiences low seed germination at Kirstenbosch National Botanical Garden (KNBG), which has the largest *ex situ* collection of the species. The cause of low seed germination is unknown and is impacting on conservation of the species. This paper investigates artificial pollination methods in relation to the formation cycle of pollination droplets by ovules and thermogenesis of male cones, as a potential cause of low seed germination.

Pollination parameters tested included: the effectiveness of wet and dry artificial pollination methods; the timing of pollination in relation to thermogenesis in pollen cones and expected synchrony with the formation of pollination droplets by ovules; and the duration that sporophylls remain loose, signalling receptivity. This study compared *E. latifrons* with a more common cycad *E. altensteinii* with a high seed viability of > 60% at KNBG.

Results show that sporophylls of *E. latifrons* remain loose up to three times longer than in *E. altensteinii* at KNBG. Pollination droplet formation and thermogenesis in both species is diurnal, predominantly occurring in the afternoon to early evening, with at least some overlap. Dry

pollinated *E. latifrons* cones had significantly higher pollen germination in the ovules, embryo presence in seeds and germinated seed compared to those that were wet pollinated ($p < 0.05$). This was contrary in *E. altensteinii*, where pollination method did not make a difference to seed germination ($p > 0.05$). However, pollination in the afternoon was significantly more effective than the morning period ($p < 0.05$). The conclusion is that the wet artificial pollination method can negatively impact on seed viability of certain *Encephalartos* species held in *ex situ* collections.

Key words: *Encephalartos latifrons; E. altensteinii; artificial wet pollination; dry pollination, pollination droplets; pollen germination, thermogenesis, seed germination.*

4.2. Introduction

One of the factors complicating the *ex situ* conservation of cycads (Cycadales) is that cycad seeds are recalcitrant (Forsyth & Van Staden, 1983) and cannot be stored for long periods. This means that living plant collections are currently the only viable *ex situ* conservation method. Kirstenbosch National Botanical Garden in South Africa has the largest *ex situ* collection of the critically endangered *Encephalartos latifrons* (da Silva *et al.,* 2012), but seeds obtained from this collection experience low viability of generally <10% (Winter, 2006). Similar observations with this species were also reported in another *ex situ* collection in Eagle Rock, California, USA (Whitelock, 2002). Chapter Three concluded that *Encephalartos* pollen is affected by environmental conditions and this response varies between species. This is particularly relevant in *ex situ* collections because of the need to artificially pollinate plants. Therefore, a general understanding of cycad pollination biology is necessary, so as to contextualise the low seed germination problem experienced by *E. latifrons* at the KNBG *ex situ* cycad collection.

Cycads are dioecious (Chamberlain, 1935) and are predominantly pollinated by insects, especially beetles (Terry, *et al.*, 2012). Pollination in cycads is a complex and drawn out process (Chamberlain, 1935; Norstog & Nicholls, 1997), which can be divided into two general stages: (a) the transfer of pollen from pollen cones to ovulate cones, where pollen is deposited on the micropyle and (b) the resorption of pollen into the ovule and its subsequent germination and development leading to fertilisation. During pollen transfer, insect vectors typically gain access to the receptive ovulate cone through loose sporophylls and deposit pollen onto the micropyle of the receptive ovules. In *ex situ* collections, artificial hand pollination is used to transfer pollen using a number of

methods (Whitelock, 2002), and it is not clear whether such artificial pollination introduces factors that could cause pollination failure.

One of the factors that should be considered is the overall period of receptivity for pollination. During pollination, the sporophylls of ovulate cones separate to allow pollinators to enter the cone and then close again after a number of days (Tang, 1986b; Grobbelaar, 2002; Whitelock, 2002). Cycad growers report that the sporophylls of different species stay open for different periods (Grobbelaar, 2002; Jones, 2002) but there has been only one formal study undertaken into the number of days sporophylls remain open in *E. inopinus,* were ovulate cones were reported to be receptive for only four days (Grobbelaar, 1996). It is not known how long *E. latifrons* sporophylls stay open, and whether this has a bearing on the formation of pollination droplets so it remains an uncertainty in the context of poor seed set for this species, especially since *E. latifrons* is the only winter (August and highest rainfall month) pollinated cycad at KNBG.

Natural pollination studies in cycads have revealed that pollen cones experience thermogenesis (Poisson, 1878; Jacot-Guillarmod, 1958; Tang, 1987b) and emit volatile odours resulting in the regulation of insect movements between cones (Terry *et al.,* 2004; Terry *et al.,* 2007; Suinyuy *et al.,* 2010; 2012). Since thermogenesis can influence the timing of insect movement between cones, it may be expected that receptivity in ovulate cones, and especially the presence of pollination droplets, would be synchronised with periods of heating and insect dispersal from male cones. Previous studies have shown some synchrony. In *E. villosus,* peaks in cone temperature and the presence of pollination droplets were not fully aligned, but between 60% and 80% of ovules had pollination droplets when male cones experienced their highest temperatures (Donaldson, 1997). In another study, pollination droplets in *Zamia pumila, Ceratozamia robusta, Dioon spinulosum* and *E.*

ferox were predominant in early morning (Tang, 1993), whereas male cone thermogenesis peaked in the afternoon to evening (Tang, 1987b). This is in contrast to *Cycas rumphii* and *Macrozamia robusta*, where the presence of droplets and thermogenesis overlap in the morning to midday (Tang, 1987b; 1993). Despite these reports, it is still not clear whether synchronicity between droplet formation and pollination activity could be a factor affecting seed viability in *E. latifrons*.

Two methods of artificial pollination have been used in cycad collections, usually referred to as the dry and the wet methods (Tang, 1986; Grobbelaar, 2002; Whitelock, 2002). For *Encephalartos* species, the dry method involves pneumatically squirting dry pollen between the loose sporophylls (Grobbelaar, 2002). In the wet method, pollen is mixed with water and the suspension is squirted with a syringe between the loose sporophylls (Grobbelaar, 2002). Cycad species differ in cone size, shape and structure and it has been recognised that different pollination methods may suit different species (Tang, 1986b). For example, Grobbelaar (2002) advocated the dry pollination method for *Bowenia, Ceratozamia, Chigua, Cycas, Dioon, Microcycas, Stangeria and Zamia* and the wet method for *Encephalartos, Lepidozamia and Macrozamia*. No reasons have been given for this. The only comparative study of pollination methods (Tang, 1986b) reported that *E. ferox* had high seed viability (82%) using a dry pollination method where the top part of the cone was cut off for better access and pollen was blown inside the cone. The current information suggests that the artificial pollination method has an impact on seed viability but the underlying mechanism is not clear so it is impossible to predict which method would work best for *E. latifrons*.

This paper investigates several aspects of artificial pollination, specifically the effectiveness of different pollination methods and the timing of artificial pollination on seed set. The focus of the

study was primarily on *E. latifrons* but cones of this species are very scarce so comparative studies were undertaken on *E. altensteinii*, which is common in the KNBG collection. The approach focuses on whether observable aspects of pollination biology, such as heating in pollen cones and presence of pollination droplets in ovulate cones, can be used to improve the effectiveness of artificial pollination.

4.3. Materials and Methods

4.3.1. Duration of sporophylls opening

Encephalartos latifrons and *E. altensteinii* ovulate cones were monitored for receptivity at KNBG while still attached to the plant. Selected cones were covered with a fine mesh material to keep out insects. Receptivity was monitored by checking for looseness of the top sporophylls and also by squirting 50ml of distilled water between the top sporophylls to determine whether fluids could penetrate between the sporophylls and run down the cone axis. Cones were monitored every second day. Cones with loose sporophylls, and where water ran through to the bottom of the cone axis, were recorded as receptive. In *E. latifrons* six cones were monitored in August, and 15 cones of *E. altensteinii* between April and May (2012 and 2013). The total number of cone receptive days in each species was statistically analysed with *ANOVA* in Statistica7® software.

4.3.2. Diurnal formation of pollination droplets

Encephalartos altensteinii and *E. latifrons* ovules were monitored for pollination droplets when the cone was receptive. Well developed ovulate cones were covered with a fine mesh material bag to prevent insect contact before sporophylls became loose. Receptive cones were partially dissected by removing a spiral of sporophylls with the ovules still attached. The sporophylls and ovules were placed in a container with a base of moist tissue paper and covered with a lid to prevent drying out, and were observed for formation of pollination droplets every hour over a 24h period. The partially dissected ovulate cones were covered with a damp cloth as well as a bag made from fine gauze to prevent drying, as suggested by Donaldson (1997). For each observation period of 24h, 10 pairs of sporophylls were extracted in a spiral from each cone. Two *E. latifrons* (August) cones and nine *E.*

altensteinii (April -May) cones were used for the experiment. One additional *E. latifrons* cone was monitored for pollen droplets after pollination was carried out. Pollination droplet data per 24h, was statistically analysed with *t* test in Statistica7® software.

4.3.3. Pollen cone thermogenesis

Pollen cone temperatures were monitored under controlled conditions with ambient temperature set at 20°C and 12 /12h photoperiod. Cone temperature was monitored using micro ibuttons (Cold Chain "Thermodynamics" ®, Fairbridge Technology®) recording at 30 min intervals. The ibutton was inserted when the sporophylls were loose, prior to pollen shedding. A second ibutton recorded the temperature of the growth room (within one meter of the cone). Eight *E. latifrons* cones and 10 *E. altensteinii* cones were monitored.

4.3.4. Efficacy of pollination methods

4.3.4.1 Efficacy of pollen delivery by different artificial pollination methods

Efficacy of pollen delivery to the micropyle was tested between the wet and dry hand pollination methods in *E. altensteinii.* Finely-sieved pollen was mixed with aniline blue dye at 10 / 70% (W/W) ratio. Receptive ovulate cones (April –May) were covered with a fine mesh material to prevent insect contact before and after pollination. In the wet pollination method, pollen was mixed with 250ml of distilled water and was squirted between the loose sporophylls using a syringe. In the dry pollination method, a hand operated pneumatic pump (rubber ball) and attached nozzle was used to squirt pollen between the loose sporophylls. Pollination was carried out three times every second day and 15g total amount of pollen was used per cone. Pollinated cones were harvested two days

after pollination and dissected in the laboratory to extract ten ovules from the top, middle and bottom of each cone per treatment. This was done for seven cones per treatment (n = 210 ovules). Extracted ovules were then viewed under a microscope and scored using a modified Likert scale from one to four, depending on the intensity of the dye observed in and around the micropyle area. Data was converted into frequencies and the $\chi 2$ test was used to statistically test data with Statistica7® software.

4.3.4.2 Pollination at different time of the day

To test for effects of timing on success of pollination, dry and wet pollination methods were applied in the morning (10h00 – 11h00) and in the afternoon (14h00 – 15h00) on *E. altensteinii*. Pollinated cones were harvested after natural disintegration began in November to December. The seed sarcotesta was cleaned off and seed kennels were dusted with a wettable powder fungicide Efekto Fungi-Nill 500 WP® Captab, (Dicarboximide). Seed was stored at ambient temperature in a cool room for eight months. After storage, seed was sown in an open germination bench heated to 28°C in silica sand (media) and watered every day for 30 weeks and the number of germinating seed was taken as the measure of pollination effectiveness. There were four treatment combinations; i) morning dry pollination, ii) morning wet pollination, iii) afternoon dry pollination and iv) afternoon wet pollination. A total of 120 seeds from 16 cones were tested. Seed germination at 30 weeks was analysed using a factorial *ANOVA* in Statistica7®.

4.3.4.3 In vivo pollen presence in the dry and wet artificial pollination methods

The presence of pollen in the ovules was tested in both *E. altensteinii* and *E. latifrons* by comparing the dry and the wet pollination methods. Ovulate cones were pollinated three times every second day with 5g of fresh pollen. Ovules were extracted from the cones twenty days after the first day of pollination, the sarcotesta was removed and the ovules were soaked in a formalin propionic acid (FPA) fixative until dissection. The nucelli were extracted from the ovules to examine for pollen germination in the pollen chamber. The nucellus was hand sectioned with a thin blade under a microscope to obtain fine slices of the pollen chamber, then placed on a 76mm x 26mm slide and covered with a drop of aniline blue dye (Donaldson & Bösenberg, 1995). The slides were examined using a Leitz Laborlux K® microscope with a fluorescent light source, and with a mounted Olympus SC® camera. In *E. latifrons*, 120 ovules from 4 ovulate cones were extracted for viewing. This process was repeated in *E. altensteinii* (n = 7 cones with 210 ovules). Sectioned nucellus was scored for presence of pollen using a number measure: 1 (germinated) and 2 (non germinated). Scores were converted into a frequency table and the χ^2 goodness of fit test was used for an inference.

4.3.4.4 Embryo presence and seed germination between the dry and wet pollination methods

Pollinated cones were harvested after natural disintegration began during the months of November - December. Seed storage and germination were handled the same way as for testing pollination at different times of the day (section 4.3.4.2). In *E. latifrons*, seed numbers tested were as follows: for dry pollination embryo test (n = 180) seeds; seed germination (n = 150), and wet pollination embryo test (n = 180); seed germination (n = 120). For *Encephalartos altensteinii* cone replicates were as

follows: dry pollination: embryo test (n = 540); seed germination (n = 150), and wet pollination embryo test (n = 480); seed germination (n = 180). Observed numbers of embryos in seeds were scored, then compared for maximum seed germination at 30 weeks, and analysed using factorial *ANOVA* in Statistica7®.

4.4. Results

4.4.1. Duration of sporophylls opening

The number of days that sporophylls remained loose ranged from 33 – 75 days in *E. latifrons* and only 14 - 25 days in *E. altensteinii,* which was significantly less than for *E. latifrons* (*ANOVA* $F_{1, 20} = 30.923$, $p < 0.05$).

4.4.2. Diurnal cycle of pollination droplet formation

Pollination droplets in both *E. altensteinii* and *E. latifrons* were observed to have a diurnal pattern. Droplet formation, especially in *E. altensteinii,* occurred predominantly from the late morning to mid afternoon with another small peak in the early evening (Fig 4.1). The highest percentage of ovules with pollen droplets present at any particular time was considerably lower in *E. latifrons* (3%) than *E. altensteinii* (14%), and the overall percentage of ovules that produced droplets over a 24h period was also significantly lower in *E. latifrons* (*t*-test for independent means $t_{48} = -2.88$. $p < 0.05$). No droplets where observed in one of the *E. latifrons* cones with loose sporophylls, up to 27 days after it was observed to be receptive.

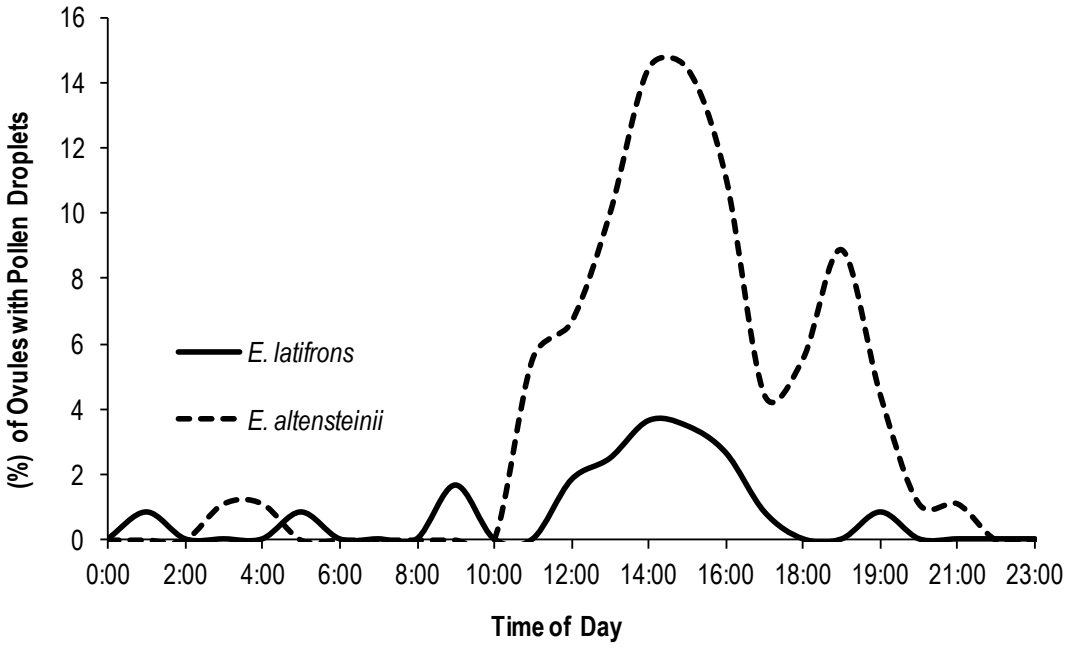


Figure 4. 1 Diurnal pattern of pollination droplet formation monitored over 24h in *Encephalartos latifrons* (*n* = 2) and *E. altensteinii* (*n* = 9).

4.4.3. Pollen cone thermogenesis

Pollen cones of both *E. altensteinii* and *E. latifrons* were observed to undergo a pattern of diurnal thermogenesis (Fig 4.2A & B). In both species, thermogenesis occurred from around midday (12h00 – 13h00), reached a peak in the mid-afternoon (14h00 – 17h00), and tapered off in the evening (20h00-22h00) (Fig 4.2A & B). In *E. latifrons* cone temperature reached a mean peak of 13°C above ambient (Fig 4.2A).

