EFFECTS OF ACID TREATMENT AND MECHANICAL SCARIFICATION ON SEED GERMINATION OF *ENCEPHALARTOS ALTENSTEINII*

By

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ABSTRACT

Cycads are one of the most threatened plant groups in the world, mainly due to their continued illegal removal from their natural habitat. While South Africa remains a diversity hotspot for cycads with 29 species out of 38 species found worldwide, seventy-eight percent of South African species remain threatened with extinction compared to the worldwide average of 62%. The increasing demand for aesthetic horticultural and medicinal use of the species have led to serious over-harvesting of wild populations. While this shortage of specimen plants presents an opportunity for potential profitable large-scale production of cycads, most species are slow growing and remain difficult to propagate with complex reproductive cycles. This study focused on the sexual propagation of the vulnerable *Encephalartos altensteinii* also known as eastern Cape giant cycad which continue to show low germination rates with prolonged germination periods and high seed viability losses at Pretoria National Botanical Garden. It was hypothesised that the low seed viability results from inadequate environmental storage conditions, desiccation intolerance of seed and germination periods which are influenced by morphophysiological seed dormancies. To investigate desiccation tolerance, seeds were subjected to hydration methods during storage to measure embryo maturity, moisture content and seed viability which were measured at 8 weeks' intervals. To break seed dormancy, seeds were pre-treated with mechanical and chemical scarification using different concentrations of sulphuric acid (H_2SO_4) and, or gibberellic acid (GA_3). The study reported that the growth of embryos of *E. altensteinii* became visible after 6 months of storage while seed hydration did not have significant effects on the embryo growth of this species. Seed moisture content was significantly different ($P < 0.01$) when compared to untreated seeds. The results also showed significantly ($P < 0.05$) high seed viability on hydrated seeds at 86.67% which decreased to 73.33% and compared to 33.33% in the control. Mechanical scarification had a significantly ($P < 0.05$) high germination of 60% on sanded seed beds with a reduced germination period when compared to other treatments. Cracking of seed coats resulted in negative effects on germination. While mechanical scarification was successful to improve germination percentage of *E. altensteinii*, the combination of GA_3 with mechanical scarification did not show a difference on seed germination. Seed pre-treatment with varying concentrations of H_2SO_4 did not influence germination either, however, H_2SO_4 in combination with GA_3 presented a significant difference ($P < 0.05$) on the final seed germination of *E. altensteinii.* The results indicated that the highest final germination of 73.33% was achieved with the $H_2SO_4$2a+$GA_3$1 treatment (25% of H_2SO_4 for 0.5hr followed by 1000ppm GA_3 for 24hrs). These results suggest that while *E. altensteinii* seeds are sensitive to desiccation, seed hydration treatment can slow down moisture content, improve viability loss and extend seed longevity. The seed pre-treatment with both mechanical and chemical scarifications can improve final

germination of *E. altensteinii*. The outcome of the study holds promising possibilities for propagation and cultivation of *E. altensteinii* as well as to reintroduce plants back into the wild.

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DEDICATION

I dedicate this work to my mother Nolungile Nomathembiso Mabuya, in loving memory of my late father and my sisters Nombini Mabuya and Nozalathiso Mabuya who has supported me throughout my academic career. You will always have a special place in my heart.

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LIST OF ACRONYMS AND ABBREVIATIONS

°C - degrees Celsius

ANOVA - Analysis of Variance

BMP - Biodiversity Management Plan

CITES - Convention on International Trade in Endangered Species

CPUT - Cape Peninsula University of Technology

DT – Desiccation tolerance

GA_3 – Gibberellic acid

H_2SO_4 - Sulphuric Acid

HD - Hydration dehydration

ISTA - International Seed Testing Association

IUCN - Union for Conservation of Nature

KZN - Kwa-Zulu Natal

KNBG- Kirstenbosch National Botanical Garden

MC – Moisture content

MPD - Morpho-physiological dormancy

NBG – National Botanical Gardens

NRF - National Research Fund

SANBI – South African National Biodiversity Institute

SE - Standard error

TTC - Triphenyltetrazolium chloride

CHAPTER 1:

INTRODUCTION, BACKGROUND TO RESEARCH PROBLEM, STATEMENT OF THE RESEARCH PROBLEM, HYPOTHESIS, OBJECTIVES AND LAYOUT OF THE THESIS

Introduction, background to research problem, statement of the research problem, hypothesis, objectives and layout of the thesis

1. Introduction

1.1. Background to the research problem

Cycads are one of the most threatened plant groups in the world, mainly due to their continued illegal removal from their natural habitat for their aesthetic horticultural and medicinal uses. *Encephalartos altensteinii* is assessed as Vulnerable (VU) on the Red List of South African plants. The species is endemic to South Africa and mainly occur in the summer rainfall areas of the Eastern Cape Province, and south coast of Kwa-Zulu Natal Province of South Africa where cool winters and hot summers prevail (Jones, 1993). Cycads are dioecious plants, with a long juvenile phase. During their reproductive phase, most species produce cones infrequently, have a poor pollination availability and distribution while also occurring in small populations which all contribute to a negative effect of their reproduction and survival in the wild (Donaldson *et al.,* 2003). Cycads are high in demand, being valued for their ornamental use in landscape designs and medicinal purposes, however due to their complex reproduction cycle most species remain commercially unavailable. Large scale nursery production of cycads remain unsuccessful as most species are difficult to propagate, due to low seed availability as well as being a slow growing group of plants (Dehgan, 1983; Hubbuch, 1987). According to Dehgan and Almira (1993) and Giddy (1996) the commercial demand for cycads could only be met with large scale nursery production of species which would also minimise illegal harvesting and destruction of populations in the wild. As a result of the vulnerability of wild cycad populations mainly due to human impacts such as collecting plants from the wild and their habitat destruction, the Cycad Specialist Group of the International Union for Conservation of Nature (IUCN) advocates for collective efforts to improve propagation and cultivation of cycads (Donaldson *et al.,* 2003).

Cycads are mainly propagated by seed and can aid in the supply of medicinal and horticultural demand for cycads. Seed propagated plants can also be reintroduced into natural habitats to prevent a genetic erosion or extinction of wild populations especially where parent plants are well documented (Walters, 1999). Germination of cycad seeds occurs over extended periods when conditions are favourable when seeds start to germinate after a few months and continue to germinate for a period of a year or more. Different pre-germination treatments have been used to speed up germination where several studies have suggested that germination can be improved by mechanical scarifying cycad seeds or with chemical

scarification using sulphuric acid (H_2SO_4) and gibberellic acid (GA_3). Smith (1978) reported that the rate and percentage of germination in seeds of *Zamia integrifolia* and *Zamia furfuracea* improved with mechanical scarification. Dehgan and Schutzman (1983) and Dehgan and Schutzman (1989) suggested improved germination in *Zamia furfuracea* and *Cycas revoluta* by chemically scarifying the seeds with H_2SO_4 and then soaking them in gibberellic acid (GA_3). However, Pérez-Farrera, *et al.* (1999) noted that the improved results for *Dioon merolae* germination could be achieved by mechanical scarification alone. Cycad seeds are sensitive to desiccation (recalcitrant) where seeds cannot be stored for extended periods of time. The conservation of species remains only successful through living plant collections and re-introductions of population in the wild. According to Broome (2001) and Grobbelaar (2004) soaking cycad seeds every month during storage prolong cycad seeds viability and storage period. To date there are limited research studies on the pre-treatments available to improve the germination of African cycads such as *Encephalartos* species. Investigating the effect of different pre-germination treatments will contribute immensely to improved propagation protocols for African cycad species and aid in the re-introduction of species to conserve the wild populations in their natural habitat. This study therefore aimed to develop a propagation protocol for *E. altensteinii* to enhance future cultivation of the species to support the economic demand and restoration of wild populations.

1.2. Statement of research problem

Cycads are one of the most threatened plant groups with a continued high demand for landscape uses. While most species remain difficult to propagate, research on seed germination of cycads, specifically the African genus, *Encephalartos* remains limited. Propagation trials at the Pretoria National Botanical Gardens where large quantities of seed are being harvested of the species such as *E. altensteinii* have shown low seed germination during extended germination periods after seed storage. Seeds are usually stored for between 6 to 12 months at room temperature for embryos to mature before sowing and germination can commence. A high level of seed viability loss is experienced during this storage period while propagation data has not been published. The purpose of this investigation is therefore to determine an optimal pre-germination treatment methodology to increase seed longevity, and to improve germination percentage, germination rate and embryo development of seed to develop a seed propagation protocol for *E. altensteinii*.

1.3. Hypotheses

1.3.1 It is hypothesised that seeds hydration dehydration treatment will have varying effects on embryo development, longevity and moisture content of *E. altensteinii* seeds while stored at room temperatures.

1.3.2 It is hypothesised that seed pre-treatments prior to sowing will have varying effects on the germination rate and germination percentage of *E. altensteinii.*

1.4. Objectives of the research

The primary aim of this study was to investigate the effects of chemical and mechanical scarification treatments to improve seed germination of *E. altensteinii.* Specific objectives were:

1) To determine the effects of seed hydration dehydration treatment on the moisture content of *E. altensteinii* seeds.
2) To determine the effects of seed hydration dehydration treatment on the embryo development of *E. altensteinii* seeds.
3) To determine the effects of seed hydration dehydration treatment on the viability of *E. altensteinii* seeds.
4) To determine the effects of mechanical scarification on germination percentage, germination rate and seedling growth of *E. altensteinii*.
5) To determine the effects of sulphuric acid (H_2SO_4) on germination percentage, germination rate and seedling growth of *E. altensteinii.*
6) To determine the effects of gibberellic acid (GA_3) on germination percentage, germination rate and seedling growth of *E. altensteinii.*

1.5. Layout of the thesis

The thesis is drafted differently to the alternative of a traditional format for a thesis. The article-format thesis examples of published, co-published and/or "ready-for-publication" articles were prepared during candidature and applies to the format prescribed by CPUT for 100% master's studies which complies to the following principles:

1. The overriding principle of the thesis is that it remains an original contribution to the discipline or field by the candidate.

2. Chapters containing the journal articles form a coherent and integrated body of work, which focused on a single project or set of related questions or propositions. All journal articles form part of the sustained thesis with a coherent theme.
3. The study does not include work published prior to commencement of the candidature.
4. The number of articles included depending on the content and length of each article and take full account of the university's requirements for the degree as well as the one article already published or "ready-for-publication" expected for a master's degree in this discipline.

The thesis should be examined in the normal way and according to the normal requirements as set out by the "Guidelines for Examiners of Dissertations and Theses" (using form HDC 1.7).

The thesis consists of the following chapters which are concisely discussed as:

Chapter One: Introduction, background to research problem, statement of the research problem, hypothesis, objectives and layout of the thesis
This chapter provides an introduction, background an overview of the research problem, aims and hypotheses of the research topic and layout of the thesis.

Chapter Two: The challenges of complex seed germination inhibitors of the endemic South African cycad, Encephalartos altensteinii: A review
This chapter provides background and review of information on what has been researched already on the topic.

Chapter Three: Effects of seed hydration and dehydration treatments on seed viability on the endemic and vulnerable South African cycad, *Encephalartos altensteinii*
This chapter provides the first experimental results, investigating the hydration on the seed viability of *Encephalartos altensteinii*.

Chapter Four: The effects of mechanical scarification on breaking seed dormancy and improving seedling growth of the *Encephalartos altensteinii*, the Eastern Cape giant cycad
This chapter provides the results of mechanical seed pre-treatment on germination percentage, germination rate and seedlings growth of *Encephalartos altensteinii.*

Chapter Five: The effects of chemical scarification on breaking seed dormancy and improving seedling growth of the Encephalartos altensteinii, the Eastern Cape giant cycad.

This chapter provides the results of the chemical seed pre-treatment on germination percentage, germination rate and seedlings growth of *Encephalartos altensteinii.*

Chapter Six: General discussion, conclusion and recommendations
This chapter comprises the general discussion which connects the previous chapters and is followed by the conclusions of the study. Recommendations are made for further work; to introduce future research topics.

Chapter Seven: References

1.6. References

Broome, T. 2001. Optimizing cycad seed germination. *The Cycad Newsletter,* 24(2): 5–7.

Hubbuch, C. 1987. Cycads: Propagation and container culture. *Fairchild Tropical Garden Bulletin,* 42: 5–8.

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

Dehgan, B. & Almira, F. 1993. Horticultural practises and conservation of cycads. In: Stevenson, D.W. & Norstog, K.J. (ed.). *Proceedings of CYCAD 90, the second international conference on cycad biology*. Palm & cycads societies of Australia Ltd, Queensland, Australia, 322-328.

Dehgan, B. & Schutzman, B. 1983. Effect of H_2SO_4 and GA_3 on seed germination of *Zamia furfuracea*. *Hortscience,* 18(3): 371–372.

Dehgan, B. & Schutzman, B. 1989. Embryo development and germination of *Cycas* seeds. *Journal of the American Society for Horticultural Science,* 114(1): 125–129.

Donaldson, J.S., Dehgan, B., Vovides, A.P. & Tang, W. 2003. Cycads in trade and sustainable use of cycad populations. In: Donaldson, J. (ed.). *Cycads: status survey and conservation action plan.* Gland, Switzerland and Cambridge, UK: IUCN, 48–57.

Grobbelaar, N. 2002. *Cycads – with special reference to the Southern African species*. Published by the author, Pretoria, South Africa, pp.331.

Giddy, C. 1996. Cycad specialist group: 6th CITES committee meeting, Tenerife, Canary Island, June 1995. *Species,* 26-27: 106-107.

Jones, D.L. 1993. *Cycads of the world*. Reed, Canberra. 180-181 & 216-217.

Pérez-Farrera, M.A., Vovides, A.P. & Alvarez-Moctezuma, J.G. 1999. A study on seed germination of the cycad, *Dioon merolae* (Zamiaceae). *The New Plantsman,* 6: 214–218.

Smith, G.S. 1978. Seed scarification to speed germination of ornamental cycads (*Zamia* spp.). *Hortscience*, 13: 436–438.

Walters, T.W. 1999. Off-site cycad preservation: what is off-site preservation? *The Cycad Newsletter,* 22(1): 10–12.

CHAPTER 2:

THE CHALLENGES OF COMPLEX SEED GEMINATION INHIBITORS OF THE ENDEMIC SOUTH AFRICAN CYCAD, *ENCEPHALARTOS ALTENSTEINII*: A REVIEW

The challenges of complex seed germination inhibitors of the endemic South African cycad, *Encephalartos altensteinii*: A review

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2.1 Abstract

The demand for cycads in the landscaping industry has increased over the years. This group of plants became one of the most valuable and collectable plants globally and due to their high demand, cycads also became one of the most threatened plants. Seed propagation of cycads to meet the market demand can support conservation efforts as it can counteract the illegal collection of cycads in the wild. Seed germination protocols would support the successful propagation of species for reintroduction programmes to succeed. Sexual propagation cycads is the only sustainable method to increase plant numbers, however seeds germination is complicated and time consuming but can possibly be overcome with mechanical and chemical scarification pre-germination treatments. *E. altensteinii* is a medium to large cycad also referred to as the giant cycad, which grows to 5 meters tall. The species is endemic to South Africa and mainly occurs in the Eastern Cape Province and south coast of Kwa-Zulu Natal Province where plants usually form clumps of two to three stems with basal suckers. Currently this species in listed as Vulnerable (VU) on the Red List of South African plants. This review aimed to highlight interventions required for successful sexual propagation of cycads. These include pollination, seed storage, viability testing, seed collection and germination methodology all to enhance the future conservation of this important species.

Keywords: cycad, dehydration, dormancy, hydration, scarification, seeds.

2.2 Introduction

South African is a diversity hotspot for cycads with 29 native species out of a total 38 species occurring South Africa (Donaldson, 2003). South African species are also the most threatened species with 78% threatened with extinction compared to the worldwide average of 62% (Raimondo *et al*., 2009). According to the Red List of South African plants, 11 species are

critically endangered while 3 species are already extinct in the wild (Bosenberg & Donaldson, 2009; Donaldson, 2008; Donaldson, 2009a Donaldson, 2009b. In efforts to to stop removal of cycads in their natural habitat, all species under genus *Encephalartos* are listed by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) to be prohibit collecting and trading of any plant material of indigenous species from the wild except for research purposes or when plants are artificially propagated. The aim of CITES is to regulate all international trade in endangered species, or species threatened by international trade. These regulations also promote trading of artificial propagated plants to prevent illegal collection in wild and according to CITES database 5850 artificially propagated *Encephalartos* species plants were exported from South Africa between 2017 and 2018 however, the market demand for cycads have not been fully met due to difficulty in propagation resulting from irregular coning, low seeds availability, slow growing and long germination periods. There has also been limited research done on cycad propagation, especially on the African genus *Encephalartos*.

Species recovery activities such as reintroduction were identified as main objective on the Biodiversity Management Plan (BMP) for critically endangered and endangered cycads to achieve in situ conservation for cycads and prevent extinction of wild populations. Cycads are mainly propagated by seed and can aid in the supply of medicinal and horticultural demand for cycads. Seed propagated plants can also be reintroduced into natural habitats to prevent a genetic erosion or extinction of wild populations especially where parent plants accessioned (Walters, 1999). Germination of cycad seeds occurs over prolonged periods. Most species of cycads start to germinate in a few months and continue to germinate for a period of a year or more.

According to Dehgan and Almira (1993), and Giddy (1996) the commercial demand for cycads could only be met with bulk scale nursery production of species which could also reduce illegal harvesting and habitat destruction of populations in the wild. As a result of the vulnerability of wild cycad populations mainly due to human impacts such as collecting plants from the wild, habitat destruction, the Cycad Specialist Group of the International Union for Conservation of Nature (IUCN) promotes for collaborative efforts to improve propagation and cultivation of cycads (Donaldson *et al*., 2003).

The aim of this research is to determine pre-germination treatment that is likely to improve germination percent and reduce germination period, and to determine whether hydration and dehydration treatment can improve seed viability in order to develop propagation protocol for *E. altensteinii*. The results from this study could be potentially used as a baseline study for other *Encephelartos* species, increase production at Pretoria National Botanical Garden and other propagation nurseries in order to meet the demand for cycads and also prevent illegal

harvesting of wild populations with an increase in the germination on seeds used for reintroduction projects.

2.2.1 Overview of cycads

Cycads are a group of gymnosperms, the oldest seed plants, with more than 300 million years, dioecious plants, occur mainly in subtropical and tropical regions (Whitelock, 2002; Donaldson, Hill & Stevenson, 2003). Cycad groups consisting of three families: *Cycadaceae*, *Stangeriaceae* and *Zamiaceae*, altogether made up of 10 genera with 357 species. *Cycadaceae* has only one genus, *Cycas* with 117 species that only occur in Australasia, except for *Cycas thouarsii,* Madagascar species that occurs in South Africa (Donaldson, 2003). The *Stangeriaceae* consist two genera: *Bowenia*, which has two species, endemic to Australia and *Stangeria* consist of one species, only occurs in South Africa and Mozambique. The *Zamiaceae* consists of seven genera: *Cerotozamia, Dioon, Microcycas* and *Zamia* (occurs in America), *Lepidozamia*, *Macrozamia* (occurs in Australia), and *Encephalartos* (endemic to Africa) (Whitelock, 2002; Donaldson *et al*., 2003; Colanje, *et al*., 2020). The African continent is the only continent where all three families occur and South Africa is a diversity hotspot for cycads globally, with 38 species in total (Goode, 2001; Donaldson, 2003).

2.2.2 The genus *Encephalartos*

The African genus*, Encephalartos* comprises of 65 taxa (species and subspecies) which occur primarily from the eastern part of the continent from South Africa to Sudan, extending through the Democratic Republic of Congo and Angola, as well as through Nigeria, Benin and Ghana in the west. Cycads occur in 16 African countries and the islands, Madagascar, Comoros, Seychelles and Zanzibar along the east coast of the continent (Donaldson, 2003). In South Africa, the genus mainly occurs naturally on the eastern part of the country from the Eastern Cape to KwaZulu-Natal as well as in Gauteng, Mpumalanga and Limpopo provinces. There are 37 *Encephalartos* species that occur in South Africa, of which 29 are endemic to the country (Donaldson, 2008). Cycads remains the most threatened plant group, with 78% of South African cycads are listed as “Vulnerable”, “Endangered” or “Critically Endangered” (VU, EN or CR) (Retief, *et al.,* 2014; Fragniere *et al*., 2015; Marler & Marler, 2015; IUCN, 2016), while 3 species (*E. brevifoliolatus*, *E. woodii* and *E. nubimontanus*) have become extinct in the wild (Bosenberg & Donaldson, 2009; Donaldson, 2008; Donaldson, 2009a, 2009b).

Figure 2.1: (A) Cycad plants seized at Pretoria east, (B) illegal wild collection and plant damages of *Encephalartos middelburgensis* in Mpumalanga province. (Picture: A+B Mabuya)

2.2.3 The species *Encephalartos altensteinii*

Encephalartos altensteinii is a medium to large cycad which grows to 5 meters tall. Mature plants usually form clumps of two to three stems with basal suckers. The leaves form a wide spreading crown, 1 to 3 meters long with a leaf-stalk of about 10 to 30 cm long. Mature stems can produce two to five cones with male cones of 40 to 50 cm long and 12 to 15 cm in diameter and female cones 40 to 55 cm long and 25 to 30 cm in diameter.

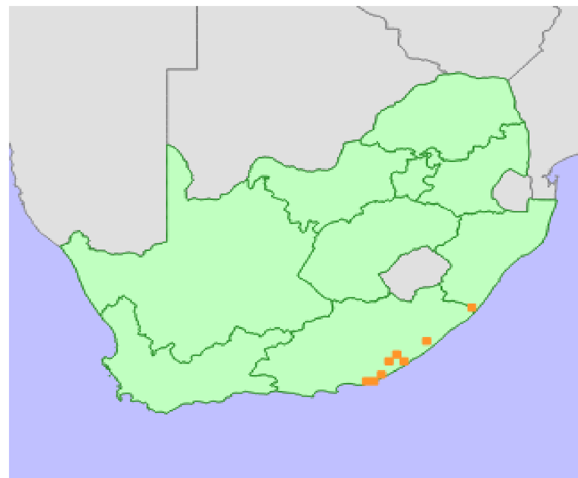

Figure 2.2: The distribution of *E. altensteinii* are highlighted where the species occur naturally in the Eastern Cape Province of South Africa (adapted from Donaldson, 2009c)

Figure 2.3: Female cones of *E. altensteinii* (adapted from Winter, 2004)

Figure 2.4:: Male cones of *E. altensteinii*
(https://davesgarden.com/guides/pf/showimage/57616/)

Figure 2.5: *E. altensteinii* plants in their natural habitat, Eastern Cape Province (Picture: Calonje *et al.*, 2020).

The species, *E. altensteinii* is endemic to South Africa and mainly occurs in the Eastern Cape Province and south coast of Kwa-Zulu Natal Province (Jones, 1993) in wide range of vegetation types such as of Bhisho thornveld, Eastern valley bushveld, Umtiza forest thicket, Fish valley thicket and Albany valley thicket usually in rocky slopes along river banks (Winter, 2004; Donaldson, 2009c). The habitat receives rainfall mainly in summer, ranging from 800 mm to 1000 mm a year. The temperatures are cool in winters with average of 5.3 ºC and hot summers with maximum average temperature of 32.3 ºC where the species have adapted to loamy, gravelly soils in semi-shade and full sun (Mucina *et al*., 2006). The wild population has declined over the years due to habitat destruction for development and collection, currently this species in listed as Vulnerable (VU) on the Red List of South African plants, with an estimate of only 10 000 plants left in the wild (Winter, 2004; Donaldson, 2009c).

2.3 Cycads reproduction

2.3.1. Pollen storage

The short-term and long-term storage for pollen remains important in ex situ cycad collection where there are limited plants, no pollinators, to prevent hybridization of species and for future artificial pollination. Storage period varies from species and depends on the storage conditions and moisture content. Osborne *et al*. (1991, 1992) reported that low moisture content and low storage temperatures improve pollen longevity on cycad species a pollen can be stored between 3 to 5 years for *Encephalartos* species. Similar observations were noted by Xaba (2014) with *Encephalartos* pollen dried in silica gel. However different results were reported by Mostert (2000), suggesting that pollen for *Encephalartos* species respond differently on cold storage. There is a need for further studies on the long-term storage of cycads pollen. Research studies should focus on the effect of temperature, moisture content and drying methods to improve pollen longevity for cycads.

2.3.2. Cycad pollination

All cycad species, female and male reproductive structure are from separate individual plants (Chamberlain, 1935) and pollination require transfer of pollen from male to female plants. According to Chamberlain (1935) gymnosperms are pollinated by wind, however recent studies on cycad pollination concluded that cycads are insect pollinated. Tang (1987), and Pellmyr *et al.* (1991) reported change in cone temperature and odor which indicated similarities with insect pollinated plant species and similar observations were reported in recent studies that confirmed that cycads are insect pollinated (Donaldson, 1997 Stevenson *et al*., 1998; Terry *et al*., 2007). Artificial hand pollination is commonly used in ex situ cycad collections where there is limited matured plants, male and female plants not coning at the

same time, no pollinators in the collection and to prevent hybridization. Female cones open sporophylls for a few days for pollination and close again, ovulation period varies from species to species (Tang, 1996; Jones, 2002), Xaba (2014) observed that *E. altensteinii* cones open for a period of 14 to 25 days. There are two methods used for cycads artificial pollination, dry and wet pollination methods however, there are limited research studies on the effectiveness between these pollination methods (Grobbelaar, 2002). According to Xaba (2014) there was no significant difference between dry and wet methods however, wet artificial pollination had negative effect on seed viability on *E. latifrons*. Tang (1996) reported that the dry pollination method had high seed viability on *E. ferox* seeds. There was significantly high germination on cones *E. altensteinii* pollinated in the afternoon (Xaba, 2014). These results suggested that there is a need to conduct more studies on the effectiveness of different artificial methods on the wide range of cycad species in order to develop artificial pollination protocols for cycads. Improved pollination methods could help to increase seed availability for large scale production of cycads to meet the market demand for cycads across the globe.

2.3.3. Seed storage and germination

All species in the genus *Zamia, Dioon* and *Microcycas*, also few species of *Encephalartos* such as *E. transvenosus* and *E. manikensis,* keep their seeds in the mother plant until the embryo is fully developed which could take between 1 to 12 months for the embryo to be fully developed. In contrast most cycad species shed their seeds with underdeveloped embryos due to several factors (Dyer, 1965; Giddy, 1974; Burch, 1981; Grobbelaar, 2004; Woodenberg, *et al*., 2007; Xaba, 2014). Embryo development of *Encephalartos* is divided into four stages, the differentiation of a small mass of meristematic tissue; (2) the rapid division and elongation of cells, forming the suspensor; (3) the differentiation of the cotyledons and stem apex; (4) the development of plumular leaves and the fusion of the distal halves of the cotyledons (Saxton, 1910). Different storage conditions have been reported for cycad seed, according Forsyth and van Staden (1983) both dry and moist storage are effective for *E natalensis* while the same observations were reported by Witte (1977) on *Zamia integrifolia* stored at low temperatures. Dehgan and Schutzman (1989) reported improved germination on *Cycas revoluta* on seeds stored at low temperature (5 ˚C) when compared 22 ˚C. Similar observations were reported to be more effective on seeds of *C. taitungensis* stored at 5 ˚C under dry conditions (Chien, *et al*., 2012). Hendricks (1980) alternatively recommended storing the seed under cool conditions (60-75°F). Conflicting results were also reported in some studies with Vorster (1995) who observed no significant difference in germination between seed stored in cold storage compare to room temperature on *Cycas revoluta* seeds. Rapid germination was observed on *C. revoluta* when seeds were stored at 25 ˚C (Frett, 1987). The high light intensity encouraged

embryo development more rapidly than low light intensity and there was a noticeable rise on the embryo fresh weight and elongation of the embryo when they were exposed to low light intensity (Vargas–Luna, *et al.*, 2004). There is also limited research work done to understand how cycad seeds respond to different storage conditions across all the taxa and currently there are no effective methods for long term seed storage for cycads. For these reasons it remains important to conduct further studies on the seed storage behaviour in order to develop storage protocols and to conserve species for future generations.