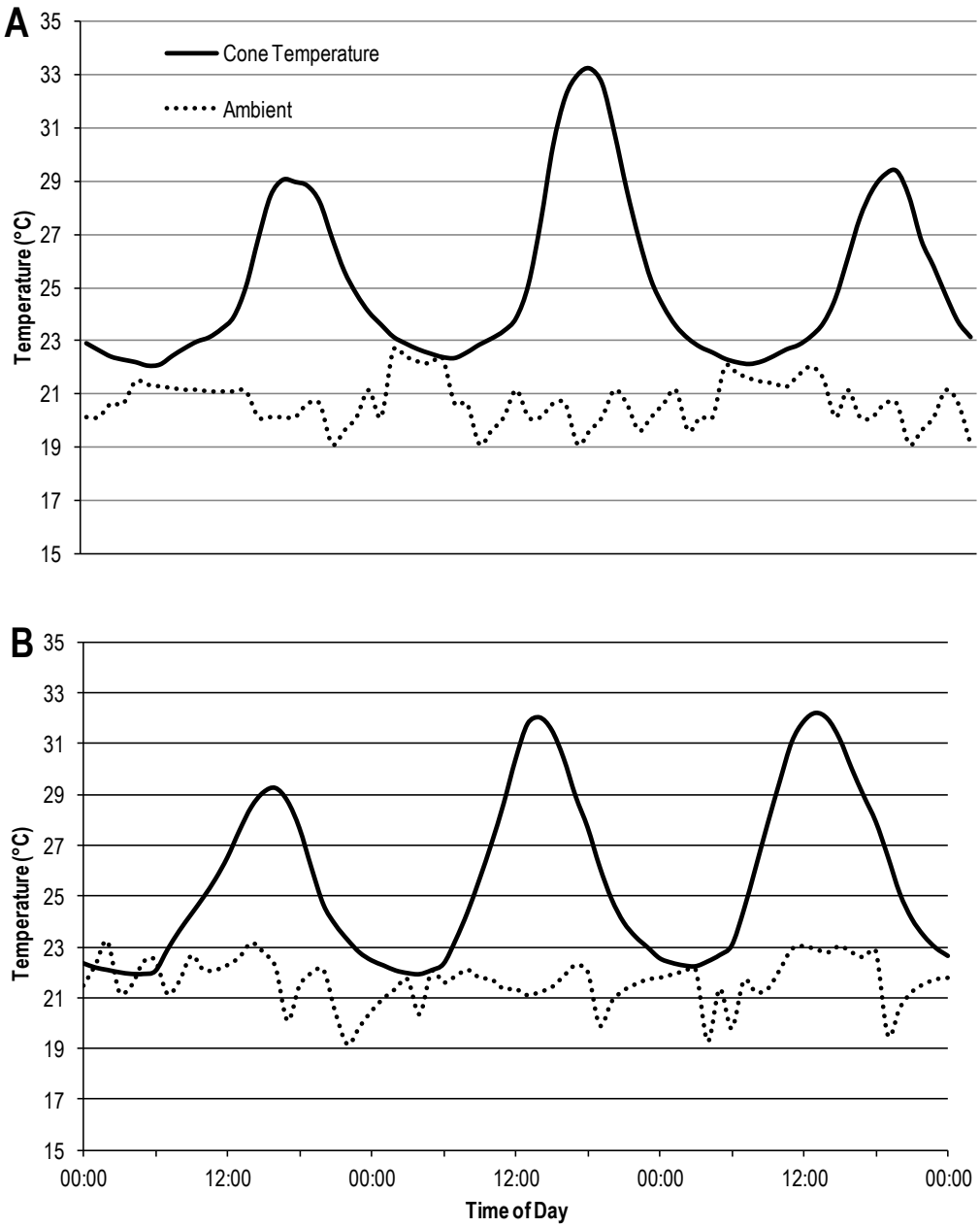


Figure 4. 2 Temperature of pollen cones in *Encephalartos* species monitored in a controlled environment set at 20°C. A = *E. latifrons* (*n* = 7); B = *E. altensteinii* (*n* = 6). Ambient room temperature fluctuated by ±3°C due to delayed responses to heating and cooling.

4.4.4. Efficacy of pollination methods

4.4.4.1 Efficacy of pollen delivery by different artificial pollination methods

The wet pollination method resulted in significantly more ovules with dye around the micropyle than the dry method ($\chi^2 = 583.60$, $p < 0.001$, $n = 560$). The wet pollination method had 57% ovules in the middle part of the cone with maximum scores (micropyle intensely blue), compared with the dry method 37% (Fig 4.3A & B). The dry method had 12% ovules with no dye compared to zero in the wet method in all different section of the cone (Fig 4.3A & B). In both treatments, there was a significant difference in the accumulation of dye between ovules from the top, middle and bottom sections of the cone using either the dry method ($\chi^2 = 279.67$, $p < 0.001$, $n = 280$), or wet method ($\chi^2 = 303.93$, $p < 0.001$, $n = 280$) (Fig 4.3B). The volume of pollen delivery to ovules was substantial for both dry and wet pollination methods, and in most cases the micropyle was filled with pollen (Fig 4.4).

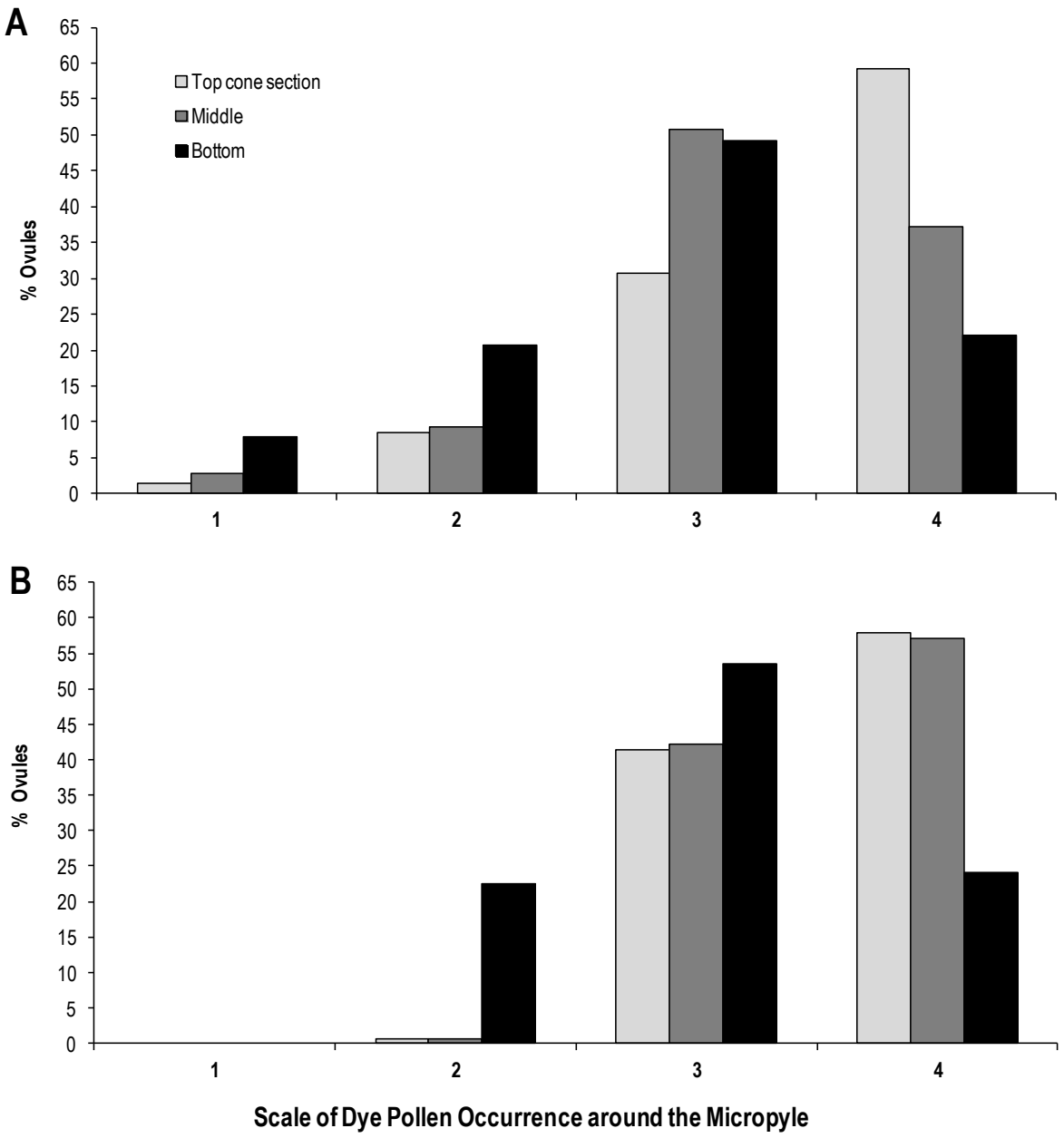


Figure 4. 3 Occurrence of aniline blue dyed pollen in the micropyle of *Encephalartos altensteinii* ovules after dry (A) and wet (B) pollination. Graph represents frequencies converted to means: 1= no dye, 2 = some dye, 3 = peak with dye and 4 = micropyle intensely blue. Each graph represents mean for 840 ovules.

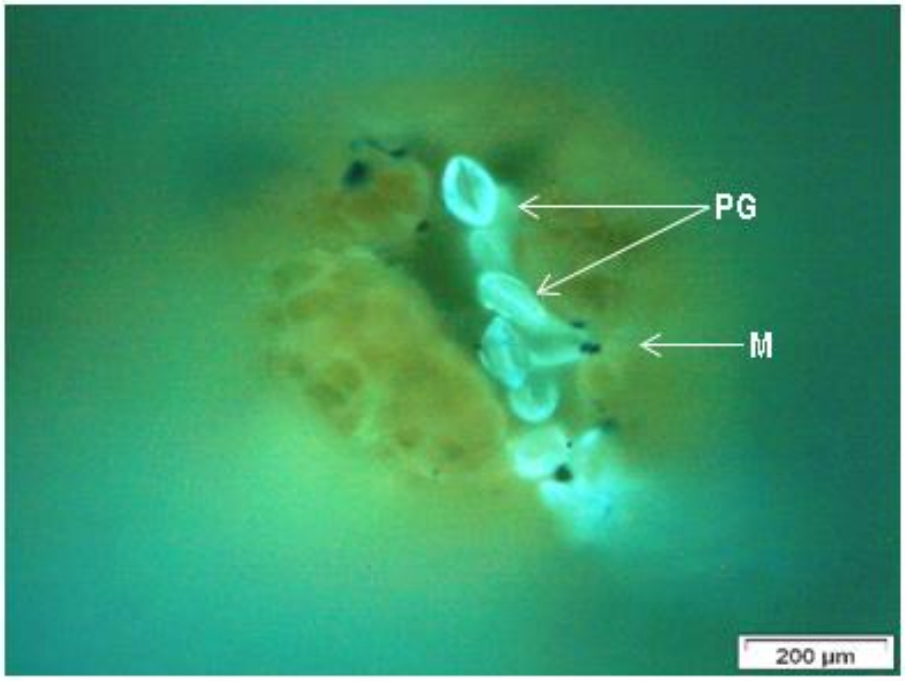


Figure 4. 4 Typical pollen accumulation in the micropyle of *Encephalartos latifrons* after pollination. PG = Pollen grain and M = Micropyle.

4.4.4.2 Pollination efficacy at different times of the day

Afternoon pollination in *E. altensteinii* resulted in significantly higher seed germination than morning pollination ($F_{1,\ 12}$ = 6.1510, $p < 0.05$) (Fig 4.5). However, seed germination was not significantly different between ovules resulting from dry or the wet pollination methods ($F_{1,\ 12}$ = .56249, $p > 0.05$) (Fig 4.5).

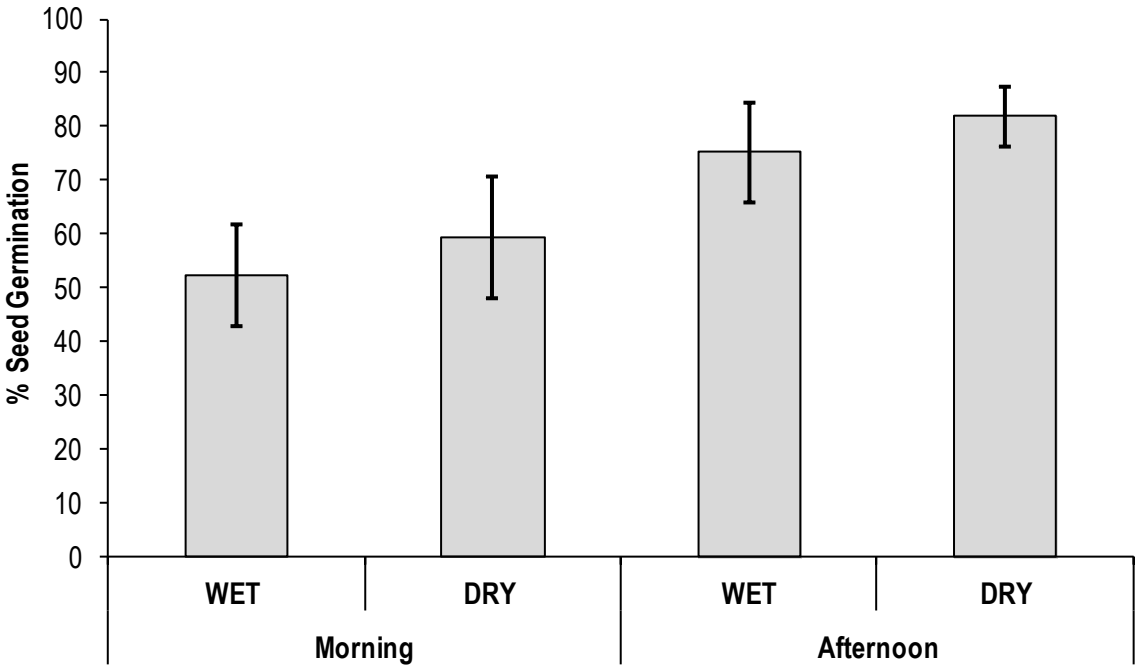


Figure 4. 5 Effects of pollination time and pollination method on *Encephalartos altensteinii* seed germination. Bars represent the Mean (±SE) of seed germination at 30 weeks after sowing 120 seeds per treatment.

4.4.4.3 In vivo pollen present in the dry and wet artificial pollination methods

In vivo pollen germination was observed in both *E. latifrons* (Fig 4.5) and *E. altensteinii* in the sectioned nucellus. Pollen germination in dry pollinated *E. latifrons* ovules was significantly greater than in ovules pollinated using the wet pollination method ($\chi^2 = 11761.20$, $p < 0.001$, $n = 120$). However, in *E. altensteinii* there was no significant difference between cones pollinated with either the dry or wet method ($\chi^2 = 0.50$, $p > 0.001$, $n = 210$).

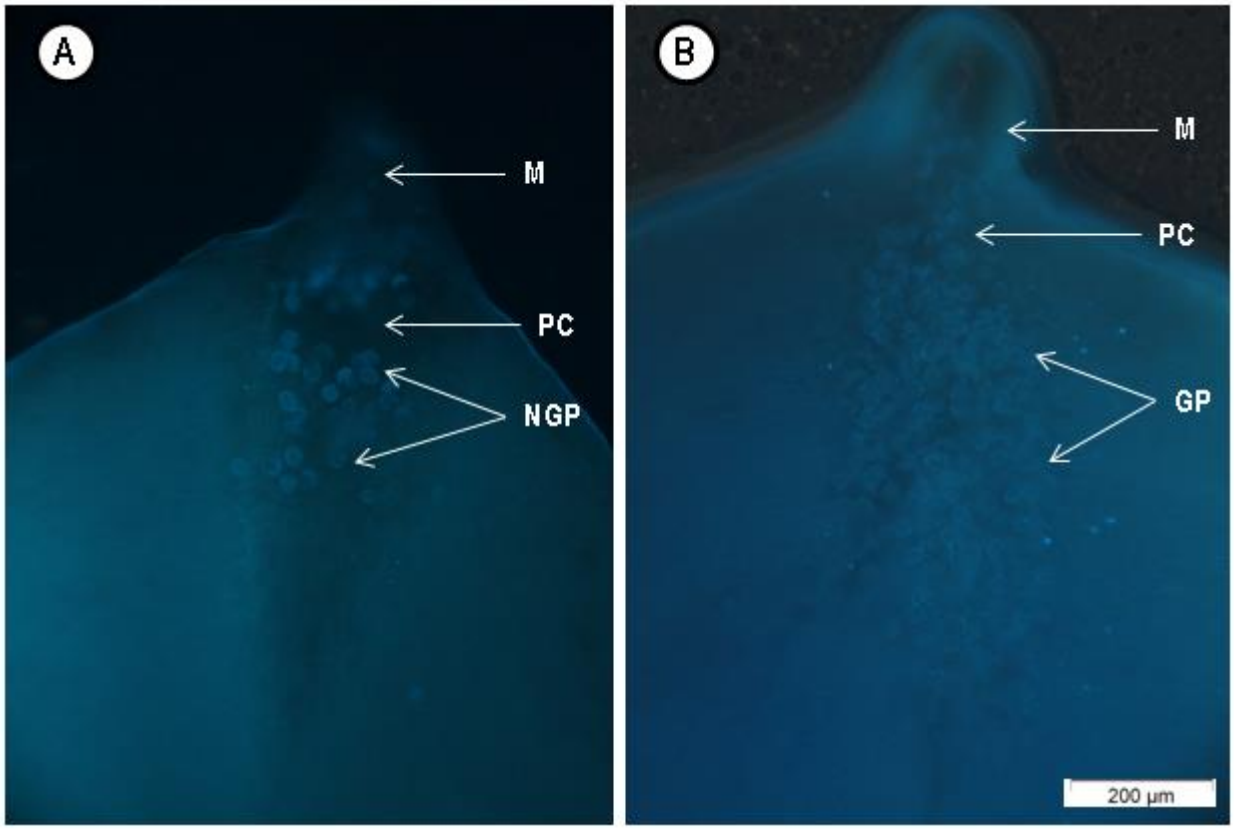


Figure 4. 6 Accumulated pollen in the pollen chamber of sectioned nucellus of *Encephalartos latifrons,* 20 days after pollination. M= micropyle; PC = pollen chamber; NGP = non germinated pollen and GP = Germinated pollen. (A) Wet pollination method with pollen but no germination (B) Dry pollination method with germinating pollen.

4.4.4.4 Embryo presence and seed germination between the dry and wet pollination methods

There was no significant difference between seed check for embryos presence prior to seed sowing and maximum seed germination at 30 weeks of both species ($F_{1, 58}$= .66723, $p > 0.05$) (Fig 4.7). Overall, seed germination and embryo presence was greater in *E. altensteinii* than *E. latifrons*

regardless of dry or wet pollination ($F_{1, 58}$= 14.132, $p < 0.05$). The Post Hoc Tukey HSD test showed that wet pollination in *E. latifrons* resulted in significantly lower seed germination and fewer embryos ($p < 0.05$) compared to all other treatments in both species (Fig 4.7 & 4.8).

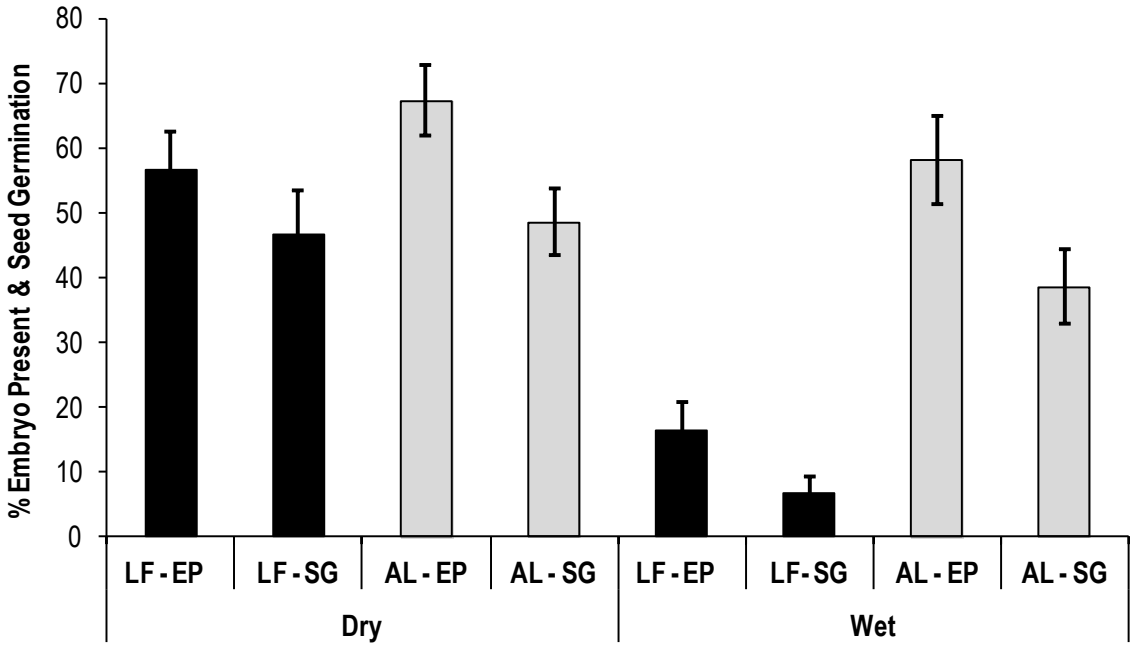


Figure 4. 7 Effects of the dry and wet artificial pollination method on seed viability in *Encephalartos latifrons* (LF = black) and *E. altensteinii* (AL = grey). Seed viability was measured through either the presence of an embryo or the proportion of seed that germinated after 30 weeks (sown after eight months warm stratification). EP = embryo presence and SG = seed germinated. Dry LF- EP (*n* = 180) and SG (*n* = 150), Dry AL- EP (*n* = 540) and SG (*n* = 150). Wet LF-EP (*n* = 180) and SG (*n* = 120), AL -EP (*n* = 480) and SG (*n* =180). (Means SE±).