2.3.4. Seed dormancy

Seed dormancy is divided into two types, morphological dormancy and physical dormancy. Morphological dormancy is a result of limiting factors in embryo germination, such as immature or developing embryo. Physical dormancy is a result of structural factors preventing germination, such as endosperm and seed coat (Nikolaeva, 1977). Cycad seeds may have morpho-physiological dormancy (MPD) (Witte, 1977; Dehgan, 1996). MPD is a combination of morphological and physiological dormancy. Some cycads species do not experience MPD, *E. transvenosus* and *E. manikensis* keep their seeds in the cones until they are fully developed and therefore able to germinate immediately after abscission (Grobbelaar, 2004). Dormancy is a common phenomenon in cycads with seeds being shed while their embryos are still immature which could take approximately 4–12 months of storage for the pro-embryo to mature for the seeds to germinate (Grobbelaar, 2004; Woodenberg *et al.*, 2007). A study conducted on the post-shedding seed behaviour of *E. villosus*, *E. gratus and E. natalensis* by Woodenberg *et al.* (2007) showed that the seeds were shed with relatively high water contents (2.3–6.0 g g^{-1}) and that a drying trial confirmed that they are recalcitrant and cannot be dried for long-term storage.

Many *Encephalartos* species in South Africa shed their seeds in spring (August-October) to summer (November-January), but due to their immature embryos they are unable to germinate before the end of the summer rainy season. This biorhythm is schedule until favourable conditions for germination return the following spring/summer season. In most cases the viability of *Encephalartos* seeds starts to decrease after 2 years of storage in open storage at room temperature, although some can remain viable for up to 5 years (Grobbelaar, 2004). For successful germination of cycad seeds under nursery conditions it is recommended that the dormant seeds are stored in open containers (Grobbelaar, 2004). Before seeds are stored, their fleshy layer (sarcotestae) is removed as documented as it inhibits embryo development and prevent germination (Grobbelaar, 2004). With the current high demand globally and the

need for ex situ and in situ conservation for cycads, it is important to understand their seed dormancy mechanisms in order to apply suitable techniques to improve seed germination.

2.3.5. Temperature and germination

The conditions under which seeds develop and mature can regulate the rate of seed germination and dormancy, which influence time of germination (Roach & Wulff, 1987; Meyer & Allen, 1999; El-Keblawy & Al-Rawai, 2006). Environmental conditions experienced by plants in the growing period have revealed to play a significant role in determining subsequent germination responses in seeds of many species (Meyer & Monsen, 1991; El-Keblawy & Al-Ansari, 2000; Galloway, 2002).

Many studies suggest that most cycad species germinate well at a temperature between 25 to 30 °C. Dandugula (2011) reported high germination rates of *Cycas revoluta* seeds sown at 25 °C and similar results were reported on *Cycas siamensis* (Umair, 2011). Xaba (2014) reported 60% germination of *E. altensteinii* seeds at sown at 28 °C and these results concur with warm temperature of 30 °C described by Witte (1977) on seeds germination of *Zamia integrifolia* and *Z. floridana,* while lower temperatures of 15 to 20 °C have been suggested for *C. revoluta* (Zarchini *et al.,* 2011).

2.3.6. Light and germination

Among cultivated plants there is very little evidence for light as a factor influencing germination as most seeds equally germinate well both in the dark and under light conditions (Fenner, 1985). In contrast, among wild plants there have been a difference observed in the behaviour of seeds toward light intensity and photoperiods as a requirement for germination. So species germinate well under light conditions compared while other are more successful in germination under dark conditions (Baskin & Baskin 1988). Light plays a major role in the breaking of dormancy especially in nature where light controls the timing of germination of seeds (Fenner, 1985). Direct sunlight may prevent seed germination of some species; research showed that high irradiances prevent germination on *Lactuca sativa* seeds (Gorski & Gorska, 1979).

Seeds may be divided into those which germinate only in the dark, seed which germinate only under continuous light, seed which germinate after being given brief illumination period and seed which are indifferent to the presence or absence of light during germination. Under natural conditions seeds may be shed to fall on the soil or be covered by leaf litter, thus creating different conditions of light during germination. Exposure to light, fluctuations in temperature, or combinations of these factors may be needed to relieve residual and induced

dormancy at times of low dormancy (Fenner, 1985). The seeds of some species may require light at one temperature, but no light at another (Pons, 1992).

According to Frett (1987) sowing seeds in the dark doubled the germination percentage of *Cycas revoluta*. As the germination light/dark requirements for *Encephalartos* is relatively unknown it remains uncertain how *E. altensteinii* seeds respond to germinating in the dark. This species occurs mainly in thicket vegetation, in semi-arid areas, therefore it is important to understand how seeds respond when planted in the dark. Successful germination in the dark can help with future reintroduction projects, it will suggest that seeds can be planted in shade under the shrubs and trees to protect seeds and seedlings from hot dry conditions in the natural habitat.

2.3.7. Seeds hydration and germination

Many plants species experience stress in the natural habitat such as irregular variation in temperature, water availability during the germination period while excess salinity can also be problematic, however many plants have a range of mechanisms to adapt to these stressful conditions during germination of seed (Ingram & Bartels, 1996). In semi-arid and arid areas discontinuous seed imbibition occur without any intervention on the natural habits (Lima & Meiado, 2017). All species of *Encephalartos,* seeds are covered with fleshy layer (sarcotestae) that helps to prevent seed desiccation, while some studies suggested that the sarcotestae should be removed as it inhibits embryo development and prevent successful germination (Grobbelaar, 2004).

Seed hydration and dehydration treatments during storage could have positive effects on seed desiccation tolerance, germination percent and longevity. According to Lima and Meiado (2017) hydration dehydration (HD) treatments showed improvement on germination and environmental stress tolerance of *Pilosocereus catingicola* subsp. *salvadorensis* seeds. Lima *et al*. (2018) also reported the same observations on the HD treatments of *Senna spectabilis* var. *excels* seeds. Demir and Mavi (2003) reported an increase in the longevity of *Solanum melongena*, and according to Grobbelaar (2004) and Broome (2001), HD treatments improved viability and storage period for cycad seeds. However, conflicting results on similar studies were observed with Lima *et al*. (2018) that HD treatments had negative effect on germination of *Macroptilium atropurpureum* and with the same results being reported on the germination of *Echinocereus engelmannii* (Santini *et al*., 2017), these studies suggest that HD treatments may have positive and or negative effect on seed longevity and germination, depending on the species and the maturity of the seeds. It is therefore not clear what the effect of seed

hydration on *E. altensteinii* seed germination. Most *Encephalartos* species require 6 to 12 months of storage for embryo development and many cycad growers in general and in particular the Pretoria NBG do not have cold storage facilities for seed storage and seeds are stored at room temperatures. Under these conditions many seeds lose their viability within short period of time which has a negative impact on the production of rare and threatened species. Considering these discussions on the influence of temperature and moisture content of seeds, it remains important that further studies being conducted to improve the knowledge of seed biology on the effects of hydration on seed viability of seeds stored at a varied range of conditions to enhance the reproduction of cycad species.

2.3.8. Mechanical scarification and germination

Cycad seeds have a fleshy outer layer that covers hard seed coat. A study on *Cycas revoluta* showed that the outer fleshy layer inhibits embryo development and germination. The removal of cycad seeds fleshy layer before seed storage could improve embryo development and germination (Burch, 1981; Frett, 1987; Dehgan, 1983). Most *Encephalartos* species shed their seeds with underdeveloped embryos and germination can be improved by storing the seeds for a period of 6 months before sowing. Cycad seeds also have water-impermeable seed coats and dormancy can be broken by physically making seed coat water-permeable for seeds to germinate. The Smith (1974) and Witte (1977) reported that removal and cracking hard seed coat improved germination rate of *Zamia* seeds. However, Broome (2001) reported that cracking seed coat on cycad seeds is only effective when seeds are sown under aseptic conditions. There is limited research on the effect of mechanical scarification on germination of *Encephalartos* species, therefore it remains important to test these methods to improve germination of which could indirectly allowed increase propagation success to support the demand for cycads and conservation of this threatened plant group.

2.3.9. Chemical scarification and germination

Breaking physical seed dormancy of cycad species has been achieved by chemical scarification with H_2SO_4 (Frett, 1987; Zarchini *et al.,* 2011), and also soaking seeds in hot water at 100 ˚C for 1 hour and a 25% diluted H_2SO_4 for 2 hours (Zarchini *et al.*, 2011). Pre-treatment of cycad seeds with GA_3 has been reported to stimulate rapid embryo growth and seed germination (Dehgan, 1983). Different soaking periods in GA_3 solutions (24 h or 48 h) in *Encephalartos* species are reported to improve the germination period and uniform germination (Dehgan, 1999). Combinations of chemical scarification with H_2SO_4 and soaking in GA_3 have resulted to 100% germination within four weeks of *Zamia floridana* (Dehgan & Johnson, 1983). *Cycas revoluta* showed improved germination when treated with H_2SO_4

(Zarchini *et al.*, 2011). According to Frett (1987) *Cycas revoluta* seed treated with GA_3 at 500, 1000 and 5000 pm for 12 h resulted to lower seed germination when compared with untreated (control). Xaba (2014) reported that there was no significant difference in seed pre-treatment with GA_3 on *E. latifrons* and *E. altensteinii* respectively, and these results disagree with the other studies on cycad seed germination and this raised a question whether scarification or a combination of scarification of seed coat and growth regulator (GA_3) treatments could improve the germination of selected *Encephalartos* species. It is uncertain how *E. altensteinii* seed would respond to a pre-germination treatment with growth regulators, as growth regulators have shown an improvement on improved germination. Testing this methodology would support future propagation of the species for commercial and conservation purposes.

2.3.10. Conclusion

The need to conserve and safeguard South African cycad species have increased over the years as wild populations continue to decline and the demand for speciality plants as ornamentals in horticulture industry medicinal uses have value surpasses supply worldwide. Currently there is limited baseline knowledge on the reproduction and propagation of *Encephalartos* species to support conservation programmes. Challenges of poor seed germination, viability loss and slow growing of *E. altensteinii* necessitate the need to investigate the effects of different propagation methods to increase the production of the species. While different techniques to improve germination of cycads have been tested in some species, there remain limited knowledge on seed biology, seed storage behaviour and sexual propagation of *E. altensteinii.* Future research could also explore asexual propagation methods such as in vitro culture and long-term seed storage. Future research on *Encephalartos* seed behaviour will not only contribute to improve propagation methodology to increase cultivation, but also support conservation of this threatened group of plants.

2.4. Acknowledgement

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2.5. References

Baskin, C.C. & Baskin, J.M. 1988. Germination of ecophysiology of herbaceous plant species in a temperate region. *American Journal of Botany*, 75: 286-305.

Bosenberg, J.D. & Donaldson, J.S. 2009. *Encephalartos brevifoliolatus* Vorster. National Assessment: Red List of South African Plants version 2017.1. http://redlist.sanbi.org/species.php?species=823-44. [Accessed on 2018/07/05].

Broome, T. 2001. Optimizing cycad seed germination. *The Cycad Newsletter,* 24(2): 5–7.

Burch, D. 1981. Propagation of Florida *Zamia* from Stem Pieces. SNA Research Conference, 221-223.

Calonje, M. Stevenson, D.W. & Osborne R. 2013-2020. The World List of Cycads. online edition.https://www.cycadlist.org/gallery.php?scientific_name=encephalartos+altensteinii&genus=-1&country=-1. [Accessed 2020/08/06].

Chamberlain, C.J. 1935. Gymnosperms: Structure and Evolution. University of Chicago Press.

Chien, C.T., Chen, S.Y., Chang, S.H. & Chung, J.D. 2012. Seed Germination and Storage of *Cycas taitungensis* (Cycadaceae). *Taiwan Journal for Science*, 27(1): 1-11.

Convention on International Trade in Endangered Species. 2019. The CITES Trade Database. https://trade.cites.org/en/cites_trade/download/view [Accessed on 2020/06/19].

Dadugula, S.R. 2011. Assessment of desiccation and freezing sensitivity towards development of cryopreservation protocols for three cycad species: *Cycas revoluta*, *Dioon edule* and *Zamia furfuracea*. MSc. Thesis, University and Bedfordshire and Royal Botanic Gardens Kew, UK.

Dehgan, B. & Almira, F.C. 1993. Horticultural practices and conservation of cycads. In: Stevenson, D.W & Norstog, K.J. (eds.), The biology, structure, and systematics of the Cycadales: *Proceedings of CYCAD 90, the Second International Conference on Cycad Biology*. Palm & Cycad Societies of Australia Cycads, Milton, Queensland, Australia. 332–338.

Dehgan, B. 1996. Permian permanence. *American Nurseryman*, 183(2): 66.

Dehgan, B. & Schutzman, B. 1989. Embryo development and germination of *Cycas* seeds. *Journal of the American Society for Horticultural Science,* 114(1): 125–129.

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

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

Dehgan, B., & Johnson, C.R. 1983. Improved Seed Germination of *Zamia floridana* with Sulphuric Acid and GA3. *Scientia Horticulturae*, 19: 357-361.

Department of Environmental Affairs. 2017. Biodiversity Management Plan for 11 critically endangered and 4 endangered *Encephalartos* species. https://cer.org.za/wp-content/uploads/2004/09/National-Environmental-Management-Biodiversity-Act-10-2004-Biodiversity-Management-20170428-GGN-40815-00315.pdf. [Accessed on 2020/07/22].

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

Donaldson, J. S. 2008. South African *Encephalartos* species. NDF workshop case studies: Case study 4: *Encephalartos*. Mexico.

Donaldson, J.S. 2009a. *Encephalartos woodii* Sander. National Assessment: Red List of South African Plants version 2017.1. http://redlist.sanbi.org/species.php?species=823-42. [Accessed on 2018/07/05].

Donaldson, J.S. 2003. Regional Overview: Africa. In: Donaldson, J. (ed.). *Cycads: status survey and conservation action plan.* Gland, Switzerland and Cambridge, UK: IUCN, 9–19.

Donaldson, J.S. 2009b. *Encephalartos nubimontanus* P.J.H. Hurter. National Assessment: Red List of South African Plants version 2017.1. http://redlist.sanbi.org/species.php?species=823-49. [Accessed on 2018/07/05].

Donaldson, J.S. 2009c. *Encephalartos altensteinii* Lehm. National Assessment: Red List of South African Plants version 2017.1. http://redlist.sanbi.org/species.php?species=823-2. [Accessed on 2018/06/19].

Donaldson, J.S., Hill, K.D. & Stevenson, D.W. 2003. Cycads of the World: An Overview. In:

Donaldson, J. (ed.). *Cycads: status survey and conservation action plan.* Gland, Switzerland and Cambridge, UK: IUCN, 3–8.

Dyer, R. A. 1965. The cycads of Southern Africa. *Bothalia,* 8: 405- 515.

El-Keblawy, A. & Al-Ansari, F. 2000. Effect of site of origin, time of seed maturation and seed age on germination behavior of *Portulaca oleracea* L. from old and new world. *Canadian Journal of Botany*, 78: 279-287.

El-Keblawy, A. & Al-Rawai, A. 2006. Effects of seed maturation time and dry storage on light and temperature requirements during germination in invasive *Prosopis juliflora*. *Flora*, 201: 135-143.

Fenner, M. 1985. *Seed Ecology.* London: Chapman and Hall.

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

Fragniere, Y., Betrisey, S., Cardinaux, L., Stoffel, M. & Kozlowski, G. 2015.Fighting their last stand? A global analysis of the distribution and conservation status of gymnosperms. *Journal of Biogeography,* 42: 809– 820.

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

Galloway, L.F. 2002. The effect of maternal phenology on offspring life history in the herbaceous plant *Campanula americana. Journal of Ecology* 90: 851-858.

Giddy, C. 1974. *Cycads of South Africa*. Purnell and Sons Ltd, Cape Town, South Africa.

Giddy, C. 1996. Cycad Specialist Group: Cycad action plan. Species 26–27: 106–107.

Goode, D.G. 2001. *Cycads of Africa.* Volume I. D & E Cycads of Africa Publishers, Sandton.

Gorski, T. & Gorska, K. 1979. Inhibitory effects of full daylight on the germination of *Lactuca sativa* L. *Planta,* 144: 121-124.

Grobbelaar, N. 2002. *Cycads* – with special reference to the Southern African species. Published by the author, Pretoria, South Africa, 331.

Grobbelaar, N. 2004. Cycads: With special reference to the southern African species. Published by the author. Pretoria.

Hendricks, I.G. 1980. Germination of cycad seed. *The Cycad Newsletter,* 3: 3-8.
Ingram, J. & Bartels, D. 1996. The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 47: 377–403.

IUCN 2016. The IUCN red list of threatened species. Version 2016.3. http://www.iucnredlist.org. [Accessed 05 July 2020]

Jones, D.L. 1993. *Cycads of the world.* Reed, Canberra, 180-181 & 216-217.

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

Lima, A.T. & Meiado, M.V. 2017. Discontinuous hydration alters seed germination under stress of two populations of cactus that occur in different ecosystems in Northeast Brazil. *Seed Science Research*, 27: 292-302.

Marler, P.N. & Marler, T.E. 2015. An assessment of red list data for the Cycadales. Tropical Conservation Science 8: 1114–1125. www.tropicalconservationscience.org. [Accessed on 2018/06/19].

Meyer, S.E. & Allen, P.S. 1999. Ecological genetics of seed germination regulation in *Bromus tectorum* L. II. Reaction norms in response to a water stress gradient imposed during seed maturation. *Oecologia*, 120: 35-43.

Meyer, S.E. & Monsen, S.B. 1991. Habitat-correlated variation in mountain big sagebrush (*Artemisia tridentate* ssp. *vaseyana*) seed germination patterns. *Ecology*, 72: 739-724

Mostert, C. 2000. Aspekte van die Keimingsgedrag en Fynstruktuur van *Encephalartos stuimeel.* MSc Thesis. University of Pretoria, South Africa.

Mucina, L. & Rutherford, M.C. (eds) 2006. *The vegetation of South Africa, Lesotho and Swaziland.* Strelitzia 19. South African National Biodiversity Institute, Pretoria.

Nicolaeva, M.G. 1977. Factors controlling the seed dormancy pattern. In: Khan AA (Ed) *The Physiology and Biochemistry of Seed Dormancy and Germination*. North Holland, Amsterdam, Netherlands, 51–74.

Osborne, R., Robbertse, P.J. & Claassen, M.I. 1992. The longevity of cycad pollen in storage. *South African Journal of Botany*, 58(4): 250–254.

Palmbob. 2004. *Encephalartos* Species, Altenstein's Cycad, Bread Tree, Eastern Cape Giant Cycad, Prickly Cycad (*Encephalartos altensteinii*). Dave's Garden. https://davesgarden.com/guides/pf/showimage/57616/. [Accessed 20 July 2020]

Pellmyr, O., Tang, W., Groth, I., Bergstrom, G. & Thien, L.B. 1991. Cycad cone and angiosperm floral volatiles: inferences for the evolution of insect pollination. *Biochemical Systematics and Ecology*, 19: 623– 627.

Pons, T. 1992. The role of temperature in the regulation of dormancy and germination. In: M. Fenner, ed. *Seeds: The ecology of regeneration in plant communities.* Wallingford: CABI Publishing, 261-292.

Raimondo, D., Von Staden, L., Foden, W., Victor, J.E., Helme, N.A., Turner, R.C., Kamundi, D.A. & Manyama, M.A (eds). 2009. *Red list of South African Plants*. Strelitzia 25. South African National Biodiversity Instituted. Pretoria

Retief, K., West, A.G. & Pfab, M.F. 2014. Can stable isotopes and radiocarbon dating provide a forensic solution for curbing illegal harvesting of threatened cycads? *Journal Forensic Science,* 59(6): 1541-1551.

Roach, D.A. & Wulff, R. 1987. Maternal effects in plants. Annual Review of Ecology, *Evolution, and Systematics*, 18: 209-235.

Saxton, W.T. 1910. The Development of the Embryo of *Encephalartos*. *Botanical Gazette*, 49(1): 13-18.

Smith, G.S. 1974. Seed treatments to speed germination of *Zamia integrifolia*. *Florida State Horticultural Society,* 436–438.

Stevenson, D.W., Norstog, K. & Fawcett, P. 1998. Pollination biology of cycads. In: Owen, S. & Rudall, P. (ed) *Reproductive biology: In systematics, conservation, and economic botany*. Royal Botanic Gardens, Kew, UK.

Tang, W. 1986. Pollinating cycads. *Encephalartos*, 8: 16–19.

Tang, W. 1987. Heat production in cycad cones. *Botanical Gazette*, 148: 165–174.

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

Umair, S. 2011. Cycad Seed Biology and Embryo Cryopreservation: Especially *Cycas siamensis*, *Dioon edule* and *Zamia floridana*. MSc Thesis, University of Bedfordshire and Royal Botanic Gardens Kew, UK.

Vargas–Luna, I., Ortiz–Montiel, G., Chávez, V.M., Litz, R.E. & Moon, P.A. 2004. Biochemical characterization of developmental stages of cycad somatic embryos. *The Botanical Review,* 70(1): 54-62.

Vorster, P. 1995. Aspects of reproduction of cycads. An annotated review of known information in Vorster, P. (ed.). *Proceedings of the Third International Conference of Cycad Biology.* Stellenbosch, South Africa: cycad Society of South Africa, 379–387.

Walters, T.W. 1999. Off-site cycad preservation: I. what is off-site preservation? *The Cycad Newsletter*, 22(1): 10–12.

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

Winter, J. 2004. *Encephalartos altensteinii* Lehm. South African National Diversity Institute. (Online). http://pza.sanbi.org/encephalartos-altensteinii. [Accessed 20 July 2018].

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

Woodenberg, W.R., Erdey, D., Pammenter, N., and Berjak, P. 2007. Post-shedding seed behaviour of selected *Encephalartos* species. Abstracts from the 5th International Workshop on Desiccation Tolerance and Sensitivity of Seeds and Vegetative Plant Tissues. *South African Journal of Botany,* 73(3): 496.

Xaba, P.M.A. 2014. Pollination and Germination as Limiting Factors in the Propagation of Threatened Cycads, *Encephalartos (Zamiaceae*). MSc Thesis, University of Western Cape, South Africa.

Zarchini, M., Hashemabadi, D., Kaviani, B., Fallahabadi, P.R. & Negahdar, N. 2011. Improved germination conditions in *Cycas revoluta* L. by using sulphuric acid and hot water. *Plant Omics Journal*, 4 (7): 350-353.

CHAPTER 3:

EFFECTS OF SEED HYDRATION DEHYDRATION TREATMENTS ON SEED VIABILITY ON THE ENDEMIC AND VULNERABLE SOUTH AFRICAN CYCAD, *ENCEPHALARTOS ALTENSTEINII*

Effects of seed hydration dehydration treatments on the seed viability of endemic and vulnerable, *Encephalartos altensteinii*

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3.1 Abstract

Encephalartos altensteinii is a medium to large cycad which grows to 5 meters tall. Mature plants usually form clumps of two to three stems with basal suckers. The species is listed as vulnerable, and endemic to South Africa, mainly occurs in the Eastern Cape Province and south coast of Kwa-Zulu Natal Province in thicket biome, usually in rocky slopes along riverbanks. Seeds of most cycad species are sensitive to desiccation and cannot be stored for long-term period. High seed viability loss at Pretoria NBG were observed on seeds stored at room temperature. To improve seed longevity of *E. altensteinii*, the effects of hydration dehydration treatments on embryo development, moisture content and viability were studied. Seeds were hydrated (24 hrs) and dehydrated (96 hrs) every month during storage for 8 months. During this study matured embryos were only observed after 6 months of storage and embryo length had a high increase after 12 months of storage for both treated and untreated seeds. This study showed that hydration dehydration had positive effects on the moisture content and viability on *E. altensteinii* seeds stored at room conditions and that further decrease on moisture content was detrimental.

Key words: dehydration, desiccation tolerance, discontinuous seed inhibition, hydration, moisture content, viability

3.2 Introduction

Many plant species experience stress conditions in their natural habitat, such as fluctuating temperatures, prolonged drought and excess saline levels while several species have developed a wide range of tolerance mechanisms to survive under these conditions (Ingram & Bartels, 1996; Ntuli, 2012; Dinakar & Bartels, 2013). In semi-arid and arid areas discontinuous seed imbibition is a common phenomenon in natural habits due to a lack of water availability. Experimental work completed on plant structures, such as pollen and bulbs

have measured water loss and tolerance levels. In seeds there have been described as a test for desiccation tolerance (DT). DT ability in plants depends on the species, type and maturity of seeds, with some seeds become DT at the last stages of seed development (Leprince *et al.*, 1993; McCarty, 1995). All species in the genus *Zamia, Dioon* and *Microcycas*, also few species of *Encephalartos* such as *E. transvenosus* and *E. manikensis,* keep their seeds in the mother plant until the embryo is fully developed, however most cycad species shed their seeds with underdeveloped embryos after which it could take between 1 to 12 months for the embryo to be fully developed (Dyer, 1965; Giddy, 1974; Burch, 1981; Grobbelaar, 2004; Woodenberg, *et al.*, 2007; Xaba, 2014). During the developmental period, seeds undergo embryonic development during storage period where higher moisture levels are required during these developmental stages such as recalcitrant seeds which should be exposed to continuous moisture content (MC) (Pammenter & Berjak, 1999). Many studies have reported that seeds loose DT during germination (Roberts 1973; Buitink *et al.*, 2003; Rodriguez *et al.*, 2010; Maia *et al.*, 2011; Maia *et al.*, 2014;), however over time DT can be re-established in germinated seeds (Buitink, 2003; Maia *et al.*, 2011; Maia *et al.*, 2014).

Storage period for seeds is influenced by seed moisture content (MC) and the regulation of storage temperatures (Roberts, 1972; Forsyth & van Staden, 1983; Chien *et al.,* 2012). Viability of *Ginkgo biloba* seeds stored at 25 ˚C decreased to 53% in six months and to 0 % in 9 months, compared to seeds stored at 4˚C which only declined to 87% after twelve months (Tommasi *et al.*, 2006). A similar study also suggested that high storage temperatures had a negative effect on the seed viability in *Euphorbia esula* where seeds were stored at 30˚C showed faster decline on viability compared to seeds stored at 5 ˚C (Foley, 2008). Chien *et al.* (2012) observed no significant difference in germination of *Cycas taitungensis* seeds stored at 5 ˚C compared to fresh seeds, however early germination was observed on stored seeds compared to germination after 20 weeks for fresh seeds. According to Frett (1987) *Cycas revoluta* seeds stored at 25 ˚C showed a high mean time germination, with 36% of germination after three months. Dehgan and Schutzman (1989) observed faster embryo growth on *Cycas revoluta* seeds stored at room temperatures than seeds stored at 5 ˚C, however viability loss was high, with a decline of 58% compared to seeds stored at 5 ˚C with only 5% decline.

Most plant species with recalcitrant seeds usually drop their seeds with a high MC (Roberts, 1973; Forsyth & Van Staden, 1983; Woodenberg *et al.,* 2007) while sensitive to desiccation, in contrast to orthodox seeds where low MC is more beneficial for long term storage. A high percentage decrease on seed viability of *Citrus reticulate* was observed when seeds were desiccated to 15% 0r 7% MC (Khan *et al.*, 2001). According to De Andrade (2001), *Euterpe edulis* seeds indicated similar results in seeds stored at a high (40-44%) MC with high

germination rates where seeds remained viable only during the first nine months of storage compared to seeds stored at a lower MC (36%). Other studies showed that a high MC had a negative effect on viability and growth while a low MC (7-5%) improved longevity in seeds of *Cannabis sativa* (Parihar *et al*., 2014). Tompsett and Pritchard (1998) reported that reducing seed MC resulted in an increase in germination of *Aesculus hippocastanum* L.