Figure 4. 8 Seed germination of *Encephalartos latifrons* pollinated with different artificial methods at 30 weeks. A = wet pollination and B = dry pollination.

4.5. Discussion and Conclusions

The observations and experiments carried out in this chapter were designed to investigate if methods of artificial pollination contribute to low seed germination in *E. latifrons* at KNBG. Aspects of artificial pollination, specifically the effectiveness of different pollination methods and the timing of artificial pollination on seed set were studied.

In Chapter Three it was demonstrated that *E. latifrons* pollen was sensitive to desiccation after wetting compared to *E. altensteinii*, and that this was the cause of low pollen germination. In this chapter, low pollen germination in the nucellus as a result of wet pollination is identified as having a significant impact on seed viability, and subsequent low seed germination in *E. latifrons* at KNBG (Fig 4.7 & 4.8). This is contrary to reports of pollination of wild cycads, where wet artificial pollination methods results in greater than 60% seed germination (Fletcher, 2008). Given that it is well documented that pollen is affected by environmental factors (Stanley & Linskens, 1974; Stone *et al.*, 1995 and Acar & Kakani, 2010), this provides additional evidence that environmental conditions influence pollination, and that conditions in KNBG can affect seed set in *E. latifrons*.

These results need to be discussed in the context of our hypotheses, but also within the general understanding of cycad pollination biology and artificial pollination.

Sporophylls opening in relation to pollination droplet formation and cone thermogenesis

During pollination, ovulate sporophylls become loose indicating cone receptivity (Tang, 1986b; Grobbelaar, 2002). Results show that the period of sporophyll separation in *E. latifrons* ranged from

33 – 75 days, and was significantly longer than in *E. altensteinii* where sporophylls were loose for 14 - 25 days ($p < 0.05$). Both periods were longer than the four day period reported in *E. inopinus* (Grobbelaar, 1996). During this period, pollination droplet formation in both *E. latifrons* and *E. altensteinii* was observed predominantly in the afternoon to early evening. However, there was considerable difference in the proportions of ovules where pollination droplets were present, with a maximum of 3% of ovules in *E. latifrons* and a maximum of 14% in *E. altensteinii* (Fig 4. 1).

This is contrary to findings reported in *Cycas rumphii*, *Ceratozamia robusta*, *Dioon spinulosusm*, *Macrozamia lucida* and *Zamia pumila*, where pollination droplets are predominantly formed in the early morning and in *E. ferox* where droplets form only in the mid-morning (Tang, 1993). Yet, in *E. villosus* droplets occur throughout the day (> 50%) and peaked in the mid to late morning (> 90%). This means that droplets were present during pollen cone thermogenesis from 15h00 in the afternoon to 21h00 when pollinators were active (Donaldson, 1997). This period of co-occurrence of pollination droplets in ovulate cones and increased pollen cone temperature was also observed at different periods of the day in *C. rumphii* and in *M. robusta*, where the presence of droplets and thermogenesis overlapped in the morning to midday (Tang, 1987b; 1993). During this period when both the pollination droplets and thermorgenesis were recorded, insect pollen vectors are more active (Donaldson, 1997), and therefore, pollination potentially more likely to occur. Given that in both *E. latifrons* and *E. altensteinii* pollination droplets formation and thermogenesis are prevalant in the afternoon (Fig 4.1 & 4.2), this is possibly the the optimal period for artificial pollination for both species. This is further corroborated by significantly greater seed germination results from the afternoon pollination treatments, compared with the treatements where pollination occurred in the morning in *E. altensteinii* ($p < 0.05$) (Fig 4. 5).

Although *Encephalartos* species seem to have variable periods that sporophylls remain loose, the long period that sporophylls remain loose in *E. latifrons* at KNBG is an anomaly. It may be related to environmental conditions at KNBG since in the wild *E. latifrons* sporophylls are reported to remain loose for a much shorter period of two weeks (Fletcher, *Per Com*). Furthermore, no pollination droplets were observed in *E. latifrons* after 27 days following the sporophylls becoming loose. This suggests that pollination droplets do not continually form after a period of time, regardless of the sporophylls remaining loose.

Efficacy of pollination methods

Results show that seed set after dry artificial pollination is significantly higher than after wet pollination in *E. latifrons* ($p < 0.05$); this is contrary to the recommendation of the use of the wet pollination method for *Encephalartos* (Grobbelaar, 2002; Whitelock, 2002). However, dry pollination was also reported to result in 75% more seed germination than the wet method in a studyon *E. ferox* (Tang, 1986b). These high seed viability results using dry pollination on *E. ferox,* are also supported by Grobbelaar (2002). This means that it is probable that *E. ferox* pollen is also sentivitive to desiccation after wetting as demostrated in *E. latifrons*. This raises question that, what other cycads species are negatively affected by wet pollination. These question can be answered by a species wide test of pollen viability after wetting and drying.

It was also observed that pollen delivery to ovules after wet pollination was significantly greater compared to the dry pollination method tested in *E. altensteinii* ($p < .0001$). Furthermore, the wet pollination method in *E. altensteinii* was significantly better at delivering pollen to different parts of

the cone (top, middle and bottom) compared to the dry method (Fig 4.3). Despite these apparent differences, more direct tests based on pollen germination, presence of embryos and seed germination failed to show any significant difference between dry and wet pollination methods in *E. altensteinii* ($p > 0.05$) (Fig 4. 7). This means that *E. altensteinii* at KNBG, which is pollinated predominantly in autumn (April and May), is not negatively affected by either method of pollination. In contrast, results from *E. latifrons* show that pollen germination and seed germination using dry pollination is significantly greater than from wet pollination at KNBG ($p < 0.05$), corroborating findings from the Chapter Three, where wet pollination showed significantly less pollen germination than in the dry pollination method ($p < 0.05$).

Dry artificial hand pollination in *Encephalartos* is a less risky method as a potential cause of low seed viability in *ex situ* collection. This is in view of the fact that dry pollination mimics natural pollination and does not require any pre-wetting of pollen. In addition, afternoon pollination is also recommended, given that seed germination was greater after pollination in the afternoon than morning pollination. This is also recommended for *E. latifrons* since both species have similar diurnal pattern of pollination droplets formation and thermogenesis.

CHAPTER 5. EFFECT OF SEED STORAGE AND PRE-TREATMENT ON GERMINATION IN SPECIES OF THREATENED AFRICAN CYCADS (*Encephalartos:* ZAMIACEAE)

5.1. Abstract

The critically endangered cycad *Encephalartos latifrons,* experiences low seed viability in *ex situ* living collections at Kirstenbosch National Botanical Garden (KNBG). This is the largest *ex situ* collection of *E. latifrons* and conservation programmes are negatively impacted by low seed set. This study tested the hypothesis that seed storage factors and the lack of appropriate pre-germination treatment are the cause of low seed germination.

Seeds derived from artificially pollinated cones were subject to different treatments to test the effects of storage period (six, eight and 12 months), storage conditions (ambient temperature and RH or 15 °C / 15% RH for eight months) and pre-treatment (scarification with or without soaking in GA_3 for 48h or 96h). For comparison, treatments were repeated on *E. latifrons* and *E. altensteinii,* a related species with high seed viability of > 60%.

Maximum seed germination after dry storage for six months in both species was significantly lower compared to seed stored for eight and 12 months (*ANOVA* $p < 0.05$). *Encephalartos latifrons* seed stored for eight months had a mean time to germination (MTG) of 19 weeks whereas those stored for six months had an MTG of 15 weeks. Although maximum seed germination at 30 weeks, was significantly lower in seeds stored for six months compared to those stored for eight months

(*ANOVA* $p < 0.05$). However, seed storage under controlled or ambient conditions had no impact on presence of embryos, seed MTG, maximum germination (*ANOVA* $p > 0.05$), and t_{G50} (time taken for 50% of seeds to germinate). Furthermore, pre-treatment of seed had no impact on maximum seed germination or MTG (*ANOVA* $p > 0.05$) or t_{G50}. Dry seed storage for 12 months increased MTG by 80% compared to eight month storage control period used at KNBG. *Encephalartos latifrons* and *E. altensteinii* has similar seed response to storage period, environmental conditions and pre-treatments similar. Furthermore, seed germination vigour is enhanced by > 8 months dry seed storage.

Key words: *Encephalartos latifrons; E. altensteinii; seed storage; GA_3 pre-treatment; mechanical scarification, seed germination*

5.2. Introduction

The background relating to poor seed viability in *E. latifrons* was outlined in Chapters One and Three. Although Chapters One and Three identified the wetting and subsequent drying of pollen as one of the factors that could influence poor seed viability, this does not rule out other factors associated with seed germination. This chapter covers a series of experiments designed to test whether methods of seed storage and seed treatment, prior to germination, may also affect the overall viability of *ex situ* seeds.

Seed development and dormancy in the Cycadales is not well understood, especially because cycad seed is diverse with regards to size, seed coat thickness and development rate (Dehgan & Yuen, 1983; Tang, 1987; Osborne, 1988; Norstog & Nicholls, 1997; Grobbelaar, 2002; Whitelock, 2002). After a lengthy and complex zoodigamous pollination process lasting several months (Norstog, 1993), cycad ovules are retained in ovulate cones for several more months until the cone disintegrates and seeds are dispersed (Chamberlain, 1935). Dyer (1965) reported that embryo development in *Encephalartos* occurred prior to cone disintegration but Osborne (1986) later reported that embryo development coincides with cone disintegration. Nevertheless, both authors agreed that seeds need a period of embryo development before germination. Within the genus *Encephalartos*, the duration that cones are held on the plant after pollination varies from as little as three months in *E. cycadifolius*, to six months in species such as *E. natalensis* (Giddy, 1984), and 12 months in *E. arenarius, E. longifolius* and *E. manikensis* (Xaba, unpublished data). Furthermore, in *E. gratus, E. natalensis* and *E. villosus* seed germination can take from four to six months after cone shedding (Woodenberg *et al.*, 2007), indicating that either embryo development is incomplete at

the time of seed shed or there is some form of dormancy. In either case, the lengthy and complex seed development process in cycads, when compared to most other seed plants, poses an increased risk of reproductive failure.

During the lengthy seed development process in cycads, seeds undergo non deep simple morphophysiological dormancy (ND-MPD), a condition which occurs in many seed plants from temperate regions and requires time after seed shed for embryo development (Baskin & Baskin, 1998). Reported periods for embryo development, when seed would need to be stored, varies in different genera (Calonje *et al.*, 2011), from one month to two years with some extreme records of up to six years (Witte, 1977; Giddy, 1984; Vorster, 1995; Grobbelaar, 2002; Whitelock, 2002). *Encephalartos* seed can tolerate storage for two years but generally, a six month period is recommended (Grobbelaar, 2002). On the other hand, *E. transvenosus* and *E. manikensis* do not appear to require any additional time from seed shed for the embryo to develop - germination may occur while seeds are still enclosed in the cone (Vorster, 1995; Grobbelaar, 2002). In addition, a similar trend was also observed to be inconsistent in *E. altensteinii*, *E. arenarius*, *E. natalensis* and *E. longifolius* (pers. obs.). Therefore, it unclear how what storage period is required in *Encephalartos* species, or whether different species require different periods of storage. In addition, reports on environmental conditions for seed storage of cycads are also varied, from as low as 5°C to ambient temperatures, in moist or dry storage conditions (Witte, 1977; Dehgan & Schutzman 1989; Grobbelaar, 2002). At KNBG, the sarcotesta is removed and the seed is dusted with fungicide before storage for eight months at dry ambient room temperature. Given that there are so many varied reports on cycad seed storage periods and environmental conditions, it is not possible to determine optimal conditions for seed storage in *E. latifrons* at KNBG..

After a period of seed storage to allow for embryo development, most cycad growers report success with seed germination in a heated environment from 27°C – 35°C (Forsyth and Van Staden, 1983; Grobbelaar, 2002; Jones, 2002; Whitelock, 2002). During this period, germination can be inhibited if the sarcotesta is not removed (Smith, 1978; Forsyth and Van Staden, 1983). Even though the embryo is fully developed, some cycad species with a thick sclerotesta are still mechanically hindered from germination as a result of the thick seed coat. Cycad species with a thick sclerotesta, which stops water from entering (physical dormancy), include *C. revoluta, C. circinalis, Z. floridana; Z. furfuracea* (Dehgan, 1996) and these might be examples of MPD in cycads. Scarification breaks physical dormancy by allowing the rapid exchange of water and oxygen (amongst other factors) with the embryo and megagametophyte (Salisbury and Ross, 1978). Breaking physical dormancy of some cycad species has been achieved by chemical scarification with sulphuric acid (H_2SO_4) (Dehgan and Schutzman, 1983; Frett, 1987; Dehgan, 1999 and Zarchini *et al.*, 2011), and also by treating the seed with boiling water (100 °C) for 1h and with 25% H_2SO_4 for 2h (Zarchini *et al.*, 2011). Mechanical scarification (sanding down the coronula at the apex of the seed kernel) of the hard seed kernel in *Dioon merolae* (Perez-Farrera *et al.*, 1999) and *Encephalartos* is reported to enhance germination (Jones, 2002). Since other cycads benefit from pre-treatment by scarification, it raises the question whether scarification in *E. latifrons* could also improve the low seed germination at KNBG.

Pre-treatment of cycad seed with growth regulators such as gibberrelic acid (GA_3) is also reported to promote rapid embryo growth and germination in cycads (Dehgan, 1983). Different periods of treatment with GA_3 (24h or 48h) in *Encephalartos* are reported to also improve time to seed germination and result in uniform seed germination (Dehgan, 1999). In other cycad species, pre-

treatment consisting of a combination of seed scarification and growth regulators result in high seed germination results - in *Z. floridana*, scarification with H_2SO_4 and soaking for 24h in GA_3 (1000pm) resulted in 100% germination within four weeks (Dehgan and Johnson, 1983). Similar results were also obtained in *Z. furfuracea* and *Macrozamia communis* (Ellstrand *et al.*, 1990). *Cycas revoluta* showed improved seed germination by 15% with half an hour H_2SO_4 scarification compared with the control. In the same study, GA_3 was applied at 500, 1000 and 5000 pm for 12h on cracked seed kernels, resulting in low seed germination compared to the control (50%) (Frett, 1987). The success of scarification alone or a combination of scarification and growth regulator treatment in some cycad species raises questions about whether these treatments could improve germination of *E. latifrons* seed from KNBG.

This chapter tests four treatments: the duration of storage; environmental conditions during storage; the effects of pre-treatment by scarification; and lastly the effects of scarification and GA_3 treatment on seed germination.

5.3. Materials and Methods

5.3.1. Seed storage

5.3.1.1 Effect of seed storage duration on germination

Hand pollination was carried out at KNBG on *E. altensteinii* and *E. latifrons* ovulate cones using the dry pollination method (Grobbelaar, 2002). A hand operated pneumatic pump (rubber ball) and attached nozzle was used to squirt 5g of fresh pollen between the loose sporophylls (receptive), three times every second day. Seeds were harvested after cones naturally disintegrated (*E. altensteinii* October – December; *E. latifrons* November - December). The sarcotesta was cleaned off and seeds subjected to a floating test where floating seed kernels were regarded as non viable and sinkers as potentially viable. Potentially viable seed was smeared with Efekto Fungi-Nill 500 WP® Captab (Dicarboximide) and placed in a breathable plastic mesh bag. Seeds were then stored under dry conditions at ambient temperature for six, eight or twelve months. After storage, seeds were sown in an open air germination bench at 28°C, with silica sand as the germination medium. Seeds were watered every day and germination was scored once a week for 30 weeks. Germination was defined as the point when the radicle was pushed out from the coronula. The numbers of seeds used to test storage in *E. altensteinii* were: six months (n = 210); eight months (n = 180) and 12 months (n = 90). In *E. latifrons*: six months (n = 240) and eight months (n = 180). Since *E. latifrons* cones infrenqeuntly, there were not enough cones and seed to conclude the 12 month seed storage experiments.

Mean time to germination (MTG) is an index of seed germination vigour and was calculated using the formula:

$$MTG = \sum nt / \sum n$$

Where *n* is the number of germinated seeds at time t expressed in weeks. The MTG was calculated for all seeds until 30 weeks (end of the experiment) and analysed using factorial *ANOVA* in Statistica7®. The mean accumulated seed germination at 30 weeks was also analysed using factorial *ANOVA* in Statistica7®.

Observed seed germination data was fitted using a binomial logistic model of the form:

$$P(G) = \frac{G_\infty}{1 + \exp(-(t - t_{G50}) / \delta_t)}$$

where *P* (G) is the predicted proportion of germinated seeds at time *t*, G_∞ is the estimated maximum proportion of seed germination (i.e. proportion of seed germination approached if the experiment had gone on), t_{G50} is the time taken to reach 50% of G_∞ and δ_t is the steepness parameter of the logistic ogive. The model was fitted by minimizing the negated binomial log-likelihood function.

5.3.1.2 Effects of environmental storage conditions on seed germination

Seeds derived from artificially pollinated cones (dry method) were treated as above and placed into two storage facilities: one under controlled dry and cool conditions (15% RH at 15°C), and a second at ambient environmental conditions at KNBG for eight months. To observe embryo presence, a non-destructive method was initially undertaken using the Faxitron® X-ray machine set at 22Kv, 0.3mA (20s). X-rays were found to be informative only when applied to mature seed with fully developed embryos after more than 10 months in storage (Fig 5.1). Since experimental seed were stored for eight months, a more accurate estimate of embryo formation was determined by seed dissection. Seeds were also sown in similar conditions as in the seed storage test. Seed samples for the embryo presence test comprised *E. latifrons* seed (*n* = 180) and *E. altensteinii* (*n* = 540). Seed samples used for the seed germination test comprised *E. latifrons* (*n* = 180) and *E. altensteinii* (*n* = 180) seed. The presence of embryo and seed germination was analysed using factorial ANOVA in Statistica7®. Scoring for seed germination, fitting of logistic models and calculation of t_{G50} and MTG followed the same procedure as in the seed storage test.