Seed hydration dehydration (HD) treatments during storage has positive effects on desiccation tolerance, germination percent and longevity, according to Lima and Meiado (2017) HD treatment showed improvement on germination and environmental stress tolerance of *Pilosocereus catingicola subsp. salvadorensis* seeds while Lima *et al*. (2018a) also reported similar observations on the HD treatments of *Senna spectabilis var. excels* seeds. Demir and Mavi (2003) showed an increase on longevity of *Solanum melongena,* while according to Grobbelaar (2004) and Broome (2001), HD treatment prolonged the longevity for cycad seeds. Conflicting results on similar studies were observed, showing that HD treatments had a negative effect on germination of *Macroptilium atropurpureum* (Lima *et al.,* 2018b). Similar results were reported on the germination of *Echinocereus engelmannii* (Santini, *et al*., 2017). All these studies suggested that HD treatments may have positive or negative effects on seed longevity, depending on the species and the maturity of seeds.

Encephalartos altensteinii occurs in summer rainfall, semi-arid areas in South Africa where seeds experience discontinuous hydration in the natural habitat. At Pretoria National Botanical Garden cycad seeds lose viability after 6 months of storage at room temperature while limited research on the effect of HD on seed MC, viability and embryo growth of cycad seeds have been done. The aim of this study was to measure the effect of HD on seed MC, viability and embryo growth in order to develop a protocol for *E. altensteinii* seed storage to improve longevity and enhance future cultivation of the species to support the economic demand and restoration of wild populations.

3.3 Materials and Methods

3.3.1 Seed hydration dehydration experiment

The experiment was conducted at Pretoria National Botanical Garden, East of Pretoria, in Gauteng province, South Africa, -25°44’18.2″ S 28°16’19.8″ E in the seed storeroom at the production nursery from February 2019 to March 2020. The seed storeroom environmental conditions were not controlled; seeds were stored at room temperature. Seeds were first cleaned and dusted with Efekto Fungi-Nill 500 WP Captab fungicide to prevent development

of fungi, placed in mesh bags and stored under dry conditions at room temperature during the experiment (Hendricks, 1980).

3.3.2 Artificial Pollination

Due to unavailability of commercial seed, CITES restrictions on wild seed collections, low seed viability, cross pollination in ex situ collections, cones were hand pollinated. Female cones open sporophylls for a few days for pollination and close again, ovulation period varies from species to species (Tang, 1996; Jones, 2002) and Xaba (2014) observed that *E. alternsteinii* sporophylls on female cones open for 14 to 25 days. Receptive female cones of *E. altenstienii* were hand pollinated at Kirstenbosch National Botanical Garden (KNBG), Cape Town, in the Western Cape Province (-25°59'22.24″ S 18°25'44.2″ E) in May 2018 using wet pollination method (Grobbelaar, 2002, Xaba 2014). Five grams of pollen were mixed with 500 ml of distilled water and were used to pollinate receptive female cones three times every second day in mid-morning and late evening when the micropylar droplets formed (Tang, 1993, Xaba 2014) by injecting the pollen solution between the loose sporophylls using syringe. Unused pollen was packaged in a paper envelope, sealed in a plastic container with silica gel and stored at -15 °C (Osborne *et al.*,1991, 1992).

3.3.3 Seed collection and storage

Seeds were collected when they naturally disintegrated on the cone in November 2018, six months after pollination. Collected seeds were soaked in tap water for a week, followed by removing the fleshy layer (sarcotestae), washed and air dried in plastic trays at room temperature (Burch, 1981; Frett, 1987; Meerow & Broschat, 1991) for a week. Seed were then dusted with Efekto Fungi-Nill 500 WP Captab fungicide to prevent development of fungi, placed in mesh bags and stored under dry conditions at constant 15 °C for 2 months (Hendricks, 1980). The seeds were then transported to Pretoria in mesh bags on a two-hour flight at room condition on January 2019 and stored at room temperature for 5 days before the germination experiment commenced.

3.3.4 Seed viability testing

Seed viability was tested using two methods, 1) water floating test, all seeds for this experiment were immersed in water and seeds floating were regarded as non-viable seeds, discarded and sinking seeds as potentially viable, and used to test viability percentage of the seeds (Burch, 1981; Perez-Farrera *et al.*, 1999; Broome, 2001; Grobbelaar, 2002), 2) by soaking seeds in a solution of 2,3,5-Triphenyltetrazolium chloride. A random sample of 10

seeds was selected, the hard seed coats were cracked with a nut cracker, seed coats around the endosperm were removed and cut open longitudinally using scalpel blade to expose the embryo and each seed was soaked in 250 ml glass container with a solution of 0.5 g of 2,3,5-Triphenyltetrazolium chloride obtained from Sigma-Aldrich and 500 ml of distilled water and placed in the dark for 60 minutes to determine the viability percentage. Seed embryos that changed the colour to purple were regarded as viable and seeds that remained the same as non-viable seeds (ISTA, 2003; Tommasia, *et al.*, 2006)

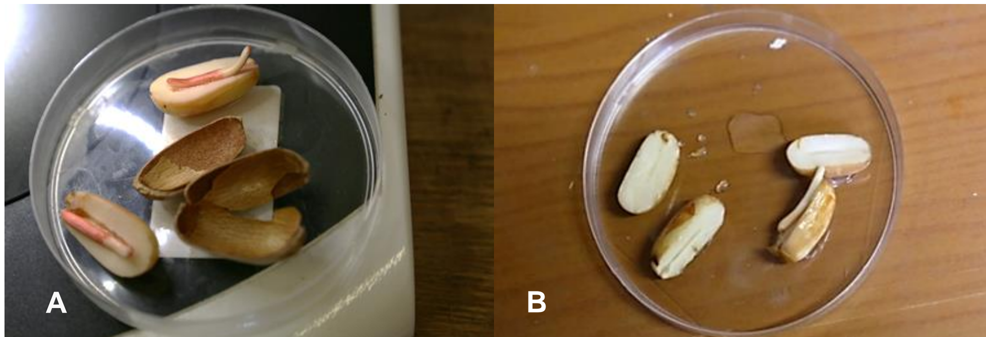

Figure 3.1: Seeds treated with 2,3,5-Triphenyltetrazolium chloride to test viability, (A) embryo with pink colour showing that seed is viable, (B) unchanged embryo indicating that seed is not viable. (Pictures A & B: Mabuya).

3.3.5 Experimental treatments

To assess the effects of seed hydration dehydration treatment on *E. altensteinii*, 360 seeds were used for this experiment, divided into 2 replications of 180 seeds each. Primarily, to evaluate the imbibition period, a sample of 15 seeds were weighed on an analytical balance scale and placed for imbibition in a 3-litre plastic container with distilled water, which were maintained in the seed storeroom at room temperature. Seeds were imbibed in water and weight was measured at 4 hrs intervals until the seed weight was stable. For each weight evaluation, the seeds were removed from the water, dried up with filter paper and weighed again. Seeds of *E. altensteinii* were subjected to HD1 (untreated) and HD2 (soaked in distilled water for 24hrs every month) treatment.

3.3.5.1 Embryo growth

To monitor the effects of seed hydration dehydration treatment on embryo growth, 360 seeds of *E. altensteinii* were used for this experiment, divided into 2 replications of 180 seeds each. Seeds were collected from ex situ collection at KNBG and cleaned as described above. 1) HD 1 (seeds placed in mesh bag and stored at room temperature); 2) HD 2 (seeds placed in mesh bag, soaked in distilled water for 24 hrs at 4 week intervals, air dried and stored at room

temperature). Embryo growth was measured at 8-week intervals. Hard seed coats were cracked with a nutcracker, the seed coat around the endosperm were removed and the endosperm were cut open longitudinally using scalpel blade to expose the embryo. Thereafter the embryo growth length of 15 seeds from the above 2 treatments were measured using electronic microscope and ZEN microscope software obtained from ZEISS.

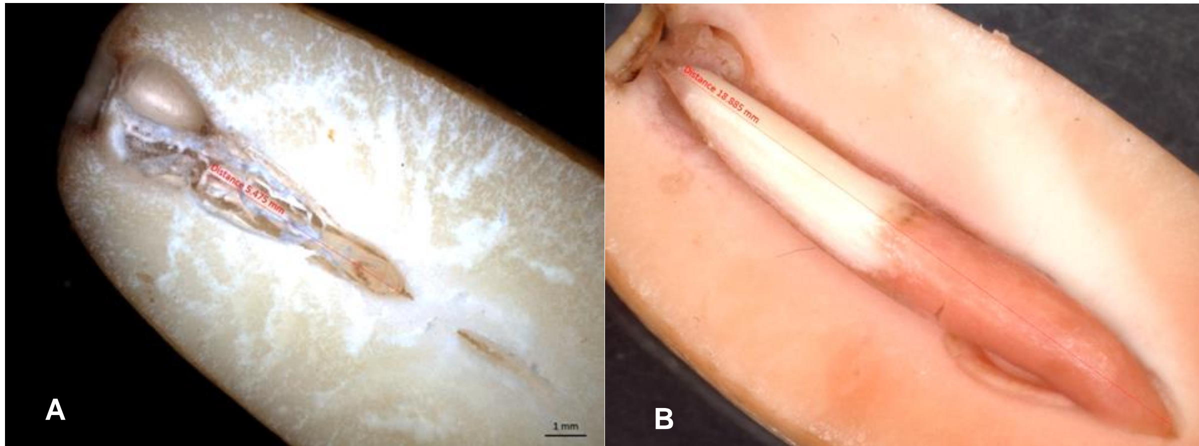

Figure 3.2: Measuring embryo growth, (A) underdeveloped embryo after 6 months of storage, (B) fully developed embryo after 14 months of storage at room temperature. (Pictures: Mabuya).

3.3.5.2 Seed moisture content

To evaluate the effects of seed hydration dehydration treatment on seed moisture content, 360 seeds of *E. altensteinii* were used for this experiment, divided into 2 replications of 180 seeds each, collected from ex situ collection at KNBG in 2018 and cleaned as described above. 1) HD 1 (seeds placed in mesh bag and stored at room temperature); 2) HD 2 (seeds placed in mesh bag, soaked in distilled water for 24 hrs at 4 week intervals and stored in mesh bags at room temperature). A random sample of 15 seeds from the above 2 treatments were used to measure seed moisture content at 8-week intervals. A sample of 15 seeds were individually weighed and their fresh weight were recorded, followed by cracking seed coat with a nutcracker, removal of the seed coat around the endosperm and cutting the endosperm longitudinally using scalpel blade. Thereafter the seed was dried in an oven at 103 °C for 17 hours, weighed again to record dry weight. Moisture contents expressed as a percentage of the wet weight of the sample, calculated to two decimal places using the following formula (ISTA, 2003, 2008):

$$\text{Moisture content} = \frac{\text{wet weight - dry weight}}{\text{wet weight}} \times 100$$

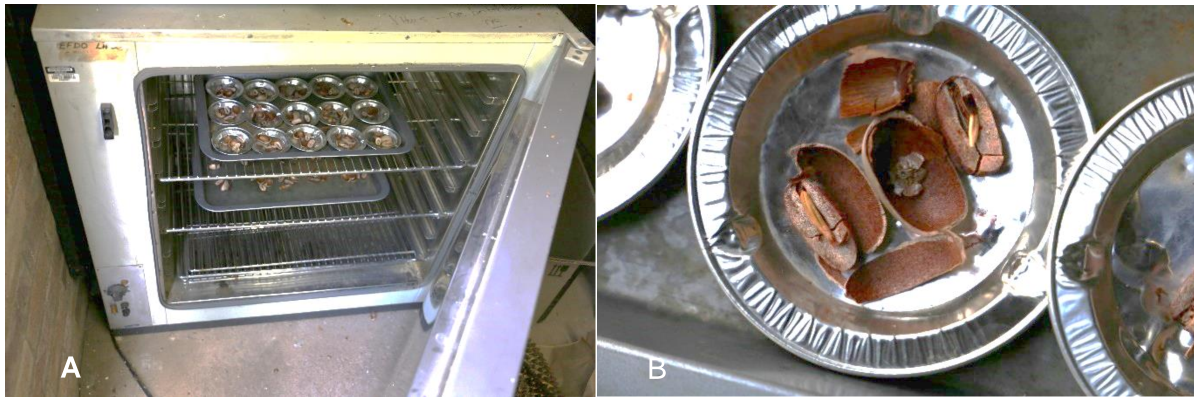

Figure 3.3: Drying seeds to measure dry weight, (A) preparing to dry seeds in an oven at 103°C, (B) dry seed after 17 hours at 103°C (B). (Picture A & B: Mabuya).

3.3.5.3 Seed viability

To study the effects of seed HD treatment on seed viability, 360 seeds of *E. altensteinii* were used for this experiment, divided into 2 replications of 180 seeds each, collected from ex situ collection at KNBG in 2018 and cleaned as described above. 1) HD 1 (seeds placed in mesh bag and stored at room temperature); 2) HD 2 (seeds placed in mesh bag, soaked in distilled water for 24hrs at 4 week intervals and stored in mesh bags at room temperature). A random sample of 15 seeds from the above 2 treatments were used to measure seed viability at 8-week intervals. Seed coat were cracked with a nutcracker and the seed coat was removed around the endosperm and cutting the endosperm longitudinally with scalpel blade to expose the embryo. Thereafter each seed was soaked 250 ml glass container with a solution of 0.5 g of 2,3,5-Triphenyltetrazolium chloride obtained from Sigma-Aldrich and 500 ml of distilled water for 60 minutes to determine viability percent. Seed embryos that changed the colour to purple were regarded as viable and seeds that remained the same as non-viable seeds (ISTA 2003; Tommasia, *et al*., 2006).

3.3.6 Data collection

Embryo growth, moisture content and viability were measured at 8-week intervals. Measurements for embryo length were done in millimetres using electronic microscope and ZEN microscope software obtained from ZEISS. Moisture contents and viability were recorded as a percentage of the sample.

3.3.7 Statistical analysis

Data was analysed using one-way analysis of variance (ANOVA), using the computing software program TIBC STATISTICA 13.5. Occurrence of statistical difference was determined by using the Fisher Least Significance Difference (L.S.D.) at values of $P < 0.05$ levels of significance (Steel & Torrie, 1980).

3.4 Results

3.4.1 Effects of hydration dehydration treatments on embryo growth

Embryo development response of *E. altensteinii* seeds on HD treatment were measured over a 14-month period. Results for this study showed that embryos started to be visible after 6 months of storage and that embryos continued to grow, with a high growth rate from month 12 onwards (Fig 3.4). Embryo length increased sharper in the control (1.27 mm) than the hydrated dehydrated (0.99) treatment from month 10, however from month 14 the embryo length in the hydrated dehydrated treatment (4.39 mm) exceeded the control (3.47 mm). The HD 2 showed no significant (ns) difference compared to HD 1 (control) seeds on embryo growing length.