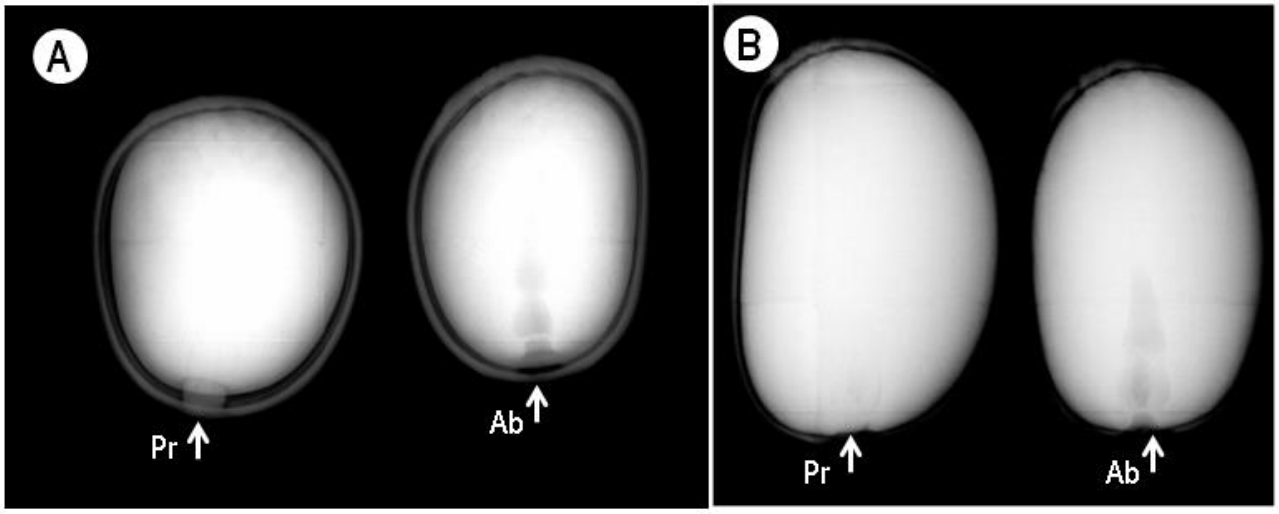


Figure 5. 1 X- rayed *Encephalarto*s seed for embryo presence. A = *E. latifrons* Pr = present embryo (full seed), Ab = absent embryo (empty embryo chamber). B = *E. altensteinii.*

5.3.2. Effect of seed pre-treatment on germination

Encephalartos altensteinii and *E. latifrons* female cones were pollinated and the seed treated as above and stored for eight months in dry controlled (15% RH at 15°C) or ambient conditions. In addition to an untreated control, there were three germination treatments: i) scarification by sanding down the projecting coronula (Fig 5.2); ii) scarified seed soaked in GA_3 (1000 pm) for 48h (Dehgan and Schutzman, 1983); iii) scarified seed soaked in GA_3 (1000 pm) for 96h. Seed were sown in similar conditions as in the seed storage test. Seed sampling was as follows: in *E. latifrons*: control (*n =180*); scarification (*n* =180); scarification & GA_3 for 48h (*n* =180) and scarification & GA_3 for 96h (*n* =180) *and* seed sampling for *E. altensteinii*: control (*n =150*); scarification (*n* =180); scarification & GA_3 for 48h (*n* =150) and scarification & GA_3 for 96h (*n* =150). Six replicates of *E. altensteinii* were removed because they were from one tree that consistently had no seed

germination. Scoring for seed germination, fitting of logistic models and calculation of t_{G50} and MTG followed the same procedure as in the seed storage test.

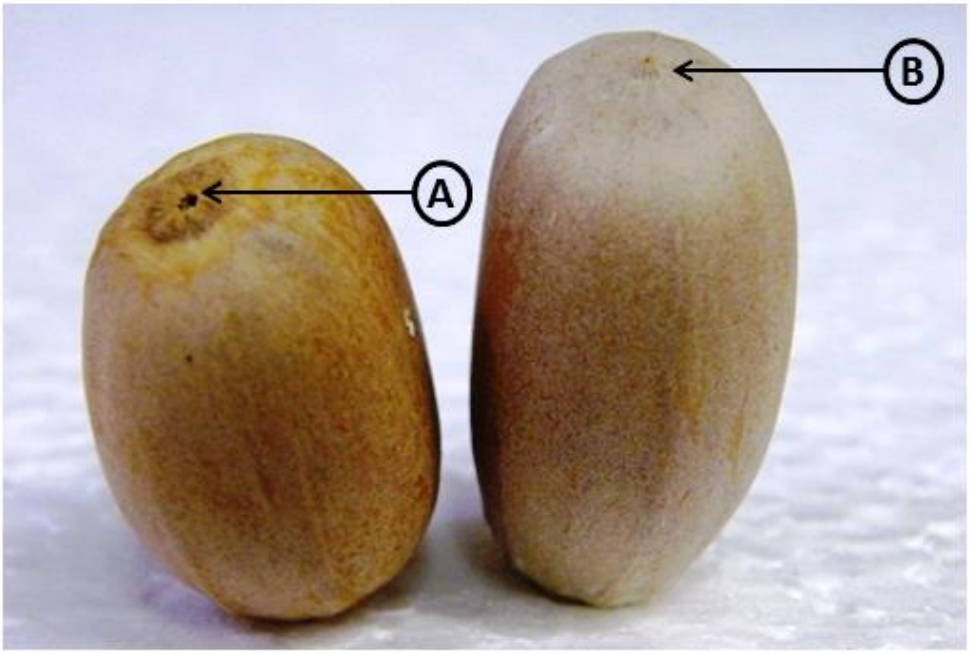


Figure 5. 2 *Encephalartos latifrons* seed. A = mechanically scarified and B = control with coronula unscarified.

5.4. Results

5.4.1. Seed storage

5.4.1.1 Effect of seed storage on germination

The duration of seed storage in *Encephalartos* had variable impacts on t_{G50} (Fig 5.3). Maximum seed germination at 30 weeks, was significantly different between seed stored for six and eight months in *E. latifrons* (*ANOVA* $F_{1,\ 16} = 20.392$, $p < 0.\ 05$) (Fig 5.4). Furthermore, the MTG was significantly longer in *E. latifrons* seed stored for six months than when stored for eight months (*ANOVA* $F_{1,\ 8} = 1.323$, $p < 0.05$) (Table 1.). *Encephalartos altensteinii* seed storage for 12 months resulted in the fastest germination, reaching t_{G50} at 6 weeks and reaching MTG at 12 weeks. The MTG from seed stored for six months was significantly higher than when stored for eight or 12 months (*ANOVA* $F_{1,\ 14} = 10.438$, $p < 0.05$) (Table1). Maximum seed germination at 30 weeks was consistently higher for both *E. altensteinii* and *E. latifrons,* which exhibited similar responses to storage at six or eight months (*ANOVA* $F_{1,\ 28} = 0.01798$, $p > 0.\ 05$) (Fig 5.4).

Table 5. 1 Effects of *Encephalarto*s seed storage period on mean time to germination (MTG).

Species	Treatment Months	MTG	Std.Dev.	Std.Err
Encephalartos latifrons	**6**	15	4.62	1.75
	8	19	3.56	1.07
E. altensteinii	**6**	21	4.52	1.71
	8	13	1.96	0.80
	12	12	3.41	1.71

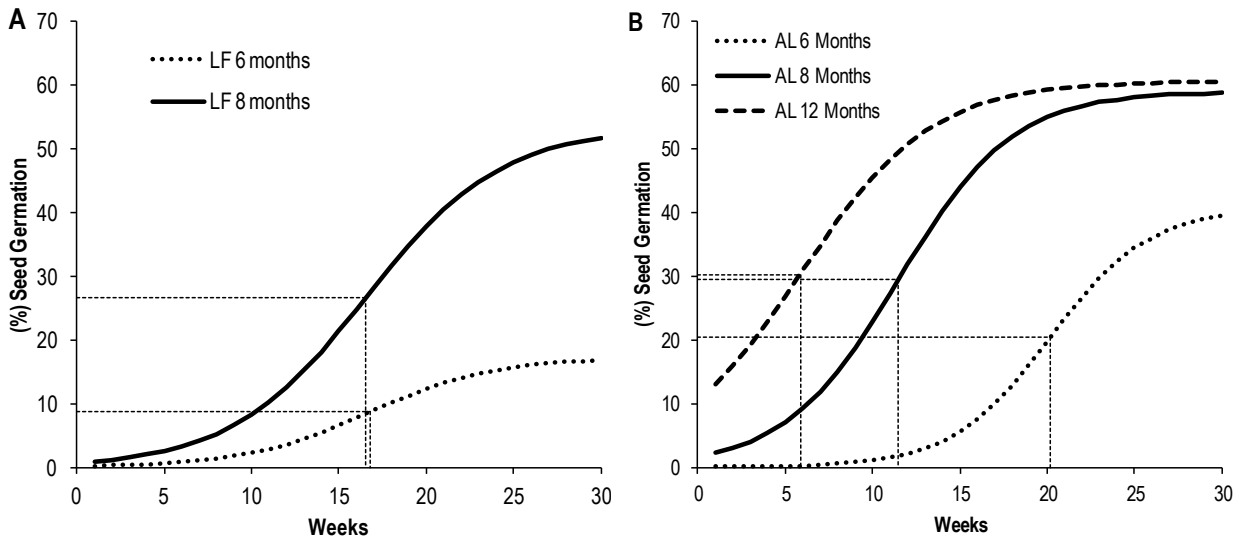


Figure 5. 3 Effects of storage time on seed germination in *Encephalartos*. Lines represent seed germination fitted into a binomial logistic model for A = *E. latifrons* (LF) and B = *E. altensteinii* (AL). Dashed straight lines denote time taken to reach 50% seed germination (t_{G50}). Data represent results for *Encephalartos altensteinii* stored for six months (*n* = 210); eight months (*n* = 180), or 12 months (*n* = 90) and *E. latifrons* for six months (*n* = 240) or eight month (*n* = 180).

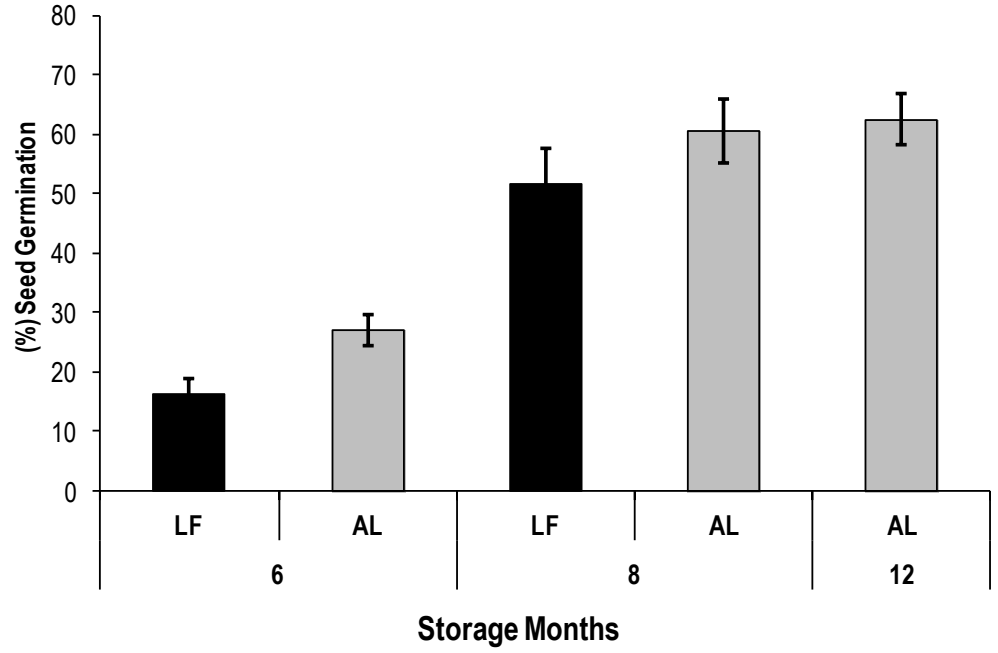


Figure 5. 4 Effect of storage duration on seed germination of *Encephalartos* seeds at 28°C. LF = *E. latifrons* and AL = *E. altensteinii*. Graph represents seed germination with mean SE ± at 30 weeks for *E. altensteinii* stored for six months (*n* = 210); eight months (*n* = 180); 12 months (*n* = 90). In *E. latifrons* seed stored for six months (*n* = 240) and eight months (*n* = 180).

5.4.1.2 Effects of storage environment on seed germination

The conditions under which *Encephalartos* seed were stored had no impact on MTG and t_{G50} (Fig 5.5 & Fig 5.6). *Encephalartos latifrons* seed stored in ambient conditions reached t_{G50} at 18 weeks with an MTG of 13 weeks, compared to seed under controlled conditions which reached t_{G50} at 17 weeks but had an MTG of 17 weeks. *Encephalartos altensteinii* seed stored in ambient conditions reached t_{G50} at 16 weeks and had an MTG of 16 weeks, compared to controlled conditions which

reached t_{G50} at 16 weeks and had an MTG of 15 weeks (Fig 5.5 B). Embryo presence was not statistically different in seed stored in controlled and ambient conditions for either species (*ANOVA* $F_{1, 28} = 0.50201$, $p > 0.05$). Mean time of germination was not significantly different in seed stored in controlled and ambient conditions for either species (*ANOVA* $F_{1, 8} = 1.323$, $p > 0.05$). Furthermore, maximum seed germination at 30 weeks, was not significantly different between ambient and control environmental conditions, and in both *E. latifrons* and *E. altensteinii* (*ANOVA* $F_{1, 28} = 0.85477$, $p > 0.05$) (Fig 5.6 A &B)

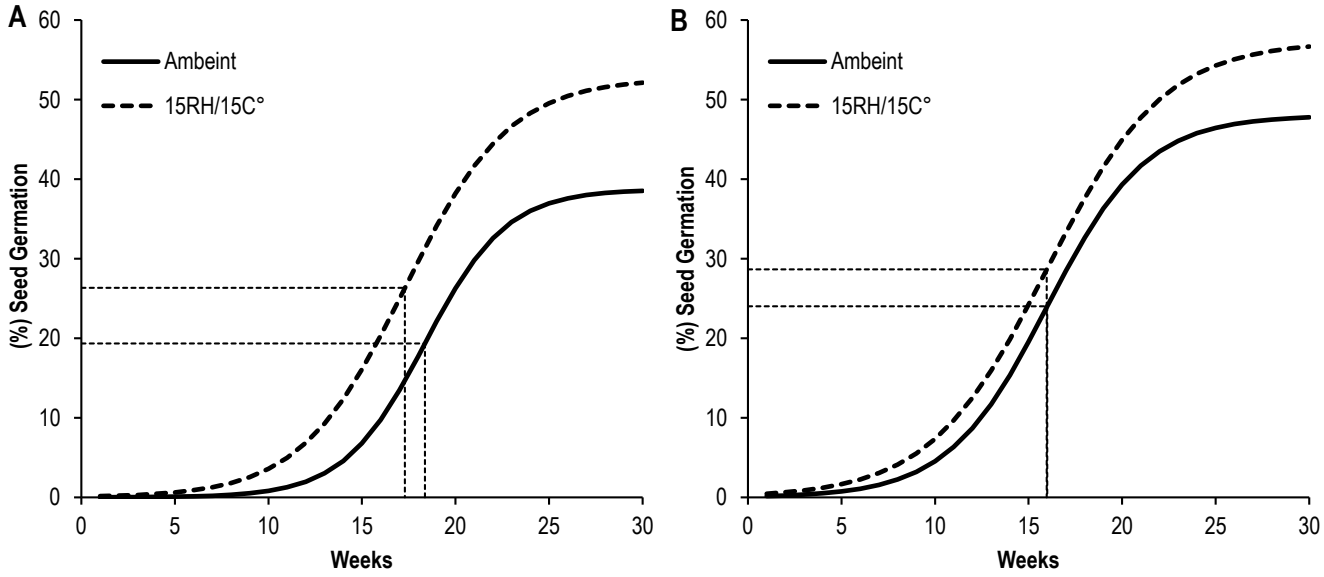


Figure 5. 5 Effects of storage conditions on seed germination in *Encephalartos*. Lines represent seed germination fitted into a binomial logistic model for A = *E. latifrons* (LF) and B = *E. altensteinii* (AL). Dashed straight lines denote time taken to reach 50% seed germination (t_{G50}). Results represent seed stored for eight months from *E. latifrons* (n = 180) and *E. altensteinii* (n = 180).

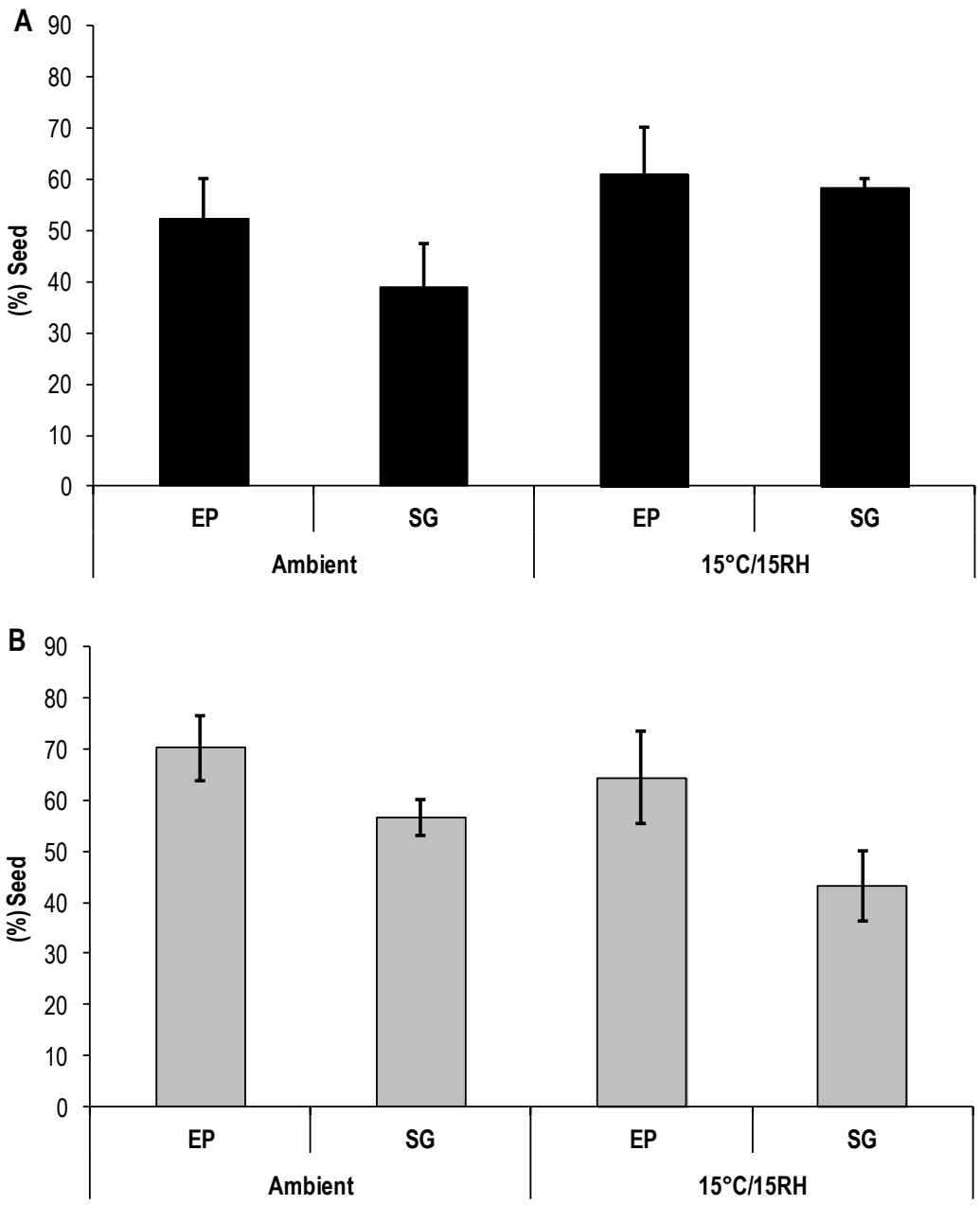


Figure 5. 6 Effects of storage conditions on *Encephalartos* seed embryo presence and seed germination, after eight months in storage at ambient room temperature and at 15°C with 15% Relative Humidity. Graph represents mean SE±. for *Encephalartos latifrons (*A) and *E. altensteinii* (B). EP = embryo presence in *E. latifrons (n =180)* and in *E. altensteinii (n = 540)*. SG = seed germination at 30 weeks for *E. latifrons (n =180)* and *E. altensteinii (n = 180)*.

5.4.2. Effect of seed pre-treatment on germination

Seed germination pre- treatments in *Encephalartos* had no impact on the t_{G50} (Fig 5.7). However, MTG in *E. latifrons* was significantly higher than *E. altensteinii* (*ANOVA* $F_{1, 36}$ =17.775, $p < 0.05$) (Table 2). Even so, effects of pre- treatments on both species, was not significantly different from each other (*ANOVA* $F_{3, 36} = 0.106$, $p > 0.05$) (Fig 5.7). Furthermore, maximum seed germination at 30 weeks of seed pre-treatments did not result in any significant difference between species (*ANOVA* $F_{1, 36} = 2{,}278$, $p > 0.05$) (Fig 5.8 A &B).

Table 5. 2 Effects of *Encephalarto*s seed pre-treatment on mean time to germination (MTG).

Species	Treatment	MTG	Std.Dev.	Std.Err
Encephalartos latifrons	**Control**	18	2.49	1.02
	Scarification	18	2.97	1.21
	Scar. GA_3 48 h	17	3.04	1.24
	Scar. GA_3 96 h	18	3.18	1.30
E. altensteinii	**Control**	14	1.89	0.85
	Scarification	13	2.60	1.16
	Scar. GA_3 48 h	14	5.25	2.35
	Scar. GA_3 96 h	14	3.10	1.39

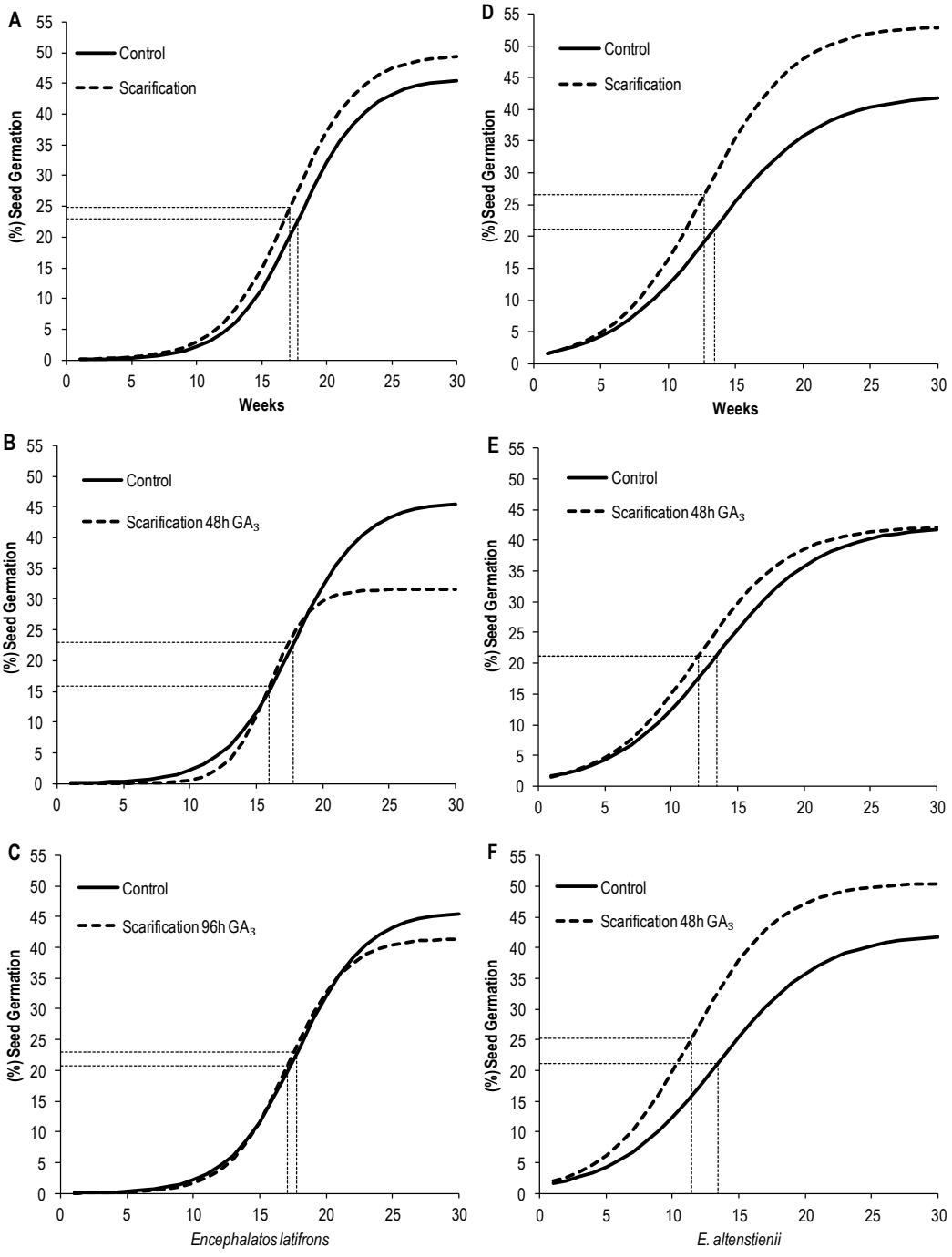


Figure 5. 7 Effects of pre-treatment on seed germination in *Encephalartos*. Lines represent seed germination fitted into a binomial logistic model. Dashed straight small lines denote time taken to reach 50% seed germination (t_{G50}). Data represents results from *E. latifrons* seeds subjected to (A) scarification, (B) scarification followed by 48h half-submerged in GA_3; (C) scarification followed by 96h half-submerged in GA_3. In *E. altensteinii* seeds subjected to (D)

scarification; (E) scarification followed by 48h half-submerged in GA_3, and (F) scarification followed by 96h half-submerged in GA_3. *Encephalartos latifrons* data based on (*n* = 180) seeds per treatment and (*n = 150)* in *E. altensteinii*.

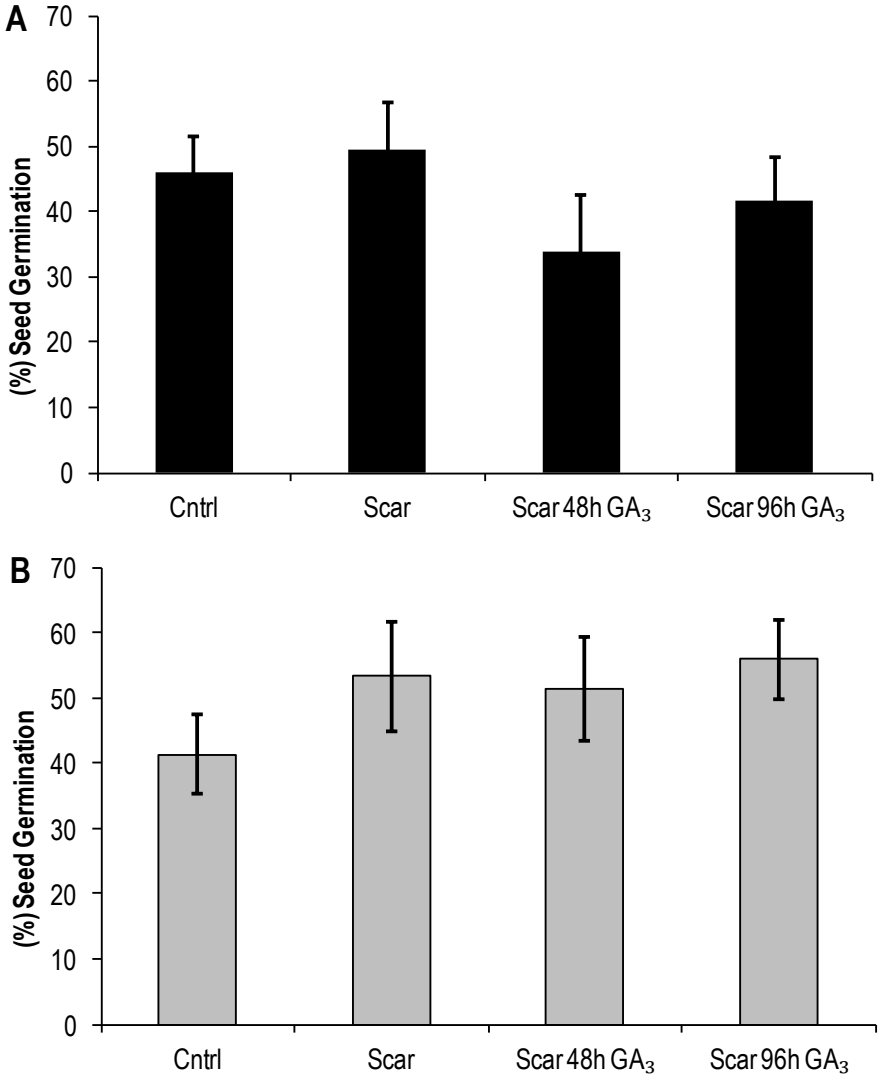


Figure 5. 8 Effects of seed pre-treatment on germination of *Encephalartos* seed after eight months in storage. A = *Encephalartos latifrons* and B = *E. altensteinii*. Cntrl = control, Scar = scarification, Scar 48h half-submerged in GA3 = scarification followed by 48h half-submerged in GA3 and Scar 96h GA3 = scarification followed by 96h half-submerged in GA3. Graph represents germination with mean SE ± at 30 weeks. *Encephalartos latifrons* data based on (n = 180) seeds per treatment and (n = 150) in *E. altensteinii*.

5.5. Discussion and Conclusions

This study investigated whether seed germination in specific *Encephalartos* species was affected by the period of storage preceding germination, the conditions under which storage occurred or the treatment of seeds at the time of sowing. The objective was to determine whether any of these factors could contribute to low seed germination in *E. latifrons* at KNBG. The results show that storing seeds for longer periods prior to germination had significant benefits for overall germination percentage as well as for measures of seed vigour such as MTG and t_{G50}. However, the storage conditions tested here, as well as the pre-treatment of seeds with scarification or GA_3 had no significant benefits. These results need to be discussed in the context of our hypotheses, but also within the general understanding of cycad seed storage, germination and dormancy.

5.5.1. Effect of seed storage on germination

The sexual reproductive period in cycads is prolonged due to the lengthy processes of pollination (Donaldson & Bosenberg, 1995), fertilisation (Styne, 1993) and embryo development (Saxton, 1910 and Chamberlain, 1935). The results presented here appear to corroborate reports that cycad seed, in particular *Encephalartos,* need a lengthy period for embryo development (Saxton, 1910; Sedgwick, 1924; Forsyth and Van Staden, 1983; Dehgan and Yuen, 1983; Giddy, 1984; Tang, 1986, Broome, 2001; Woodenberg *et al.,* 2007). Seed storage period that is > 8 months benifits is embryo maturation in both *E.latifrons* and *E. altensteinii,* by enhancing t_{G50} and maximum seed germination. This means *E. latifrons* and *E. altensteinii* seed germination responds in similar ways to storage periods. The high MTG of 21 weeks in *E. altensteinii* suggests that embryo maturity occurs while seeds are on the heated bench for the period of 30 weeks. These results suggest that

morphological limitations of an under-developed embryo is the cause of seed not releasing dormancy. This result supports reports in *E. cycadifolius* of 5% germination after six months dry storage (Dyer, 1965), which is contrary to the six months seed storage period recommended for *Cycas revoluta* and *C. circinalis* (Dehgan, 1983). In *E. natalensis* however it is reported that one week storage resulted in 40% maximum germination, and three weeks resulted in 60% germination (Forsyth and Van Staden, 1983). These reports of storage periods are not consistent with the long period required by cycad seed classed as ND morphophysiological dormancy (Baskin & Baskin, 1998). It raised the question whether different cycad genera and even species fit into the eight levels of morphophysiological dormancy identified by Baskin & Baskin (2004). Finally, given that the MTG of *E. latifrons* seed stored for eight months has a similar response to *E. altensteinii;* 12 months seed storage in *E. latifrons* could also improve seed germination vigour thus reducing the germination period.

Results from seed storage conditions were not significantly different between ambient room temperature and 15°C with 15% RH in both species (*ANOVA*, $p > 0.05$), and embryo presence was comparable to maximum seed germination (Fig 5.5 & 5.6). This means, ambient storage condition is not negatively impacting seed and seed germination of *E. latifrons.* This also indicates that seed dormancy in *E. latifrons* and *E. altensteinii* was partially removed by a longer storage period. There are variable reports from *E. natalensis*, where moist storage at 5°C - 20 °C resulted in 80- 90% seed germination (Forsyth and Van Staden, 1983). Furthermore, in *C. revoluta* and *C. circinalis* 7°C - 10°C was recommended (Dehgan, 1983). This suggests that different cycad seeds may require a range of storage conditions, possibly because cycads occur in different climatic conditions and cycad seed morphology is diverse (Osborne, 1988). This also raises further questions about whether

cycads fall under a different recalcitrant classification, since higher moisture during storage played an important role in *E. natalensis* but less so in *E. latifrons* and *E. altensteinii*.

5.5.2. Effect of seed pre-treatment on germination

The seed pre-treatments in both *E. latifrons* and *E. altensteinii* did not influence t_{G50}, MTG and overall seed germination was not significantly different (*ANOVA*, $p > 0.05$) (Fig 5.7 and Fig 5.8). This is contrary to investigations in *Zamia integrifolia* and *Z. floridana* (Witte, 1977; Dehgan & Johnson, 1983), *Cycas circinalis, C. revoluta* and *Z. furfuracea*, Dehgan, 1996), where morphophysiological dormancy is reported, with both the immature embryo (morphology) and thick seed coat (physical) restricting germination (Nikolaeva, 1977; Baskin and Baskin, 1998). Given that the sclerotesta in *Encephalartos* does not cause physical dormancy, but seed dormancy result from an undeveloped embryo (morphological dormancy), perhaps this variation indicates an evolutionary trees of young and older cycad seed. Molecular evidence suggests that *Encephalartos* recently diversified in the last 2 MY (Yessoufou *et al.*, 2014), compared to most other species that are reported to have diversified in the last 10MY (Nagalingum *et al.*, 2011 and Salas-Leiva *et al.*, 2013). Therefore, seed with thick sclerotesta and thin sclerotera in relation to embryo maturation needs to be tested to accept or refute this suggested evidence.

Since treatment of *E. latifrons* seeds with GA_3 had no statistically significant effect on germination (Fig 5.6 B and C), these results corroborate reports from *C. revolut*a (Frett, 1987) and *Dioon merolae* (Perez-Farrera *et al.*, 1999), where GA_3 application had no positive effect on seed germination. Perhaps this could be as a result of too high concentrations or the timing of application of GA_3, given that these two factors can influence seed germination negatively (Riley, 1987). On the

other hand, reports in *E. gratus* show that GA_3 treatments resulted in a rapid and uniform germination pattern that yielded 100% germination (Dehgan, 1999). Different responses in cycad seed could be as a result of varied i) seed morphology and size (Osborne, 1988), ii) development and maturation, and iii) period of dormancy (Grobbelaar, 2002 and Whitelock, 2002). These factors are reported to influence production and concentration of GA_3 in seeds in other plants (Bewley and Black, 1994). Perhaps, the standard dosage of 1000pm may be inappropriate for *E. latifrons* and other species, and instead of stimulating germination, it inhibits it. Then again, GA_3 may not be needed in certain cycads species, since not all seed are consistent with ND morphophysiological dormancy, as in the case of *E. latifrons* and *E. altensteinii*. *Encephalartos latifrons* and *E. altensteinii* are consistent with "deep simple morphophysiological dormancy" (DS MPD), given that they only require warm stratification and no GA_3 pre-treatment.

In summary, *E. latifrons* seed response to storage period, environmental conditions and pre-treatments is similar to *E. altensteinii*. These responses are consistent with deep simple morphophysiological dormancy. Finally, storing *E. latifrons* seeds for 12 months under ambient conditions at KNBG could possibly benefit seed vigour.

CHAPTER 6. CONCLUSION AND RECOMMENDATIONS

The aim of this study was to investigate the cause of low seed germination (<10%) in the critically endangered *Encephalartos latifrons* at Kirstenbosch National Botanical Garden (KNBG), that houses the largest *ex situ* collection of this species. The cause of low seed germination in *E. latifrons* at KNBG is unknown (Winter, 2006), but the problem is not unique to KNBG since similar difficulties have been reported in at least one other collection in California, USA (Whitelock, 2002). The low level of seed germination in *E. latifrons* at KNBG is impacting negatively on the conservation programme of this species and requires practical solutions. Besides, cycads are the most threatened group of living organisms, where > 60% of the ca. 300 species are classified as threatened with extinction (IUCN Red List, 2010). Furthermore, cycad seeds are reported to be recalcitrant (Forsyth & Van Staden, 1983; Woodenberg *et al.,* 2007), and therefore cannot be conserved through conventional seed banking techniques. As a result, living plant collections and seed orchards are currently the only viable option for the *ex situ* conservation of cycads (Kluge, 1988).