Table 3.1: Embryo growth response (mean ± standard error [SE]) to monthly (0 to 14 months) hydration dehydration (HD) treatments of E. altensteinii seeds, stored at room temperature. Treatments= HD 1 (control) and HD 2 (seeds hydrated in distilled water for 24 hours (n=15).

Treatments	Month 0	Month 2	Month 4	Month 6	Month 8	Month 10	Month 12	Month 14
HD 1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.28±0.16	1.27±0.58	1.56±0.84	3.47±1.17
HD 2	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.54±0.32	0.99±0.55	1.26±0.57	4.39±1.27
One-way ANOVA F-Statistic								
	0.00 ns	0.00 ns	0.00 ns	0.00 ns	0.50 ns	0.10 ns	0.09 ns	0.30 ns

Mean values ±SE are shown in columns. The mean values followed by same letters are ns = not significant as calculated by Fisher's least significant difference.

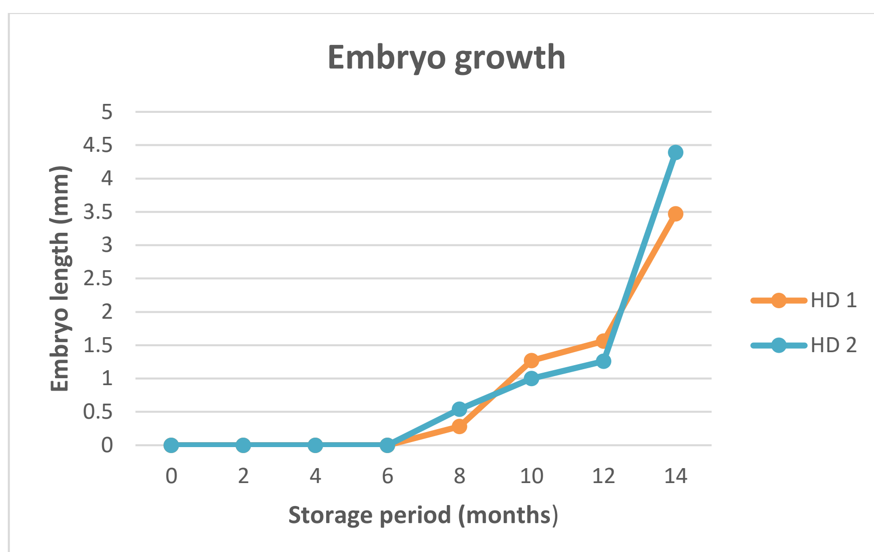

Figure 3.4: Effects of hydration dehydration treatments (HD) on embryo length growth of *E. altensteinii* seeds stored at room temperature. Treatments= HD 1 (control) and HD 2 (seeds hydrated in distilled water for 24 hours (n=15).

3.4.2 Effects of hydration dehydration treatments on seed moisture content

Seed moisture content showed a gradual decline from months 0 to 8 months' storage at room temperatures. The seed moisture content response to HD 2 treatment remained higher compared to the HD 1 treatment (control) during a storage period of 8 months. The control showed a sharper decline in moisture content from month 6 to 8 (Fig 3.4). Results showed a significant difference ($p < 0.001$) in the seed MC of the HD 2 treatment compared to the HD 1 in storage month 2 and month 8. Although between month 4 and 6 no significant difference occur the HD treatment peaked again with a significance ($P < 0.01$) at month 8. Moisture content from the control declined sharply in the HD 1 to 22.60% compared to the HD 2 treatment (29.40%) towards month 8 (Table 3.2).

Tables 3.2: Effects of hydration dehydration treatments on seed moisture content (MC) (mean ± standard error [SE]) of *E. altensteinii* seeds stored at room conditions. Treatments= HD 1 (control) and HD 2 (seeds hydrated in distilled water for 24 hours (n=15).

Treatments	Month 0	Month 2	Month 4	Month 6	Month 8
HD 1	35.13 ±0.39a	31.07 ±0.28b	29.87±1.55a	28.47 ±1.56a	22.60±1.08b
HD 2	35.13 ±0.39a	33.33 ±0.45a	32.47 ±1.38a	30.47±1.49a	29.40±1.55a
One-way ANOVA F-Statistics					
	0.00 ns	17.90 ***	1.60 ns	0.90 ns	13.00 **

Mean values ±SE are shown in columns. The mean values followed by different letters are significantly different at P < 0.01 (**), P < 0.001 (***) and ns = not significant as calculated by Fisher's least significant difference.

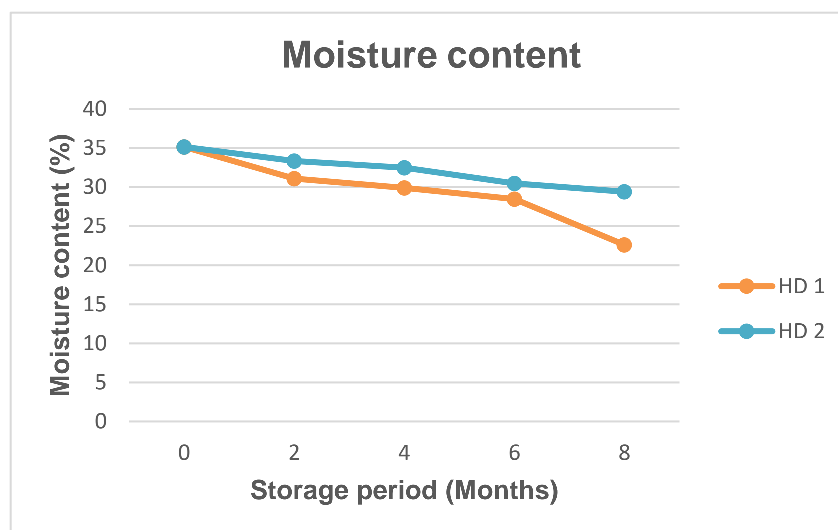

Figure 3.5: Effects of monthly hydration dehydration treatments on *E. altensteinii* seed moisture content (MC) stored at room temperature for 8 months. Treatments= HD 1 (control) and HD 2 (seeds hydrated in distilled water for 24 hours (n=15).

3.4.3 Effect of Hydration dehydration treatments on seed viability

Seed viability response to HD treatments showed interesting results during storage in treated and untreated seeds. The HD 2 treatment showed a bigger difference with a less decline (13.34%) in seed viability compared to the control which declined rapidly (53.34%) between 0 to 8 months (Fig 3.5). The results on seed viability showed a significant difference at ($P < 0.05$) between HD 2 treatments (73.33%) and HD 1 (33.33%) at the end of the experiment (8 months).

Table 3.3: Effects of hydration dehydration (HD) treatments on viability (mean ± standard error) [SE] of *E. altensteinii* seeds stored at room conditions over a period of eight. Treatments= HD 1 (control) and HD 2 (seeds hydrated in distilled water for 24 hours (n=15)

Treatments	Month 0	Month 2	Month 4	Month 6	Month 8
HD 1	86.67 ±9.09a	66.67 ±12.60a	53.33 ±13.33a	46.67 ±13.33a	33.33 ±12.60b
HD 2	86.67±9.09a	80.00 ±10.69a	80.00 ±10.69a	80.00 ±10.69a	73.33 ±11.18a
One-Way ANOVA F-Statistics					
	0.00 ns	0.70 ns	2.40 ns	3.80 ns	5.40 *

Mean values ±SE are shown in columns. The mean values followed by different letters are significantly different at P < 0.05 (*) and ns = not significant as calculated by Fisher's least significant difference.

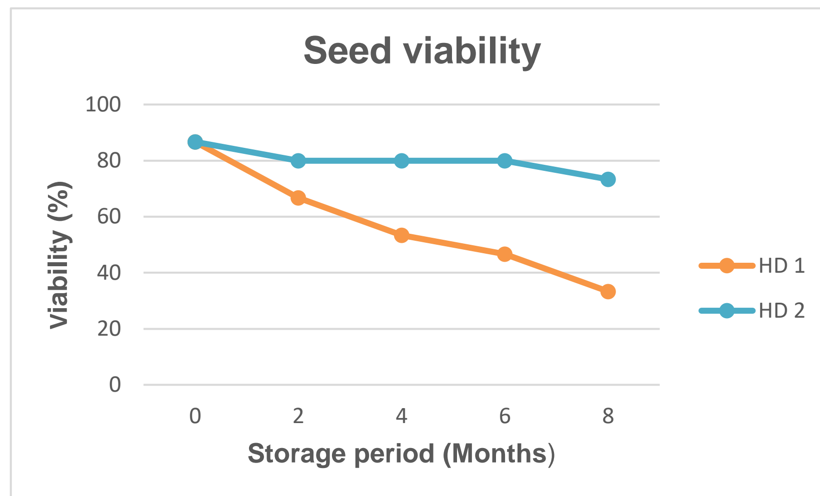

Figure 3.6: Effect of hydration dehydration (HD) treatments on *E. altensteinii* seed viability after 8 months, stored at room temperature. Treatments= HD 1 (control) and HD 2 (seeds hydrated in distilled water for 24 hours (n=15).

3.5 Discussion and conclusion

The principal objectives of this investigation were to use HD treatments to determine the effects of HD on embryo development, MC and viability on seeds stored at room conditions. To determine the effects of HD on seeds, embryo growth, moisture content and viability were measured at 8 week intervals.

3.5.1 Embryo growth

The study showed that the embryo of *E. altensteinii* seeds became visible after 6 months of storage. These results confirm results from similar studies that most cycads species shed seeds with underdeveloped embryo and require storage for 1 to 12 months for embryo to develop (Dyer, 1965; Giddy, 1974; Burch, 1981; Grobbelaar, 2004; Woodenberg, *et al.*, 2007), and similarly Xaba (2014) reported that 6 to 12 months of storage for *E. latifrons* and *E. altensteinii* improved germination rate. The findings showed that embryo growth of *E. altensteinii* sustained until a 12-month period towards maturity, embryo length continuing to increase even after the embryos had reached a germination size. This constant embryo development characterizes recalcitrant seeds, which are constantly metabolically active (Hong & Ellis, 1990; Farrant *et al.*, 1992; Tompsett & Pritchard, 1993; Finch-Savage & Blake, 1994;

Fu *et al.*, 1994; Lin & Chen, 1995), continuing from seed abscission through to germination (Berjak *et al.*, 1984; 1989; Pammenter *et al.*, 1984).

As the embryo length in the HD 1 treatment sustained until month 10 it is possible that the seed contained adequate moisture for the embryo to develop to this point. Whereas in the HD 2 treatment, too much moisture possibly resulted in a lack of oxygen which could have slowed down embryo development. However, the additional moisture after week 10 allowed the embryo to continue developing whereas the HD 1 treatment lacked further moisture for the embryo development to succeed (Baskin & Baskin, 2014). Even though the study showed comparative measurements, there were no significance measured between the HD 1 and HD 2 treatments which explains that seed development requires both oxygen and moisture to develop (Chin & Roberts, 1980; Ibrahim *et al.*, 1983; Tompsett, 1983).

3.5.2 Seed moisture content

This study revealed that seed MC content of *Encephalartos altensteinii* declined over a period of 8 months when stored at room temperature. Is was clear that the seed moisture content for HD 2 seeds retained more moisture and showed a gradual decline in the MC loss over this period. The HD 1 seed MC in contrast declined sharply over the same storage period. It has been well documented that seed viability of most species decline over time (Dehgan & Schutzman,1989; Broome, 2001; Grobbelaar, 2004; Xaba, 2014). It has also been recorded that maintaining moisture storage conditions can regulate seed viability and extend seed longevity (Roberts, 1972; Forsyth & van Staden, 1983; De Andrade, 2001; Calonje *et al.*, 2011; Chien *et al.* 2012).

3.5.3 Seed viability

An understanding is that hydrated recalcitrant seeds are metabolically active and experience germination-associated changes in storage which suggest a requirement for supplementary water to be present in the seed on shedding (Pammenter *et al.*,1994). This study showed that the HD treatment resulted in seed viability over a long period for up to 8 months during storage whereas in the no additional moisture content the DH treatment seed viability declined sharply. The availability of moisture over a long period advanced seed viability (Tompsett & Pritchard, 1998) suggesting that *E. altensteinii* is sensitive to desiccation. The viability over a period determine the longevity of seed. According to Grobbelaar (2004) and Broome (2001) HD treatments improve longevity on cycad seeds. This current study concurs with these results as the findings showed significant comparisons where moisture was added in the HD2 treatment. The study showed that where MC decreased below 25%, viability of seed deteriorated. Similar results were reported by De Andrade (2001) where the viability of

Euterpe edulis seed decreased below 30% MC compared to moisture treated seeds which remained at 80% MC for four months. In this study the moisture content was also maintained during low temperatures during the winter months indicating that low storage temperature could maintain viability in extending longevity of seeds.

Further studies for *E. altensteinii* could explore difference in temperature storage levels to investigate the effect of HD treatments in combination with different temperature ranges, explore hydration in GA_3 solution and different HD treatments and hydration periods and for longer storage periods. Due to a limited number of seed available for this study these variables could not be explored.

3.6 Acknowledgement

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3.7 Referencing

Baskin, C.C. & Baskin, J.M. 2014. *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination*. San Diego: Academic Press, 1586.

Berjak, P., Dini, M. & Pammenter, N.W. 1984. Possible mechanisms underlying the differing dehydration responses in recalcitrant and orthodox seeds: desiccation-associated subcellular changes in propagules of *Avicennia marina*. *Seed Science and Technology,* 12: 365-384.

Berjak, P., Farrant, J.M. & Pammenter, N.W. 1989. The basis of recalcitrant seed behaviour. In: Taylorson, R.B. (ed.), Recent Advances in the Development and Germination of Seeds. Plenum Publishing Corporation Inc., New York and London. 89-108.

Broome, T. 2001. Optimizing cycad seed germination. *The Cycad Newsletter,* 24(2): 5–7.

Buitink, J., Vu, B.L., Satour, P. & Leprince, O. 2003. The re-establishment of desiccation tolerance in germinated radicles of *Medicago truncatula* Gaertn. seeds. *Seed Science Research,* 13: 273–286.