At the same time, reproduction in cycads is complex and finding practical solutions to poor seed germination is dependent on improving the understanding of key aspects of cycad reproduction and germination. Cycads are dioecious gymnosperms (Chamberlain, 1935), that have a lengthy and complex zoodigamous reproduction process (Norstog & Nicholls, 1997), so it is not easy to separate problems with pollination, from those associated with fertilisation or later seed development. This is

particularly true in *Encephalartos* where ovules reach their full size before pollination and unfertilized ovules are retained in the cone and are externally identical to fertile seeds. Seed germination may be further affected by some form of morphophysiological dormancy (Witten, 1977; Dehgan & Schutzman, 1983; Dehgan, 1996) so that even apparently mature seeds may require pre-germination treatments (scarification and hormones) and a rest period to allow the embryo to mature. This means that seemingly poor germination may result from a number of factors at different stages of the reproductive process.

Based on the relevant literature, a contextual approach was adopted focusing on unique features of cycad reproductive biology, as well as features shared with other gymnosperms and even angiosperms, that generally could explain reproductive failure in seed plants. The key elements of reproduction that were identified as potentially contributing to low seed germination were: i) low pollen viability either as an inherent problem with *E. latifrons* or as result of prolonged storage; ii) inappropriate artificial pollination methods, especially methods that did not deliver pollen at a time when receptive ovules were producing pollination droplets; and finally, iii) poor seed germination linked to inappropriate storage (period, environmental conditions) or germination pre-treatments (including scarification and hormone). Although the focus was on *E. latifrons,* the study included similar tests on *E. altensteinii,* a related species that is more common and occurs in large numbers in collections at KNBG, and therefore more accessible for experimentation, and which does not experience the low levels of seed germination observed in *E. latifrons* at KNBG.

Apart from identifying and taking remedial action in addressing the cause of low seed germination in *E. latifrons,* this study has provided further insights into the reproductive biology of cycads, and

raised compelling questions about pollen, pollination and seed of cycads. Therefore, the implications and questions raised by these results need to be discussed in the broader context of cycad reproductive biology, but also in practical terms, with informed conclusions and recommendations.

6.1. Pollen viability, storage and responses to environmental conditions

Since environmental stresses and age are often cited as the primary sources of pollen viability loss (Stone *et al.*, 1995), this study tested these factors as possible causes of low seed germination in *E. latifrons* at KNBG. Overall results show clearly that pollen wetting, desiccation sensitivity and possibly the narrow germination temperature band contribute to low pollen germination, and the subsequent low seed germination in *E. latifrons*. Key published experiemental studies on cycad pollen corroborate these results. *Encephalartos latifrons* from the wild and KNBG, as well as data for 17 *Encephalartos* species tested from KNBG, were consistent with findings by Osborne *et al* (1992), where pollen retains high viability for three to five years at -15°C, before losing viability. Another study on optimal storage of cycad pollen by Mostert (2000) showed that different *Encephalartos* pollen responds differently to environmental factors. Again, corroborative results in response to temperature showed that in *E. latifrons* and *E. altensteinii* pollen responded differently, where *E. latifrons* ranged from > 15°C to 35°C with optimum at 30°C, and *E. altensteinii* was from > 10°C to 40°C, with optimum at 25°C (*ANOVA* $p < 0.05$). Furthermore, pollen germination in *E. latifrons* that was hydrated and desiccated for 24h and 15 d before testing was significantly lower than in *E. altensteinii* (*ANOVA* $p < 0.05$). It is uncertain what causes these different responses in cycad pollen, as pollen from all *Encephalartos* species pollen share similar morphological characteristics (Dehgan & Dehgan, 1988; Marshall *et al.*, 1989). Furthermore, the *Encephalartos*

pollen wall is reported to contain lipids (Mostert, 2000) that are known to be hydrophobic (water repellent) (Ben-Na'im, 1980). It is not known whether all species possess lipids, and whether the lack of lipids is the underlying cause of *E. latifrons* pollen sensitivity to wetting.

Encephalartos pollen also contains acidic polysaccharides (Mostert, 2000), which are known to be water soluble. Perhaps the combination of these structural components of living cells, or the lack thereof, determines water permeability, or even temperature sensitivity in different cycad pollens. Still, results from the wet pollination method used in the wild (*in situ*) on *E. latifrons* are contrary to KNBG's *ex situ* results, where seed germination is reported to be > 60%. One possible factor that could contribute to these differences is that ovulate cone temperature in *E. latifrons* at KNBG was generally below optimum temperatures for pollen germination - there were only four temperature peaks that reached pollen germination temperature (>23°C) over two months (August and September). In contrast, *E. altensteinii* had 13 peaks over a similar two month duration of receptivity (April to May), and *in vivo* pollen germination of *E. latifrons* was significantly lower than in *E. altensteinii* in wet pollinated ovules (χ^2 $P < 0.001$). Perhaps the environmental differences (temperature and water) may partly explain the differences between the wild and KNBG, as different conditions prevail.

This study supports the position that different *Encephalartos* pollens respond differently to environmental conditions. A possible follow-up study could test *Encephalartos* pollen to determine sensitivity to desiccation after wetting so as to categorise pollen. Furthermore, the presence of lipids in cycad pollen walls and their function in protecting pollen from hydrolysis can also be investigated.

6.2. Artificial pollination and poor germination

Artificial pollination of cycads is widely practised in botanic gardens as well as in private collections. Although there is considerable debate within cycad societies regarding the best methods for pollination, there has been only one published study testing different methods (Tang, 1986b) and there is little understanding of these differences.

The results in Chapter Four showed that the dry pollination method was more effective for *E. latifrons* at KNBG whereas no significant difference between wet and dry methods was observed in *E. altensteinii.* Three aspects of these results were particularly intriguing. Firstly, studies with dyed pollen showed that the wet method delivered more pollen to the micropyle of both species so that poor germination in *E. latifrons* after wet pollination seemed contrary to expectations. Secondly, the dry pollination method resulted in seed set >50% in both *E. latifrons* and *E. altensteinii,* even though there was a significant difference in the period that sporophylls on ovulate cones remained open during pollination droplet formation. Thirdly, regardless of the pollination method used in *E. altensteinii*, afternoon pollination resulted in greater seed set than morning pollination. This implies that the failure of the wet method is specific to *E. latifrons* growing at KNBG, and requires an understanding of what elements of *E. latifrons* biology differ from other species growing at KNBG, or in the wild.

One of the notable observations was that the period when sporophylls on ovulate cones opened ranged from 33 – 75 days in *E. latifrons* at KNBG, which was significantly longer than in either *E. altensteinii* at KNBG (14 - 25 days) or wild populations of *E. latifrons* (estimated at two weeks, C. Fletcher, pers. comm.). The long duration of sporophylls separation (an indicator of receptivity) in

female cones of *E. latifrons* was linked to a very low proportion of ovules with pollination droplets (< 3%) compared to *E. altensteinii* at KNBG or to any other published accounts for pollination droplets (Tang, 1987a; 1993; Donaldson, 1997) where ovules produced > 3% droplets within a 24h period. It is uncertain whether the duration of sporophylls separation and the low level of pollination droplets are influenced by the particular climate at KNBG.

The diurnal cycle of pollination droplet formation in *E. latifrons* and *E. altensteinii* is consistent with reports in other cycads, although the times of occurrence seems to vary in both genera and species (Tang, 1987a; 1993; Donaldson, 1997). Furthermore, pollination droplet formation in both *E. latifrons* and *E. altensteinii* overlapped with pollen cone thermogenesis, which occurred around midday (12h00 – 13h00), and reached a peak in the mid-afternoon (14h00 – 17h00). During this overlaping period, insect pollen vector activity is prominent (Donaldson, 1997), and possibly pollination is more likely. This is supported by results that ovules pollinated in the afternoon (14h00 -15h00) had higher embryo presence and seed germination compared to those pollinated in the morning (10h00-11h00) in *E. altensteinii*. This means that during this period, pollination droplets have a high chance of securing and resorbing pollen into the ovule. Therefore, the same principle could be applied to other species to improve the efficiency of artificial pollination. Still, the question persists, why is there no significant difference in seed set between the wet dry artificial methods in *E. altensteinii*? Given that *E. altensteinii* pollen was not negatively affected by wetting from wet pollination, it is reasonable that dry and wet pollination should not be different. Then the question of pollination droplets possibly being washed away during application of the wet method arises. It is possible that during the exact time of application of the wet pollination, an insignificant number of pollination droplets are washed off. This is supported by the results that wet pollination

has greater pollen delivery, and that *E. altensteinii* pollen viability is not negatively affected by desiccation after wetting. It is likely that the delivered pollen around the micropyle is absorbed by the next diurnal cycle of pollination droplets to eventually germinate in large numbers in the pollen chamber. Although this is contrary in *E. latifrons*, where formation of pollination droplets is low (χ^2 $p < 0.001$), and pollen is negatively affected by desiccation after wetting, negatively impacting on seed set and in turn seed germination (ANOVA, $p < 0.05$).These results also support findings of high seed set in *E. ferox* when using the dry method, compared with the wet pollination method (Tang, 1986b).This further confirms findings in Chapter One, that different *Encephalartos* pollen is affected differently by envrionmental conditions, and for that reason also by the pretreatment of wetting in the wet articial pollination method.

The efficiency of artificial pollination in *ex situ* collections can be improved by closely emulating natural pollination systems, as demonstrated with dry pollination in *E. latifrons* and afternoon pollination in *E. altensteinii* resulting in higher seed viability. This is supported by the assumption that in some *Encephalartos*, insect pollen vectors carry dry pollen to ovulate cones when pollination droplets are prevalent. More investigations on when pollination droplets occur in different species can be undertaken; this knowledge could further inform efficient pollination in cycads.

6.3. Seed storage and pre-treatments to release dormancy

Low seed germination (<10%) was the primary indicator of poor reproductive performance in *E. latifrons* at KNBG, although seed storage and pre-treatment factors were also investigated and eliminated as the main cause of low seed germination. Key findings in this study show that *E. latifrons* cycad seed vigour can be enhanced by increasing storage time to longer than eight months at ambient conditions. But seed pre-treatments (scarification and soaking in GA_3) are not beneficial to seed germination. In *ex situ* collections and amongst private growers there are divergent views on how long to store seed for maximal embryo development and maturation; what environmental conditions are best during seed storage; and what seed pre-treatments improve vigour in seed germination (Dyer, 1965; Giddy, 1995; Grobbelaar, 2002; Jones, 2000; Whitelock, 2000). A number of experimental studies have been carried out on various cycad species (Witten, 1977; Dehgan, 1983; Dehgan & Johnson, 1983; Forsyth & van Staden, 1983; Frett, 1987; Dehgan, 1996; Perez-Farrera *et al.,* 1999), which have also resulted in different conclusions and recommendations for different cycad genera and species. Differences in cycad seed morphology and size (Osborne, 1988) and embryo develeopment period, could be responsible for the varied results, conclusions and recommendations regarding seed handling and pre-treatment. Nonetheless, the synthesis of this study on cycad seed pre-treatments for germination needs to be explored in both a biological and also a practical context.

6.3.1. Duration of seed storage

The results showed that seed for both species stored for eight and 12 months, had significantly higher maximum seed germination (30 weeks) and seed vigour (MTG and t_{G50}) than that stored for only six months (*ANOVA* $p < 0.05$). This means that seed stored for 12 months takes nine weeks less to reach MTG, compared with seed stored for six months. These results suggest that in both *E. latifrons* and *E. altensteinii*, the embryo is still immature at six months, and thus morphologically dormant (Baskin and Baskin, 1998). This also suggests that the period of embryo development and maturity in cycads is variable, in both genera and species (Dyer, 1965; Forsyth & Van Staden, 1983; Dehgan, 1983; Giddy, 1995; Grobbelaar, 2002). This raises the question of why there is such variability in the effects of different seed storage periods in cycads, in particular *Encephalartos*. One factor that could possibly answer this question is the different duration (4 - 13 months) that seeds are held on the cone after pollination (Tang, 1987). Furthermore researchers have found that in *E. transvenosus* and *E. manikensis* seed does not require any additional time from seed shed for the embryo to develop - germination may occur while seeds are still enclosed in the cone (Vorster, 1995; Grobbelaar, 2002). A similar trend in *E. altensteinii*, *E. arenarius*, *E. natalensis* and *E. longifolius* has been observed, although this phenomenon is inconsistent in these species (pers. obs.).

Consequently, different cycads, in particular *Encephalartos*, require different period of seed storage, depending on the period that seeds are held on the cone after pollination. This needs to be investigated, and further comparisons made between cycad species.

6.3.2. Environmental conditions of seed storage

Results show that seed storage at 15% RH/15°C recommend by Ellis *et al.,* (1990) and Roberts & Ellis, (1989) were not significantly different to ambient storage at KNBG (*ANOVA* $p > 0.05$). These results present an interesting conclusion, given that both the tested seed storage conditions had no negative or positive effect on seed viability. This raises interesting questions on environmental conditions of cycad seed storage, in particular in *Encephalartos*. Firstly, can seed storage in high moisture conditions improve or hasten dormancy release? Secondly, do *Encephalartos* seeds need cold stratification, as reported in other cycads?

Cycad seeds are reported to be recalcitrant (Forsyth & Van Staden, 1983; Woodenberg *et al.,* 2007) and in *E. natalensis* storage in high moisture conditions resulted in high seed germination (> 80%) (Forsyth & Van Staden, 1983). It is conceivable that *E. latifrons* and *E. altensteinii* seed could be benefit from high moisture storage; given that seed morphology is similar to that of *E. natalensis* (Osborne, 1988).

It is uncertain whether cold treatment (cold stratification) of *E. latifrons* seed could benefit seed dormancy release and germination. Although in the wild, temperatures can go down > 4°C during embryo development in the winter period. Perhaps a combination of cold and warm stratification at higher moisture conditions could release dormancy as in other seeds that show morphorphysiological dormancy (Baskin & Baskin, 2004).

The effects of cold stratification and high moisture storage on seed storage need to be investigated to determine the impact on seed development and dormancy release.

6.3.3. Seed pre-treatment for germination

Results from seed pre-treatment by scarification, or scarification followed by treatment with GA_3 for 48h or 96h revealed that neither *E. latifrons* nor *E. altensteinii* responded in a significant way to these treatments (*ANOVA* $p > 0.05$). Although seed germination in *E. latifrons* and *E. altensteinii* had no response to GA_3 pre-treatment, some seed characteristics are consistent with morphological dormancy, because of an under developed embryo (Baskin & Baskin, 1998; 2004). Moreover, seed characteristics are also consistent with physiological dormancy, given that the sarcotesta is reported to promote dormancy (Forsyth & Van Staden, 1983). Both characteristics are contray in *Encephalartos* seed that is held in the cones, and on the plant for a longer period (> 8 months) (Vorster, 1995; Grobbelaar, 2002; *Pes. Obs*). This implies that, not all *Encephalartos* display similar type of morphophysiological dormancy. *Encephalartos latifrons* and *E. altensteinii* are consistent with deep simple morphophysiological dormancy (DS MPD), given that they only require warm stratification and no GA_3 pre-treatment.

Cycad seed morphology is varied in both genera and species (Osborne, 1988), and seed undergoes different types of morphophysiological dormancy (Dehgan & Johnson, 1983; Dehgan & Schutzman, 1989), therefore, different cycads may require different seed storage periods conditions and pre-treatments for optimum seed germination.

6.4. Practical outcomes and protocols arising from the structured analysis

Four important conclusions have emerged from this study that can be used to improve the propagation of *E. latifrons* at KBNG. The first is that *E. latifrons* pollen is sensitive to wetting and subsequent drying, which may lead to desiccation. As a result, the wet (water-based) method for artificial pollination may not be appropriate in circumstances where subsequent drying is likely to occur. At KNBG, the dry method of pollination yielded better results for *E. latifrons* and it is advisable to adopt it. The second conclusion is that the timing of pollination can make a difference and pollination in the afternoon yielded better results. Thirdly, *E. latifrons* pollen germinated within a relatively narrow temperature band and these optimal temperatures were seldom reached during the pollination period at KNBG. There is relatively little that can be done at KNBG to remedy this but it does suggest that *ex situ* collections should be located in places where there is a better match with temperatures prevailing at the time of pollination. Finally, increased germination and better MTG and t_{G50} results were obtained from seeds stored for more than eight months as this allowed the seed to mature properly with a fully formed embryo. Based on the results from *E. altensteinii,* a period of 12 months is likely to yield the best results.

The recommended protocol for the propagation of *E. latifrons* is as follows:

- Artificial pollination using dry pollen. A total of five pollen applications, each using 5g of pollen should be carried out, starting two days after sporophylls open. Pollination should be carried out in the afternoon (14h00 – 16h00).

- After natural cone disintegration, the fleshy sarcotesta should be cleaned off the seed, the seed then dusted with fungicide, packed in a breathable material and stored at dry ambient room temperature for 12 months.

- No treatment of the seed is required prior to sowing.

In conclusion, this study, of the critically endangered *E. latifrons* has contributed in identifying the most effective artificial pollination and propagation methods for use at KNBG. Since this collection is the largest *ex situ* collection of the species and contains unique genotypes, the improved seed set should benefit conservation projects and inform future pollination in *ex situ* collections.

REFERENCE LIST

Ainsworth, C. 2000. Boys and girls come out to play: the molecular biology of dioecious plants. Annals of botany. 86: p. 211-221.

Anderson, J. M., Anderson, H. M. & Cleal, C. J. 2007. Brief history of the gymnosperms: classification, biodiversity, phytogeography and ecology. 20 ed. Strelitzia, Pretoria.

Barrow, J. R. 1983. Comparisons among pollen viability measurement methods in cotton. Crop Science. 23: p. 734-736.

Baskin, C. C. & Baskin, J. M. 2001. SEEDS ecology, biogeography, and evolution of dormancy and germination. 1st ed. Academic Press, San Deigo and London.

Ben-Na'im, A. 1980. Hydrophobic Interaction. Plenum Press, New York.

Bewley, D. J. & Black, M. 1994. Seeds "physiology of development and germination". 2nd ed. Plenum Press, New York.

Bewley, J. D. 1997. Seed germination and dormancy. Plant Cell. 9: p. 1055-1066.

BGCI. 2012. International agenda for botanic gardens in conservation. Botanic Gardens Conservation International, London.

Bino, R. J., Dafni, A. & Meeuse, A. D. 1984a. Entomophily in the dioecious gymnosperm *Ephedra aphylla Forsk.* (Epalte C.A.Mey.),with some notes on *E. Campylopoda* C.A. Mey. I. Aspects of the

entomophilous syndrome. In Proceedings of the Koninklijke Nederlandse Akadamie van Wetenschapen, Series C. 87: p. 1-13.

Bino, R. J., Devente, N. & Meeuse, A. D. 1984b. Entomophily in the dioecious gymnosperm *Ephedra aphylla* Forsk. (Epalte C.A.Mey.),with some notes on *E. Campylopoda* C.A. Mey. II. Pollination droplets, nectarial secretion in *Ephedra*. In the Proceedings of the Koninklijke Nederlandse Akadamie van Wetenschapen, Series C. 87: p15–24.

Bold, H. C. 1967. Morphology of plants. 2nd ed. Harper & Row, Evaston & London and John Weatherhill, New York & Tokyo.

Bonner, F. T. 2008. Seed biology. In: F. T. Bonner & R. P. Karrfalt, eds. The woody plant seed manual. Washington: United States of America, Department of Agriculture Forestry Services. p. 3-38.

Broome, T. 2001. Optimising cycad seed germination. The Cycad Newsletter. 24: p. 5-7.

Calonje, M., Kay, J. & Griffith, P. 2011. Propagation of cycad collections from seed: Applied reproductive biology for conservation. The Journal of Botanic Garden Horticulture, Sibbaldia. 9: p77-96.

Carmichael, J. S. & Freidman, W. E. 1995. Double fertilization in *Gnetum gnemon*: the relationship

between the cell cycle and sexual reproduction. Plant Cell. 7: p. 1975-1988.

Chamberlain, C. J. 1909. Spermatogenesis in *Dioon edule*. Botanical Gazzette, 47. p. 215-236.

Chamberlain, C. J. 1919. The living cycads, New York and London: Hafner Publishing company.

Chamberlain, C. J. 1926. Hybrids in cycads. Botanical Gazette. 81: p. 401-418.

Chamberlain, C. J. 1935. Gymnosperms structure and evolution. 1st ed. University of Chicago, Chicago.

Chavez, V. M. & Litz, R. E. 1992b. In vitro morphogenesis of *Ceratozamia hildae* and *C. mexicana* from megagametophyte and zygotic embryos. Plant Cell Tissue Organ Culture. 30: p. 93-98.

Chavez, V. M., Litz, R. E.; Monroy, M., Moon, P. A. & Vovides, A. 1998. Regenaration of *Ceratozamia euryphyllidea* (Cycadales, Gymnospermea) plants from embryogenic cultures derived from mature phase trees. Plant Cell Reports. 8: p. 612-616.

Chavez, V. M., Litz, R. E., Moon, E. A. & Norstog, K. 1992c. Somatic embryogenesis from leaf callus of a mature gymnosperm *Ceratozamia mexicana vat. robusta* (Miq.) Dyer (Cycadales). In vitro cell. Development Biology. 28: p. 59-63.

Chavez, V. M., Litz, R. E., Moon, P. A. & Norstog, K. J. 1995. Somatic embryogenesis and organigenesis from leaf and embryo explants of *Ceratozamia and Zamia.* The Cycad Society of South Africa, Stellenbosch. p. 91.

Chavez, V. M., Litz, R. E. & Norstog, K. 1992a. Somatic embryogenesis and organogenesis in *Zamia fischeri, Z. furfuracea and Z. pumila*. Plant Cell, Tissue and Organ Culture. 30: p. 99-105.

Chawick, C. E. 1990. The roles of *Tranes Lyterioides and T. sparsus Boh* (Col., Curculionidae) in the pollination of *Macrozamia communis* (Zamiaceae). Palm and Cycad Society of Australia Ltd, Milton. p. 77-80.

Ching, T. M. & Ching, K. K. 1964. Freeze-drying pine pollen. Plant Physiology. 39(5): p. 704-709.

Cohen, J. I., Williams, T. J., Plucknett, D. L. & Shands, H. 1991. *Ex situ* conservation of genetic resources: Global development and environmental concerns. Science. 253: p. 866-872.

Convention on Biological Diversity. 2009. The convention on biological diversity plant conservation report : a review of progress in implementing the global strategy of plant conservation (GSPC), Montreal: Secretariat of the Convention on Biological Diversity.

Daly, B., Donaldson, J.S., Friedmann, Y., Hahndiek, Q., King, N. Newton, D. and Southwood, A. (Eds). 2006. Albany Cycad (*Encephalartos latifrons*) Population and Habitat Viability Assessment Workshop Report. Conservation Breeding Specialist Group (SSC / IUCN) / CBSG Southern Africa. Endangered Wildlife Trust, Johannesburg.

da Silva, J. M., Donaldson, J. S., Reeves, G. & Hedderson, T. A. 2012. Population genetics and conservation of critically small cycad populations: a case study of the Albany cycad, *Encephalartos latifrons* (Lehmann). Biological Journal of Linnean Society.105: p. 293-308.

Dehgan, B. 1983. Propagation and growth of cycads a conservation strategy. Proceedings of Florida State Horticultural Society. 96: p. 137-139.

Dehgan, B. 1996. Permian permanence. American Nurseryman. 183: p. 66.

Dehgan, B. 1999. Propagation and culture of cycads: a practical approach. Acta Horticulture. 486: p. 123-131.

Dehgan, B. & Almira, F. 1993. Horticultural practises and conservation of cycads. Palm & Cycad Society of Australia Ltd, Queensland. p. 322-328.

Dehgan, B. & Dehgan, N. B. 1988. Comparative pollen morphology and taxonimic affinities in Cycadales. American Journal of Botany. 75: p. 1501-1516.

Dehgan, B. & Johnson, C. R. 1983. Improved seed germination of *Zamia floridana* (sense lato) with H2S4O and GA3. Horticultral Science. 19: p. 357-361.

Dehgan, B. & Schutzman, B. 1983. Effect of H2SO4 and GA3 on seed germination of *Zamia furfuracea*. Horticultural Science. 18: p. 371-372.

Dehgan, B. & Schutzman, B. 1989. Embryo development and germination of cycas seeds. Journal of the American Society for Horticultural Science. 114: p. 125-129.

Dehgan, B. & Yuen, C. K. 1983. Seed morphology in relation to dispersal, evolution, and propagation of *Cycas* L.. Botanical Gazette. 144: p. 412-418.

Dhiman, M., Sharma, V. & Moitra, S. 2010. Somatic embryogenesis and plant regeneration in *Ephedra foliata* (Boiss.); a non non coniferous gymnosperm. Plant Tissue Culture. 20: p. 133-143.

Donaldson, J. S. 1997. Is there floral parasite mutualism pollination in cycads? pollination biology of *Encephalartos villosus* (Zamiaceae). American Journal of Botany. 84: p. 1398-1406.

Donaldson, J. S. 2003. Regional overview:Africa. In: J. S. Donaldson, ed. Cycads. status survery and conservation action plan. IUCN/SSC Cycad Specialist Group, Gland and Cambridge. p. 9-19.

Donaldson, J. 2009. Botanic gardens science for conservation and global change. Trends in Plant Science. 14: p. 608-613.

Donaldson, J. S. 2012. *Encephalartos latifrons.* in: IUCN 2012. IUCN red list of threatened species. version 2012.2.. [Online] Available at: www.iucnredlist.org[Accessed 28 February 2013].

Donaldson, J. S. & Bösenberg, J. D. 1999. Changes in the abundance of South African cycads during the 20th century: preliminary data from the study of matched photographs. Academic Publisher, Beijing. p. 240-247.

Donaldson, J. S. & Bösenberg, J. D. 1995. Duration of development stages of the male gametophyte in the interval between pollination and the release of spermatozoids in *Encephalartos altensteinii*. Cycad Society of South Africa, Stellenbosch. p. 415-424.

Donaldson, J. S., Nänni, I. & Bösenberg, J. D. 1995. The role of insects in pollination of *Encephalartos cycadifolius.* Cycad Society of South Africa, Stellenbosch. p. 423-434.

Doyle, J. A. & Donoghue, M. J. 1992. Fossil and seed plant phylogeny reanalyzed. Brittonia. 44: p. 89-106.

Duffield, J. W. & Snow, J. A. G. 1941. Pollen longevity of *Pinus strobus* and *Pinus resinosa* as controlled by humidity and temperature. American Journal of Botany. 28: p. 175-177.

Dyer, R. A. 1965. The cycads of Southern Africa. Bothalia, VIII: p. 405-515.

Edlund, A. F., Swanson, R. & Preuss, D. 2004. Pollen and stigma structure and function: the role of diversity in pollination. Plant Cell. 16: p. S84-S97.

El-Ghazaly, G. & Rowley, J. R. 1997. Pollen Wall of *Ephedra foliata*. Palonology. 21: p. 7-18.

Ellis, R. H., Hong, T. D. & Roberts, E. H. 1990. An Intermediate category of seed storage behaviour. Journal of Experiemental Botany. 1: p. 1167-1174.

Ellstrand, N. C., Ornduff, R. & Clegg, J. M. 1990. Genetic Structure of the Australian Cycad, *Macrozamia Communis* (Zamiaceae). American Journal of Botany. 77: p. 677-681.

Fernando, D. D., Lazzaro, M. D. & Owens, J. N. 2005. Growth and development of conifer pollen tubes. Sex Plant Reproduction. 18: p. 149-162.

Fernando, D. D., Quinn, C. R., Brenner, E. D. & Owens, J. N. 2010. Male gametophyte developement and evolution an extant gymnosperms. International Journal of Plant Developmental Biology. p. 47- 63.

Fletcher, C. 2008. *Encephalartos latifrons* [Interview] (06 June 2008).

Ford, C. S.; Fischer, J., Jones, N. B., Nigro, S.A., Makunga, P. & van Standen, J. 2005. Somatic embryogenesis in *Pinus patula*. In: S. M. Jain & P. K. Gupta, (eds). Propocols for somatic embryogenesis in woody plants. Springer, Dordrecht. p. 121-139.

Forsyth, C. & Van Staden, J. 1983. Germination of cycad seeds. South African Journal of Science. 79: p. 8-9.

Freeman, D. C., Doust, J. L.t, El-Keblawy, A., Miglia, K. J. & McArthur, E. D. 1997. Sexual specialization and inbreeding avoidance in the evolution of dioecy. Botanical Review. 63: p. 65-92.

Freeman, D. C., Harper, K. T. & Charnov, E. L. 1980. Sex change in plants: old and new observations and new hypotheses. Oecologia. 47: p. 229.

Freidman, W. E. 1990. Sexual reproduction in *Ephedra nevandensis* (Ephedraecea): further evidence of double fertilization in a non flowering seed plant. American Journal of Botany. 77: p. 1582-1598.

Freidman, W. & Gifford, E. M. 1997. Development of the male gametophyte of *Ginkgo biloba*: a window into the reproductive biology of early seed plants. In: T. Hori *et al.,* eds. *Ginkgo biloba* a Global Treasure.. Springer, Tokyo. p. 26-49.

Freidman, W. E. 1993. The evolutionary history of the seed plant gametophyte. Trends in Ecology and Evolution. 8: p. 15-21.

Freidman, W. E. & Gifford, E. M. 1988. Division of the Generative Cell and Late Development in the Male Gametophyte of *Ginkgo biloba*. American Journal of Botany. 75: p. 1431-1442.

Freidman, W. E. 1987. Growth and Development of the Male Gametophyte of *Ginkgo biloba* within the Ovule (inVivo). American Journal of Botany. 74: p. 1797-1815.

Frett, J. J. 1987. Seed germination of *Cycas revoluta*. Journal of Environmental Horticulture. 5: p. 105-106.

Giddy, C. 1984. Cycads of South Africa. 1st ed. Cape Town, Struik.

Giddy, C. 1995. Cycad conservation - a third world perspective. In: Proceedings of Cycad 93, the Third International Conference on Cycad biology (Ed. Vorster, P. 1995.).The Cycad Society of South Africa, Stellenbosch. p. 19-25.

Givnish, T. 1980. Ecological constraints on the evolution of breeding systems in seed plants: dioecy and dispersal in gymnosperms. Evolution. 34: p. 959-972.

Goode, D. 1989. Cycads of Africa. 1st ed. Struik, Cape Town, Winchester.

Grobbelaar, N. 1999. Growth of cycad female cones and their omnule after the cones have become receptive to pollination. In: Proceedings of Cycad 96, the Fourth International Conference on Cycad biology (Ed. Chen, C-J. 1999). International Academic Publishers, Beijing. p. 276-279.

Grobbelaar, N. 1999. Coning frequency, gender ratio and pollination of *Encephalartos travenosus* (Zamiaceaea) at Modjadji Nature Reserve, South Africa and the germination of this cycad's seed. In: Proceedings of Cycad 96, the Fourth International Conference on Cycad biology, (Ed. C-J. 1999) International Academic Publishers, Beijing. p. 309-318.

Grobbelaar, N. 2002. Cycads: with special reference to the Southern African species. 1st ed. Privately Published, Pretoria.

Gullvåg, B. N. 1966. The fine structure of some gymnosperm pollen walls. Grana Palynologia. 6: p. 435-475.

Hall, J. A., Walter, G. H., Bergstromc, D. M. & Machin, P. 2004. Pollination ecology of the Australian cycad *Lepdiozamia peroffskyana* (Zamiaceae). Australian Journal of Botany. 52: p. 333-343.

Ikeno, S. 1896. The spermatozoid of *Cycas revoluta*. Botanical Magazine, Volume 10, p. 367-368.

Ikeno, S. & Hirase, S. 1897. Spermatozoids in gymnosperms. Annals of Botany: 11: p. 344-345.

IUCN Red List. 2014. The IUCN Red List of Threatened Species "October 2010 update, Cycad facts". IUCN, Gland.

Jacot-Guillarmod, A. 1958. Temperature variation in male cones of *Encephalartos*. Nature. 182: p. 474.

Jager, A. K. & van Staden, J. 1996a. Somatic Embryogenesis and organagenesis in *Encephalartos dyerianus* and *Encephalartos cycadifolius*. Plant Tissue and Organ Culture. 45: p. 99-102.

Jager, A. K. & van Staden, J., 1992b. Somatic embryogenesis in *Encephalartos cycadifolius*. Plant Cell Tissue and Organ Culture. 15: p. 437-440.

Jones, D. L. 2002. Cycads of the world. 2nd ed. Washington, D.C: Smithsonian Institution Press.

Kakani, V. G., Reddy, K. R., Koti, S., Wallace, T. P., Prasad, P. V., Reddy, V. R. & Zhao, D. 2005. Differences in invitro pollen gernimation and pollen tube growth of cotton cultivars in response to high temperature. Annals of Botany. 96: p. 59-67.

Kato, M., Inoue, T. & Nagamitsu, T. 1995. Pollination biology of *Gnetum* (Gnetaceae) in a lowland mixed dipterocarp forest in Sarawak. American Journal of Botany. 82: p. 862-868.

Kay, J. A., Strader, A., Murphy, V., Nghiem-Phu, L., Calonje, M. & Griffith, M. P. 2011. Palma Corcho: a case study in botanic garden conservation horticulture and economics. HortituculturеTechology. 21: p. 474-481.

Keller, T. & Beda, H. 1984. Effects of $S0_2$ on the germination of conifer pollen. Enviromental Series a, Ecological and Biology. 33: p. 237-243.

Klavins, S. D., Taylor, E. L., Krings, M. & Taylor, T. N. 2003. Gymnosperms from the Middle Triassic of Antarctica: The first structurally preserved cycad pollen cone. International Journal of Plant Science. 164: p. 1007–1020.

Kluge, J. 1988. Lowveld Botanical Garden. *Encephalartos.*15: p. 3.

Knudsen, J. T., Tollsten, L. & Bergstrom, L. G. 1993. Floral scent- a check list of volatile compounds isolated head space tecnhiques. Phytochemistry. 33: p. 253-280.

Kono, M. & Tobe, H. 2007. Is *Cycas revoluta* (Cycadaceae) wind or insect pollinated? American Journal of Botany. 94: p. 847-855.

Kormutak, A., Galgoci, M., Vookova, B., Salaj, T. & Gomory, D. Longevity of Abies pollen inder deep-freeze storage. Acta Pruhoniciana. 94: p. 35-38.

Kucera, B., Cohn, M. A. & Leuber-Metzger, G., 2005. Plant hormone interactions during seed dormancy release and germination. Seed Science Research.15: p. 281-307.

Labandeira, C. C., Kvaček, J. & Mostovski, M. B. 2007. Pollination drops, pollen, and insect pollination of Mesozoic gymnosperms. Taxon. 56: p. 663-695.

Lanteri, S., Belletti, P. & Lotito, S. 1993. Storage of pollen of Norway spruce and different pine species. Silvae Genetica. 42: p. 2-3.

Lee, C. L. 1955. Fertilization in *Ginkgo biloba*. Botanical Gazette. 117: p. 79-100.

Li, D. Z. & Prichard, H. W. 2009. The science and economics of *ex situ* plant conservation. Trends in Plant Science. 14: p. 614-621.

Lindstrom, A. J. 2012. International Union for Conservation of Nature (IUCN)/ Species Survival Commision (SSC) Cycad Specialist Group *Ex Situ* Conservation and Recovery Programme (CSG-ECRP). In: Proceedings of Cycad 2008, International Conference on Cycad biology, (Eds. Stevenson, Wm., Osborne, R., Blake, A. S. T. 1999). Memoirs of the New York Botanical Garden, Panama City. 106: p. 9-19.

Litz, R. E., Chavez, V. M. & Moon, P. A. 1995b. Somatic embryogenesis in the Cycadales. In: S. M. Jain, P. K. Gupta & R. J. Newton, eds. Somatic Embryogenesis in Woody Plants. Dordrecht: Kluwer Academic Plublishers. p. 1-15.

Litz, R. E., Moon, P. A., Benson, E. M., Stewart, J. & Chavez, V. M. 2004. A biotechnology strategy for medium- and long-term conservation of cycads. Botanical Review. 70: p. 39-46.

Litz, R. E., Moon, P. A. & Chavez, V. M. 1995a. Somatic embryogenesis from leaf callus derived from mature trees of the cycad *Ceratozamia hildae* (Gymnospermae). Plant cell Tissue and Organ Culture. 40: p. 25-31.

Marshall, J., Grobbelaar, N., Coetzee, J. & Osborne, R. 1989. Pollen morphology of cycadales with special reference to the *Encephelartos* species. Pollen Spores. 31: p. 229-249.

Maunder, M., Hughes, C., Hawkins, J. A. & Culham, A. 2004. Hybridization in *ex situ* plant collections: consevation concerns, liabilities, and oportunities. In: E. O. Guerrant, K. Havens & M. Maunder, eds. *Ex situ* plant conservation: supporting species survival in the wild. Washington: Island press Washington. p. 325-364.

Minorsky, P. V. 2003. The hot and the classic. Plant Physiology. 132: p. 25-26.

Mostert, C. 2000. MSc thesis, Aspekte van die keimingsgedrag en fynstruktuur van *Encephalartos* stuimeel. University of Pretoria.

Nagalingum, N. S., Marshall, C. R., Quental, T. B., Rai, H. S., Little, D. P. & Mathews, S. 2011. Recent synchronous radiation of a living fossil. Science. 334: p. 796-799.

Namoff, S., Husby, C., Francisco-Ortega, J., Noblick, L.R., Lewis, C. E. & Griffiths, M. P. 2010. How well does a botanical garden collection of a rare palm capture the genetic variation in a wild population? Biological Conservation. 143: p. 1110-1117.