Burch, D. 1981. Propagation of Florida *Zamia* from Stem Pieces. SNA Research Conference, 221-223.

Chien, C.T., Chen, S.Y., Chang, S.H. & Chung, J.D. 2012. Seed Germination and Storage of *Cycas taitungensis* (Cycadaceae). *Taiwan Journal for Science*, 27(1): 1-11.

Chin, H.F. & Roberts, E.H. 1980. *Recalcitrant Crop Seeds*. Tropical Press. SDN. BHD. Kuala Lumpur, Malaysia.

Calonje, M., Kay, J. & Griffith, M. P. 2011. Propagation of Cycad Collections from Seed. *Sibbaldia: The International Journal of Botanic Garden Horticulture*, 9: 79-96.

De Andrade, A.C.S. 2001. The effect of MC and temperature on the longevity of heart of palm seeds (*Euterpe edulis*). *Seed Science and Technology,* 29: 171-182.

Dehgan, B. & Schutzman, B. 1989. Embryo development and germination of *Cycas* seeds. *Journal of the American Society for Horticultural Science,* 114(1): 125–129.

Demir, I. & Mavi, K. 2003. Effect of controlled hydration treatments on storage longevity of aubergine seeds during development. *Israel Journal of Plant Sciences*, 51(4): 291-295.

Dinakar, C. & Bartels, D. 2013. Desiccation tolerance in resurrection plants: new insights from transcriptome, proteome, and metabolome analysis. *Frontiers in Plant Science,* 4: 482.

Donaldson, J.S. 2009. *Encephalartos altensteinii* Lehm. National Assessment: Red List of South African Plants version 2017.1. http://redlist.sanbi.org/species.php?species=823-2. [Accessed on 2018/06/19].

Dyer, R. A. 1965. The cycads of Southern Africa. *Bothalia,* 8: 405- 515.

Farrant, J.M., Pammenter, N.W. & Berjak, P. 1992. Development of recalcitrant (homoiohydrous) seeds of *Avicennia marina*: Anatomical, ultrastructural and biochemical events associated with development from histodifferentiation to maturation. *Annals of Botany,* 70:75-86.

Finch-Savage, W.E. & Blake, P.S. 1994. Indeterminate development in desiccation sensitive seeds of *Quercus robur* L. *Seed Science Research,* 4:127-133.

Foley, M.E. & Chao, W.S. 2008. Growth Regulators and Chemicals Stimulate Germination of Leafy Spurge (*Euphorbia esula*) Seeds. *Weed Science*, 56(4): 516-522.

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

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

Fu, J.R., Jin, J.P., Peng, Y.F. & Xia, Q.H. 1994. Desiccation tolerance in two species with recalcitrant seeds: *Clausena lansium* L. and *Litchi chinensis* S. *Seed Science Research,* 4: 257-261.

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

Grobbelaar, N. 2004. Cycads: With special reference to the southern African species. Published by the author. Pretoria.

Giddy, C. 1974. *Cycads of South Africa*. Purnell and Sons Ltd, Cape Town, South Africa.

Hendricks, I.G. 1980. Germination of cycad seed. *The Cycad Newsletter*, 3: 3-8.

Hong, T.D. & Ellis, R.H. 1990. A comparison of maturation drying, germination, and desiccation tolerance between developing seeds of *Acer pseudoplatanus* L. and *Acer platenoides* L. *New Phytologist,* 116: 589-596.

Khan, M.M., Thompson, K., Usman, M. & Fatima, B. 2001. Role of MC and controlled atmosphere in *Citrus* seed storage. *International Journal of Agriculture & Biology*, 04(2): 259–266.

Jones, D.L. 1993. *Cycads of the world*. Reed, Canberra, 180-181 & 216-217.

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

Leprince, O., Hendry, G.A.F. & McKersie, B.D. 1993. The mechanisms of desiccation tolerance in developing seeds. *Seed Science. Research,* 3: 231–246.

Lima, A.T. & Meiado, M.V. 2017. Discontinuous hydration alters seed germination under stress of two populations of cactus that occur in different ecosystems in Northeast Brazil. *Seed Science Research*, 27: 292-302.

Lima, A.T., Cunha, P.H.J., Dantas, B.F. & Meiado, M.V. 2018a. Does discontinuous hydration of *Senna spectabilis* (DC.) H.S. Irwin & Barneby var. *excelsa* (Schrad.) H.S. Irwin & Barneby (Fabaceae) seeds confer tolerance to water stress during seed germination? *Journal of Seed Science*, 40(1): 036-043.

Lima, A.T., Oloveira, D.M. & Meiado, M.V. 2018b. Effect of hydration and dehydration treatments on *Macroptilium atropurpureum* seeds germination under water deficit conditions. *Communications in plant sciences*, 08: 55-61.

Lin, T.P. & Chen, M.H. 1995. Biochemical characteristics associated with the development of the desiccation-sensitive seeds of *Machilus thunbergii*. *Annals of Botany,* 76: 381-387.

Maia, J., Dekkers, B.J.W., Provart, N.J., Ligterink, W. & Hilhorst, H.W.M. 2011. The Re-Establishment of Desiccation Tolerance in Germinated *Arabidopsis thaliana*. Seeds and Its Associated Transcriptome. *Plos One,* 6(12): e29123.

Maia, J., Dekkers, B.J.W., Dolle, M.J., Ligterink W. & Hilhorst HWM. 2014. Abscisic acid (ABA) sensitivity regulates desiccation tolerance in germinated *Arabidopsis* seeds. *New Phytologist*, 203: 81–93.

McCarty, D.R. 1995. Genetic control and integration of maturation and germination pathways in seed development. *Annual review plant physiology and plant molecular biology,* 46: 71–93.

Meerow, A.W. & Broschat, T.K. 1991. *Palm seed germination.* University of Florida IFAS Cooperative Extension BUL274, Gainesville, FL.

Ntuli, T.M. 2012. Drought and Desiccation-Tolerance and Sensitivity in Plants. *Botany*, 67: 435-441.

Ibrahim, A.E., Roberts, E.H. & Murdoch A.J. 1983. Viability of lettuce seeds. II Survival and oxygen uptake in osmotically controlled storage. *Annals of Botany,* 34: 631-640.

Ingram, J. & Bartels, D. 1996. The molecular basis of dehydration tolerance in plants. *Annual review plant physiology and plant molecular biology,* 47: 377–403.

ISTA. 2003. *ISTA Working Sheets on Tetrazolium Testing.* Vol I Agricultural, Vegetable & Horticultural Species. International Seed Testing Association (ISTA), Bassersdorf, CH Switzerland.

ISTA. 2008. *International Rules for Seed Testing, Edition 2008.* International Seed Testing Association (ISTA), Bassersdorf, CH-Switzerland.

Osborne, R., Robbertse, P.J. & Claassen, M.I. 1991. The longevity of cycad pollen – Results of some preliminary experiments. *Journal of Cycad Society of South Africa*, 28: 10–13.

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

Pammenter, N.W., Farrant, J.M. & Berjak, P. 1984. Recalcitrant seeds: short-term storage effects in *Avicennia marina* (Forsk.) Vierh. may be germination-associated. *Annals of Botany,* 54: 843-846.

Pammenter, N., Berjak, P., Farrant, J., Smith, M., & Ross, G. (1994). Why do stored hydrated recalcitrant seeds die? *Seed Science Research,* 4(2): 187-191.

Pammenter, N.W. & Berjak, P. 1999 A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms. *Seed Science Research*, 9: 13–37.

Parihar, S.S., Dadlani, M., Lal, S.K., Tonapi, V.A., Nautiyal, P.C. & Basu, S. 2014. Effect of seed MC and storage temperature on seed longevity of hemp (*Cannabis sativa*). *Indian Journal of Agricultural Sciences,* 84 (11): 1303–1309.

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

Roberts, E.H. 1972. Storage environment and control of viability. *Viability of Seeds*. In Roberts E H (Ed.). Chapman and Hall, London, 14–58.

Roberts, E.H. 1973. Predicting the storage life of seeds. *Seed Science and Technology*, 1: 499–514.

Rodriguez, M.C.S., Edsgard, D., Hussain, S.S., Alquezar, D., Rasmussen, M., Gilbert, T., Nielsen B.H., Bartels, D. & Mundy, J. 2010. Transcriptomes of the desiccation-tolerant resurrection plant *Craterostigma plantagineum, The Plant Journal,* 63: 212–228.

Santini, B.A., Rojas-Arechiga, M. & Morales, E.G. 2017. Priming effect on seed germination: Is it always positive for cacti species? *Journal of Arid Environments*, 1-4.

Steel, R.G.D. & Torrie, J.H. 1980. Principle and procedures of statistics: a biometrical approach, 2nd edition. McGraw-Hill, New York, NY.

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

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

Tommasi, F., Paciolla, C., Pinto, M.C. & De Gara, L. 2006. Effects of storage temperature on viability, germination and antioxidant metabolism in *Ginkgo biloba* L. seeds. *Plant Physiology and Biochemistry,* 44: 359–368.

Tompsett, P.B. 1983. The influence of gaseous environment on the storage life of *Araucaria hunsteinii* seed. *Annals of Botany,* 52: 229-237.

Tompsett, P.B. & Pritchard, H.W. 1993. Water status changes during development in relation to the germination and desiccation tolerance of *Aesculus hippocastanum* L. seeds. *Annals of Botany*, 71: 107-116.

Tompsett, P.B. & Pritchard, H.W. 1998. The effect of chilling and moisture status on the germination, desiccation tolerance and longevity of *Aesculus hippocastanum* L. seed. *Annals of Botany,* 82: 249-261.

Winter, J. 2004. *Encephalartos altensteinii* Lehm. South African National Diversity Institute. (Online). http://pza.sanbi.org/encephalartos-altensteinii. [Accessed 20 July 2018].

Woodenberg, W.R., Erdey, D., Pammenter, N. & Berjak, P. 2007. Post-shedding seed behaviour of selected *Encephalartos* species. Abstracts from the 5th International Workshop on Desiccation Tolerance and Sensitivity of Seeds and Vegetative Plant Tissues. *South African Journal of Botany,* 73(3): 49.

Xaba, P.M.A. 2014. Pollination and Germination as Limiting Factors in the Propagation of Threatened Cycads, *Encephalartos* (*Zamiaceae*). MSc Thesis, University of Western Cape, South Africa.

CHAPTER 4:

THE EFFECTS OF MECHANICAL SCARIFICATION ON BREAKING SEED DORMANCY AND IMPROVING SEEDLING GROWTH OF THE *ENCEPHALARTOS ALTENSTEINII*, THE EASTERN CAPE GIANT CYCAD

The effects of mechanical scarification on breaking seed dormancy and improving seedling growth of the *Encephalartos altensteinii*, the Eastern Cape giant cycad

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4.1. Abstract

Germination percentage and germination rate are generally low in most cycad species and this has a negative effect on the production and conservation efforts for this most endangered plant group. *Encephalartos altensteinii* is a medium to large cycad which grows to 5 meters tall with mature plants usually form clumps of 2 to 3 stems with basal suckers, only occurs in the Eastern Cape province and south coast of KZN province in South Africa. Germination percentage and germination rate of *E. altensteinii* are generally low, and in an effort to improve seed germination of *E. altensteinii*, mechanical scarification (sanding and cracking), and gibberellic acid (GA_3) (1000ppm) for different soaking period (24 hrs or 48 hrs) were tested. Seeds were sown in silica sand over an eight-month period using 11 treatments and 15 replicates. Results indicated that there was significant difference among treatments. Significant difference ($P < 0.05$) was observed on MS1 (sanding) when compared with MS0 (control) and MS 2 (cracking), with final germination (60%) and germination rate, more than 50% of seeds germinated in 3 months and completed germination in 4 months when compared with 8 months for untreated seeds. Results showed that MS2 treatment had low final germination (13.33%) and only germinated on month 1 with no further germination. Combination of mechanical scarification and GA_3 did not further improve final germination percent and germination rate, it was also observed that soaking seeds in GA_3 for 48hrs prolonged germination when compared with 24 hrs and mechanical scarification without GA_3. The results suggested that sanding down the projected coronula seed is sufficient to improve germination rate and final germination percent of *E. altensteinii*.

Key words: dormancy, germination rate, germination percent, gibberellic acid (GA_3), mechanical scarification

4.2. Introduction

Encephalartos altensteinii is the one of the largest African cycad species which mainly occurs in the Eastern Cape region of South Africa. *E. altensteinii* is a medium to large cycad which grows to 5 meters tall. Mature plants usually form clumps of 2 to 3 stems with basal suckers (Jones, 1993). The wild population has declined over the years due to habitat destruction for development and collection with the species currently listed as Vulnerable (VU) on the Red List of South African plants and an estimate of only 10 000 plants left in the wild (Winter, 2004; Donaldson, 2009).

Cycads are listed as possibly one of the most threatened plant groups in the world (Donaldson, 2003; IUCN, 2016). Most species have become threatened due to poor and slow reproduction and illegal harvesting of plants from the wild (Dyer 1965; Giddy 1974; Goode, 1989; Donaldson & Bösenberg, 1999), habitat destruction, alien vegetation (Donaldson, 2003) While extensive research is necessary to address conservation related aspects of individual species, the landscape design use of cycads has rapidly increased world-wide (Giddy, 1993; Whitelock, 1995; Donaldson & Bösenberg, 1999). Due to the continued decrease of natural populations, resulting in the threatened status of *E. altensteinii*, there is an urgent need for cultivation to support conservation programmes of the species. The high commercial value of the cycads further necessitates a need for methodology to increase the cultivation of these species.

E. altensteinii is mainly propagated from seed, a slow growth process which is usually hampered with complex dormancy conditions that are not well understood to support conservation projects. Amongst cultivated plants there is little evidence of factors that influence the germination of seed (Fenner, 1985). Most cycads may have morpho-physiological dormancy (MPD), explained as a combination of morphological and physiological dormancy (Witte, 1977; Dehgan, 1996). Storage periods for cycad seeds vary depending on the genus and the species to allow embryos to mature to aid successful seed germination (Giddy, 1974; Grobbelaar, 2004; Woodenberg *et al.*, 2007; Calonje, *et al.*, 2011). *Encephalartos species* have hard seed coats and most species including *E. altensteinii* shed the seeds from the mother plant with an immature embryo, with exception of few species that shed their seeds with fully developed embryos such as *E. transvenosus* and *E. manikensis* (Grobbelaar, 2004).