Nepi, M., von Aderkas, P., Wagner, R., Mugnaini, S., Coulter, A. & Pacini, E. 2009. Nectar and pollination drops: how different are they? Annals of Botany. 109: p. 205-219.

Newcomer, E. H. 1939. Pollen longevity of *Ginkgo*. Bulletin of the Torrey Botanical Club. 66: p. 121-123.

Niklas, K. J. 1985. The Aerodynamics of Wind Pollination. Botanical Review. 5: p. 328-386.

Nikolaeva, M. G. 1977. Factors controlling the seed dormancy pattern. In: A. A. Khan, ed. The Physiology and Biochemistry of Seed dormancy and Germination. North-Holland, Amstredam. p. 51-71.

Norstog, K. 1987. Cycads and the origin of insect pollination. American Scientist. 75: p. 270-279.

Norstog, K. J. 1993. Spermatogenesis in *Microcycas*: evolutionary significant of male gametes of seed plants. In: Proceedings of Cycad 90, the Second International Conference on Cycad biology (Eds. Stevenson, D. Wm., Norstog, K. J. 1995.). The Cycad and Palm Societies of Australia Ltd, Queensland. p. 270-278.

Norstog, K. J. & Fawcett, P. K. 1989. Insect-cycad symbiosis and its relation to pollination of *Zamia furfuracea* (Zamiaceaea) by *Rhopalotria mollis* (Curculionnidae). American Journal of Botany. 76: p. 1380-1394.

Norstog, K. J., Gifford, E. M. & Stevenson, D. W. 2004. Comparative development of spermatozoids of cycads and *Ginkgo biloba*. Botanical Review. 70: p. 5-15.

Norstog, K. J. & Nicholls, T. J. 1997. The biology of the cycads. 1st ed. Cornell University Press, Ithaca, London.

Norstog, K. J., Stevenson, D. W. & Niklas, K. 1986. The role of beetle in the pollination of *Zamia furfuracea*. Biotropica. 18: p. 304.

Norstog, K. & Rhamstine, E. 1967. Isolation and culture of haploid and diploid cycad tissue. Phytomorphology. 17: p. 374-381.

Nygaard, P. 1969. Studies on the germination of pine pollen (*Pinus mugo*) *in vitro*. I. growth conditions and effects of pH temperature on germination, tube growth and respiration. Physiologia Plantarum. 22: p. 338-346.

Ornduff, R. 1987. Sex ratios and coning frequency of the cycad *Zamia pumila* L. (Zamiaceae) in the Dominican Republic. Biotropica. 19: p. 361-364.

Ornduff, R. 1991. Coning phenology of the cycad *Macrozamia riedlei* (Zamiaceae) over a five- year interval. Torrey Botanical Society. 118: p. 6-11.

Osborne, R. 1986. Sex life of cycads. *Encephalartos,* Issue 8, p. 26.

Osborne, R. 1988. Cycad seed kernel morphology. *Encephalartos*. 13: p. 26-30.

Osborne, R. 1989. Cycad pollen survives ultra-low temperature. *Encephalartos*. 17: p. 25.

Osborne, R., Calonje, M. A., Hill, K. D., Stanberg, L. & Stevenson, D.Wm. 2012. The world list of cycads. In: Proceedings of Cycad 2008, the p International Conference on Cycad biology, (Eds.

Stevenson, Wm., Osborne, R., Blake, A. S. T. 1999.). Memoirs of the New York Botanical Garden, Panama City. 106: p. 480-508.

Osborne, R., Robbertse, P. & Claassen, N. 1991. The longevity of cycads Pollen "results of some preliminary experimenation". *Encephalartos.* 28: p. 10-13.

Osborne, R., Robbertse, P. J. & Claassen, M. I. 1992. The longevity of cycad pollen in storage. South African Journal of Botany. 58: p. 250- 254.

Ouyang, H., Zhang, S., Li, N. & Wu, H. 2004. Microsporogenesis of *Cycas elongata* and its systematic implication. Acta Phytotaxonomica Sinica. 42: p. 500-512.

Owens, J. 2006. The reproductive biology of lodgepole pine. FGC extension note 07.

Owens, J. N. & Blakes, M. D. 1985. Forest tree seed production. Canadian forestry Service, Chalk river.

Owens, J. N., Simpson, S. J. & Caron, G. E. 1987. The pollination mechanism of Engelmann spruce (*Picea engelmannii*). Canadian Journal of Botany. 65: p. 1439-1450.

Owens, J. N., Simpson, S. J. & Molder, M. 1980. The pollination mechanism in yellow cypress (*Chamaecyparis nootkatensis*). Canadian Journal of Forest Research. 10: p. 564-572.

Owens, J. N., Takaso, T. & Runions, J. C. 1998. Pollination in conifers. Trends in Plant Science Review. 3: p. 479.

Pacini, E. & Franchi, G. G. 1999. Pollen grain sporoderm and types of dispersal units. Acta Societatis Botanicorum Poloniae. 68: p. 299-305.

Pacini, E., Franchi, G. G. & Ripaccioli, M. 1999. Ripe pollen structure and histochemistry of some gymnosperms. Plant Systematics and Evolution. 217: p. 81-99.

Pearson, H. 1909. Futher observation on *Welwitchia.* London, Royal Society of London. p. 331-402.

Pearson, H. H. 1916. The Kirstenbosch cycads. Journal of Botanical Society. Part 2.

Perez-Farrera, M. A., Vovides, A. P. & Alavarez - Moctezuma, J. G. 1999. A study on seed germination. The New Plantsman. 6: p. 214-218.

Poisson, M. J. 1878. Heat production companing coning of males of *Dioon edule*. Botanical Soceity of France. p. 253.

Probert, R. J., Daws, M. I. & Hay, F. R. 2009. Ecological correlates of *ex situ* seed longevity: a comparative study on 195 species. Annals of Botany. 104: p. 57-69.

Proches, S. & Johnson, S. 2009. Beetle pollination of the fruit scented cones of the South African cycad Stangeria eriopus. American Journal of Botany. 96: p. 1722-1730.

Proctor, M., Yeo, P. & Lack, A. 1996. The natural history of pollination. 1st ed. Harper Collins Publishers, London, Glasgow, Sydney, Auckland.

Pullman, G. S., Johnson, S., Peter, G., Cairney, J. & Xu, N. 2003. Improving loblolly pine somatic embryo maturation: comparison of somatic and zygotic embryo morphology, germination, and gene expression. Plant Cell Reports. 20: p. 747-758.

Punt, W., Blackmore, S., Nilsson, S. & Thomas, A. L. 2014. Glossary of Pollen and Spore Terminology [Online] Available at: http://www.pollen.mtu.edu/glos-gtx/glos-int.htm[Accessed 26 January 2014].

Riley, J. 1987. Gibberellic Acid for Fruit Set and Seed Germination. California Rare Fruit Growers. 19: p. 10-12.

Robbertse, P. J. & Claassen, M. I. 1995. Vegetative and reproductive cycles of some *Encephalartos* species in Pretoria gardens. The Cycad Society of South Africa, Stellenbosch. p. 391-414.

Roberts, E. H. 1973. Predicting the storage life of seeds. Seed Science and Technology. 1: p. 299-514.

Roberts, E. H. & Ellis, R. H. 1989. Water and seed survival. Annals of Botany. 63: p. 39-52.

Russell, D. A. 1982. The mass extinctins of the late Mesozoic. Scientific American. 246: p. 370-380.

Salas-Leiva, D.E., Meerow, A. W., Calonje, M., Oterga, J. F., Nakamura, K., Stevenson, D. W., Lewis, C. E. & Namoff, S. 2013. Phylogeny of the cycads based on multiple single-copy nuclear genes: congruence of concatenated parsimony, likelihood and species tree inference methods. Annals of Botany. 112: p. 1263–1278.

Salisbury, F. B. & Ross, C. W. 1978. Plant physiology. 2nd ed. Wadsworth Publishing Company, Belmont.

Saxton, W. T. 1910. The development of embryo of *Encephalartos*. Botanical Gazette. 49: p. 13-18.

Schneckenburger, S. 1993. Embrology and germination in gymnosperms. Academia Nacional De Ciencias. 91.

Sedgwick, P. J. 1924. Life history of *Encephalartos*. Botanical Gazette. 77: p. 300-310.

Seymour, R. S. & Schultze-Motel, P. 1997. Heat-producing flowers. Endeavour. 21: p. 125-129.

Singh, H. 1978. Embryology of gymnosperms. Berlin: Gebruder Borntraegar.

Siregar, I. Z. & Sweet, G. B. 2000. The impact of extraction and storage conditions on the viability of radiata pine pollen. Silvae Genetica. 49: p. 10-14.

Smith, G. S. 1978. Seed scrarification to speed up germination of ornamental cycads (*Zamia sp*.). Hort-Science. 13: p. 436-438.

Sporne, K. R. 1969. The morphology of gymnosperms. London: Hutchison University Library.

Sporne, K. R., 1974. The morphology of angiosperms "the structure and evolution of flowering plants". 1st ed. London: Hutchington & Co.

Stanley, R. G. & Linskens, H. F. 1974. Pollen biology biochemistry management. Springer-Velag, Berlin, Heidelberg, New York.

Steyn, E. M., Strydom, D. J. & Botha, A. 1996. Fertilization and rejection of spermatozoids by egg cells in artifially pollinated ovules of *Encephalartos* (Zamiaceae). Sex Plant Reproduction. 9: p. 175-185.

Stone, J. L., Thomson, J. D. & Dent-Acosta, S. J. 1995. Assessment of pollen viability in hand-pollination experienments: A review. American Journal of Botany. 82: p. 1186-1197.

Suinyuy, T. N., Donaldson, J. S. & Johnson, S. D. 2010. Scent chemistry and patterns of thermogenesis in male and female cones of the African cycad *Encephalartos natalensis* (Zamiaceae). South African Journal of Botany. 76: p. 717-725.

Suinyuy, T. N., Donaldson, J. S. & Johnson, S. D. 2012. Geographical variation in cone volatile composition among population of the Africa cycad *Encephalartos*. Biological Journal of the Linnean Society. 106: p. 514-527.

Suinyuy, T. N., Donaldson, J. S. & Johnson, S. D. 2013. Variation in the chemical composition of cone volatiles within the African cycad genus *Encephalartos*. Phytochemistry. 85: p. 82-91.

Tang, W. 1986a. Collecting and storing pollen. *Encephalartos*. 7: p. 4-6.

Tang, W. 1986b. Pollinating cycads. *Encephalartos*. 8: p. 16-19.

Tang, W. 1987a. Insect pollination of *Zamia pumila* (Zamiaceae). American Journal of Botany. 74: p. 98.

Tang, W. 1987b. Heat production in cycad cones. Botanical Gazette. 148: p. 165-174.

Tang, W. 1987c. Cycad seeds and seedlings. *Encephalartos*. 9: p. 8-11.

Tang, W. 1990. Reproduction in the cycad *Zamia pumila* in a fire-climax habitat: an eight-year study. Bulletin of the Torrey Botanical Club. 117: p. 368-374.

Tang, W. 1993. Nectar-Like Secretions in Female Cones of Cycads. The Cycad Newsletter. 16: p. 10-13.

Tekleva, M. V. & Krassilov, V. A. 2009. Comparative pollen morphology and ultrastructure of modern and fossil gnetophytes. Review of Paloeobotany and Palynology. 156: p. 1300-138.

Tekleva, M. V., Polevova, S. V. & Zavialova, N. E. 2007. On some peculiarities of sporoderm structure in members of the Cycadales and Ginkgoales. Paleontological Journal. 41: p. 1162-1178.

Terry, I. 2001. Thrips and weevils as dual, specialist pollinators of the Australian cycads *Macrozamia communis* (Zamiacea). International Journal of Plant Science. p. 1293-1305.

Terry, I., Moore, C. J., Walter, G. H., Voster, P. I., Roemer, R. B., Donaldson, J. D. & Machin, P. J. 2004. Association of cone thermogenesis and volatiles with pollinator specificity in *Macrozamia* cycads. Plant Systematics and Evolution. 243: p. 233–247.

Terry, I., Walter, Gimme H., Donaldson, J. S., Snow, E., Forster, P. & Machin, P. 2005. Pollination of *Macrozamia cycads* (Zamiaceae): effectiveness and behaviour of specialist vectors in a dependent mutualism. American Journal of Botany. 92: p. 931-940.

Terry, I., Walter, G. H., Moore, C., Roemer, R. & Hull, G. 2007. Odour-mediated push-pull pollination in cycads. Science. 318: p. 70.

Terry, I., Tang, W., Blake A. S. T., A. S., Donaldson, J. S., Sigh, R., Vovides, A. P. & Jaramillo, A. C. 2012. An overveiw of cycad pollination studies. In: Proceedings of Cycad 2008, the International Conference on Cycad biology, (Eds). Stevenson, Wm., Osborne, R. & Blake, A. S. T. 1999). Memoirs of The New York Botanical, Panama City. p. 352-394.

Tulecke, W. R. 1954. Presevation and germination of the pollen of *Gingko* under sterile conditions. Bulletin of the Torrey Botanical Club. 81: p. 509-512.

Tweddle, J. C., Dickie, J. B., Baskin, C. C. & Baskin, J. M. 2003. Ecological aspects of seed desiccation sensitivity. Journal of Ecology. 91: p. 294-304.

United Nations. 2004. World population 2300. United Nations, New york.

Urban-Econ Developmental Economists. 2009. *Part1:* Situation analysis; local economic development strategy Makana Municipality. Makana Municipality, Port Elizabeth.

Volis, S., Blecher, M. & Yuval, S. 2009. Complex *ex situ* - *in situ* approach for conservation of endangered plant species and its application to Iris atrofusca of the Northern Negev. Biorisk. 3: p. 137-160.

Vorster, P. 1995. Aspect of reproduction of cycads. 2. an annotated review of know information. In: Proceedings of Cycad 93, the Third International Conference on Cycad biology (Ed. Vorster, P. 1995.). The Cycad Society of South Africa, Stellenbosch. p. 379-387.

Walter, K. S. & Gillet, H. J. (Eds). 1998. 1997 IUCN red list of threatened plants. Gland and Cambridge: IUCN and World Conservation Monitoring Centre.

Walters, T. 2003. Off-site collection. In: J. S. Donaldson, ed. Cycads: Status Survey and Conservation Action Plan. IUCN/SSC Cycad Specialist Group, Gland. p. 48-53.

Wang, D., Wu, Y., Shih, A.C., Wu, C., Wang, Y., Chaw, S. 2007. Transfer of chloroplast genomic dna to mitochondrial genome occurred at least 300 MYA. Molecular Biology and Evolution. 24: p. 2040-2048.

Weier, E., Barbour, M., Stocking, R. & Rost, T. 1982. Botany an introduction to plant biology. 5th ed. John Wiley & Sons, New York, Chichester, Brisbane, Toronto & Singapore.

West, W. C., Frattarelli, F. J. & Russin, K. J. 1970. Effect of stratification and gibberellin on seed germination in *Ginkgo biloba*. Bulletin of the Torrrey Botanical Club. 97: p. 380-388.

Wetschinig, W. & Depisch, B. 1999. Pollination biology of *Welwitschia mirabilis* HOOK. f. (Welwitschiaeae, Gnetopsida). Phyton, Horn, Austria. 20: p. 167-183.

Whitelock, L. M. 1995. Cycad conservation in the past and the need for improvement in the furture. In: Proceedings of Cycad 93, the Third International Conference on Cycad biology (Ed. Vorster, P. 1995.), Stellenbosch, The Cycad Society of South Africa. p. 1- 8.

Whitelock, L. M. 2002. The cycads. Timber Press, Portland.

White, R. V. 2002. Earth's biggest 'whodunnit': unravelling the clues in the case of the end-Permian mass extinction. Philosophical Transactions: Mathematical, physical and engineering sciences. 360: p. 2963-2985.

Williams, J. H. 2008. Novelties of flowering plant pollen tube underline diversification of a key life history stage. Proceedings of the National Academy of Sciences. 105: p. 11259-11263.

Wilson, G. W. 1993. Intial observation of the reproductive behaviour and insect pollination in a genus of *Bowenia serrulata*. *Encephalartos*. 36: p. 13-18.

Winter, J. 2006. *Encephalartos latifrons*. PlantZAfrica.com. [Online]Available at: http://www.plantzafrica.com/plantefg/encephlati.htm[Accessed 23 February 2014].

Witte, W. T. 1977. Storage and germination of *Zamia* seed. Proceedings of Florida State Horticultural Society. 90: p. 89-91.

Woodenberg, W., Erdery, D., Pammenter, N. W. & Berjak, P. 2007. Post-shedding seed behaviour of selected *Encephalartos* species. South African Journal of Botany. 73: p. 496.

Yatomi, R., Nakamura, S. & Nakamura, N. 2002. Immunochemical and cytochemical detection of wall components of germinated pollen of gymnosperms, Grana. 41: p. 21-28.

Yessoufou, K., Bamigboye, S. O., Daru, B. & van der Bank, M. 2014. Evidence of constant diversification punctuated by a mass extinction in the African cycads. Ecology and Evolution. 4: p. 50-58.

Zarchini, M., Hashemabadi, D., Kiviani, B., Fallahabadi, P. R. & Negahdar, N. 2011. Improved germination condiction in *Cycas revoluta* L. by using acid and hot water. Plant Omics Journal. 4: p. 350-353.

Apendix 3.1 Results of pollen germination assay (hand drop technique) of 17 *Encephalartos* species, 32 samples from 0 to eight years of freezer storage (-15°C).

Pollen Source	Species	Years of Storage	(%) of Viability
KNBG	*Encephalartos altensteinii*	0	91
KNBG	*E. altensteinii*	0	83
KNBG	*E. altensteinii*	0	74
KNBG	*E. altensteinii*	0	73
KNBG	*E. altensteinii*	0	70
KNBG	*E. altensteinii*	0	63
KNBG	*E. caffer*	0	91
KNBG	*E. latifrons*	0	79
KNBG	*E. manikensis*	0	74
KNBG	*E. princeps*	0	83
KNBG	*E. altensteinii*	1	53
KNBG	*E. caffer*	1	76
KNBG	*E. caffer*	1	50
KNBG	*E. ghellinckii*	1	84
KNBG	*E. lehmannii*	1	77
KNBG	*E. princeps*	1	81
KNBG	*E. villosus*	1	76
KNBG	*E. friderici-guilielmi*	2	27
KNBG	*E. horridus*	2	54
KNBG	*E. humilis*	2	64
WILD	*E. latifrons*	2	94
WILD	*E. latifrons*	2	91
KNBG	*E. latifrons*	2	81
KNBG	*E. latifrons*	2	72
WILD	*E. latifrons*	2	68
KNBG	*E. longifolius*	2	76
KNBG	*E. paucidentatus*	2	57
KNBG	*E. transvenosus*	2	91
KNBG	*E. trispinosus*	2	91
KNBG	*E. latifrons*	3	86
WILD	*E. latifrons*	3	84
KNBG	*E. princeps*	3	45
KNBG	*E. arenarius*	4	21
KNBG	*E. caffer*	4	21
KNBG	*E. ngoyanus*	8	10