Several problems have been identified at Pretoria, National Botanical Garden (PNBG) who reported the species difficult to propagate (undocumented research). These include aspects such as lack of availability seed, poor viability of seed, low germination rates and poor seedling survival success of *E. altensteinii*. Several studies suggested that mechanical scarification

and chemical scarification with H_2SO_4 and GA_3 are effective in breaking seed dormancy for cycads (Dehgan, 1983; Frett, 1987; Dehgan, 1999; Zarchiniand *et al*., 2011), however Xaba (2014) observed no significant difference on the seed germination of *E. altensteinii* and *E. latifrons*. With these treatments that have been tested, it remains unclear if mechanical scarification and GA_3 soaking periods could improve germination of *E. altensteinii*. This study aimed to determine the effects of mechanical scarification and soaking in GA_3 for varying periods on breaking seed dormancy of seed and to report successful seedling growth of *E. altensteinii* to develop a propagation protocol for this species.

4.3. Materials and Methods

Mechanical and chemical seed scarification methods were used to test germination and seedling growth of the Eastern Cape giant cycad.

4.3.1. Artificial pollination

Receptive female cones of *E. altensteinii* were hand pollinated at Kirstenbosch National Botanical Garden (KNBG), Cape Town, in the Western Cape Province (-25°59'22.24″ S 18°25'44.2″ E) in April 2017 using a wet pollination method (Grobberlaar, 2004). Five grams of pollen were mixed with 500 ml of distilled water and used to pollinate receptive female cones three times every second day during the mid-morning and early evening when the micropylar droplets have formed (Tang, 1993; Xaba, 2014) The pollen solution was injected between the loose sporophylls using a syringe. Pollen used was collected and stored in a paper envelope and sealed in a plastic container with silica gel and stored at -15°C (Osborne *et al*., 1991, 1992).

4.3.2. Seed collection and storage

Seeds took about 7 months from pollination to shedding and were collected at KNBG when they naturally started disintegrating on the cone. Collected seeds were soaked in tap water for a week, followed by removing the fleshy layer (sarcotestae), washed and air dried at room temperature (Burch, 1981; Frett, 1987; Meerow & Broschat, 1991). Seed were then dusted with Efekto Fungi-Nill 500 WP Captab fungicide, placed in mesh bags and stored in dry condition at a constant 15 °C and 15% relative humidity for 17 months (Hendricks, 1980). The seeds were transported to Pretoria in mesh bags on a 2-hour flight at room conditions during March 2019 and stored at room conditions for 5 days before the experimental work commenced.

4.3.3. Seed viability testing

Seed viability was tested using two methods, 1) the water floating test, seeds were immersed in water. Seed that floated in water were regarded as non-viable seeds, discarded and all sinking seeds accepted as potentially viable (Burch, 1981; Perez-Farrera *et al*, 1999; Grobbelaar, 2002; Broome, 2001), 2) by soaking seeds in a solution of 2,3,5-triphenyltetrazolium chloride (TTC). A random sample of 15 seeds were selected, the hard seed coat cracked with a nutcracker, the seed coat removed around the endosperm and the endosperm cut open longitudinally using scalpel blade to expose the embryo. Each seed was soaked in a 250 ml glass container in a solution of 0.5 g of TTC and 500 ml of distilled water placed in the dark for 60 minutes to determine viability percentage. Seed embryos that changed to a purple colour were regarded as viable and seeds that remained the same colour discarded as non-viable seeds (ISTA 2003; Tommasia, *et al.*, 2006).

4.3.4. Seed treatments and experimental design

The experiment consisted two experiments, two mechanical scarification (MS) methods with or without gibberellic acid (GA_3) using a randomized block design, with a total number of 180 seeds of *E. altensteinii.* The design consisted of 11 treatments with 15 replicates to investigate the effects of seed pre-treatments on the germination of the Eastern Cape giant cycad. All seeds used were soaked for 24 hrs in distilled water before the treatment. *Experiment 1*: The seeds were scarified using two MS methods, by sanding down projecting coronula or cracking seed coat with nut cracker (Smith, 1978; Pérez-Farrera *et al.*, 1999): *Experiment 2*: The seeds were scarified using two MS methods, by sanding down projecting coronula or cracking seed coat followed by soaking in 1000 ppm of GA_3 for 24 hrs or 48 hrs (Xaba, 2014).

Figure 4.1: The factorial randomized block design experiment on a heated bench showing seeds planted half-way embedded in rows in silica sand.

Experiment 1: Mechanical scarification treatments

1) MS0 (control)
2) MS1 (sanding down projecting coronula)
3) MS2 (cracking seed coat)

Experiment 2: Mechanical scarification and GA_3 *treatments*

1) MS0-$GA_3$1 (control)
2) MS0+$GA_3$1 (soaking 1000 ppm GA_3 / 24hrs)
3) MS0+$GA_3$2 (soaking 1000 ppm GA_3 / 48hrs)
4) MS1- $GA_3$1 (sanded down projecting coronula without GA_3)
5) MS1+$GA_3$1 (sanded projecting coronula and soaking in 1000 ppm GA_3 / 24hrs)
6) MS1+$GA_3$2 (sanded projecting coronula and soaking in 1000 ppm GA_3 / 48hrs)
7) MS2-$GA_3$1 (cracked seed coat without GA_3)
8) MS2+$GA_3$1 (cracked seed coat and soaking in 1000ppm GA_3 / 24hrs)
9) MS2+$GA_3$2 (cracked seed coat and soaking in 1000ppm GA_3 / 48hrs)

4.3.5. Greenhouse experiment

The experiment was conducted in the greenhouse of the Pretoria National Botanical Garden, east of Pretoria, Gauteng province, South Africa, (-25°44'18.2″ S 28°16'19.8″ E) in the production nursery from April 2019 to December 2019. The greenhouse environmental conditions were controlled, with maximum day temperature which ranged between 21-28 °C and night temperature between 15-21 °C, with an average of 43% relative humidity (Umair, 2011). The roof was covered with 30% shade net provide a cooler controlled temperature. A seed sowing bench of 11 x 5 m with heating cables provided bottom heating during seed germination.

4.3.6. Germination study and data collection

After the pre-germination treatments, seeds were sown in silica sand on a germination bench by pushing them halfway into the silica sand which were controlled at a temperature of 27 °C (Burch, 1981; Xaba, 2014). The germination bench was covered with 100% shade cloth and the seed were exposed to light only during watering and data collection. The soil media was kept moist by applying hand watering once daily. Data recording for the experiment were done weekly. Germination was recorded when the growth of the seed radicle elongated to 3 mm or greater and was regarded as geminated (Tommasia, *et al.*, 2006). The germination percentage was determined by the number of seeds germinated at the end of the experiment

for each treatment. The germination rate was indicated by the number of months it took for 50% of germinated seeds to germinate. Once germinated, seeds were removed from the bench and transplanted into 12 mm X 233 mm plastic planting bags in a soil media consisting of a ratio 1:2:1, river sand, compost and fine bark. Potted seedlings were placed in a greenhouse with an average temperature of 24 °C and night temperature ranging between 15 - 21 °C. Leaf sprouting was recorded daily for the first new leaf after germination and leaf count was recorded at the end of the experiment. The leaf count and leaf sprouting rate were dependent on the seed germination and new leaves sprouting from the paired cotyledonary petiole of germinated seeds. Leaf count data collection was delayed after the germination experiment by 4 months because of slow leaf sprouting from paired cotyledonary petiole.

4.3.7. Statistical analysis

Data for experiments 1 was analysed using one-way and experiment 2 were analysed using two-way analysis of variance (ANOVA) and computed software program TIBC STATISTICA Version 13.5. Occurrence of statistical difference was determined by using the Fisher Protected Least Significance Difference (L.S.D.) at values of $P < 0.05$; $P < 0.01$ and $P < 0.001$ levels of significance (Steel & Torrie, 1980).

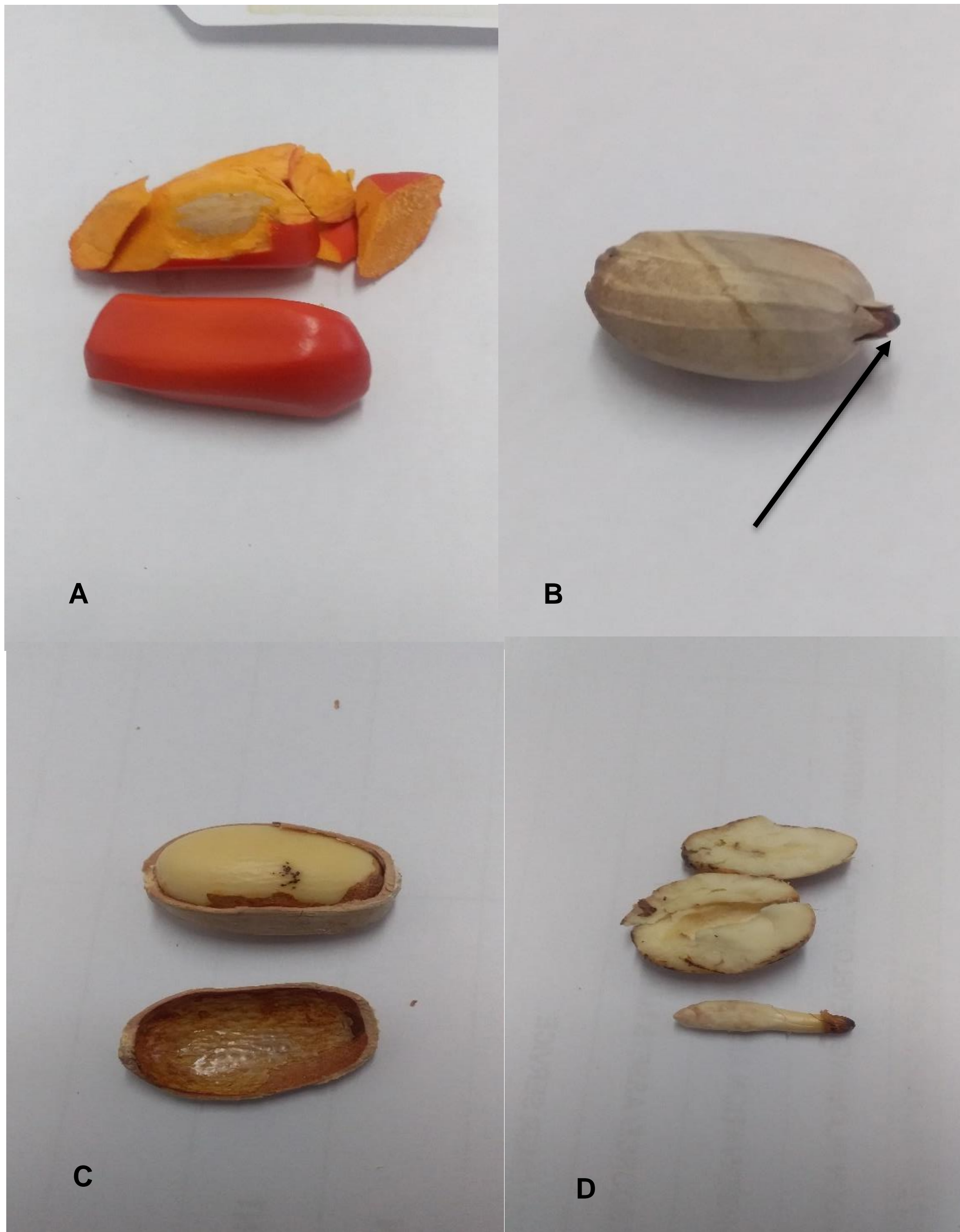

Figure 4.2: Seed morphology of *Encephalartos altensteinii*, (A) uncleaned seeds with sarcotesta (fleshy outer layer), (B) coronula, (C) sclerotesta (hard seed coat) and endosperm, (D) endosperm with fully developed embryo (Pictures: Mabuya).

Figure 4.3: Representing seedling development of *E. altensteinii*, (A) germinated seed with only paired cotyledonary petiole, (B) germinated seed with only radicle emergence, (C) fully developed taproot, and first leaf sprouting, (D) fully developed seedling with leaves and taproot with lateral roots (pictures: Mabuya).

4.4. Results and discussion

4.4.1. The effects of mechanical scarification on germination

The results for mechanical scarification in sanding down the projecting coronula (treatment MS2) showed a significance ($P < 0.05$) in germination of *E. altensteinii* seed from months 1 to 3 compared to the control. At month 4 the significance level increased at $P < 0.01$ compared to the control and from months 5 to 8 the same treatment significance level at value $P < 0.05$ were measured compared to the control (See Table 4.1). The MS treatment showed significant levels at ($P < 0.05$) in germination of the seed pre-treatment with sanding down the projecting coronula (MS 1) at 60 % after 8 months of germination compared to the 33 % in the control (MS 0) (See Table 4.1). The most rapid germination in the MS 1 treatment showed a 33.33% germination in month 1, 53.33% in month 3 which increased to 60% germination in month 8. The MS 2, cracking of the seed coat treatment was far less than the control with no treatment. (See Figure 4.4). Mechanical scarification in sanding down the projected coronula significantly increased the germination percentage throughout the study period from months 1-8. The mechanical scarification improved germination of *E. altensteinii* with 60% germination when compared with 33.33% of untreated seeds and reduced germination period to four months, suggesting that impermeable seed coat inhibit germination (Dehgan, 1983; Dehgan & Almira,1993). These results concur with results reported by Smith (1974) on the germination of *Zamia integrifolia* that indicated that seed distal scarification had 60% germination in 30 days and improved final germination percent (88%) at the end of the experiment. In a recent study Xaba (2014) observed improved germination, however there was no significant difference to untreated seeds of *E. altensteinii*. Perhaps improved germination on mechanical scarification on this study is influenced by sowing seeds in the dark (Frett, 1987; Malwane, 2019). It was thus necessary with sanding to thin the seed coat and not crack the seed coat which had a negative effect on germination percent (13.33%). These results disagree with the study on storage and germination of *Zamia* (LaRue, 1948; Hooft, 1970; Witte 1977) where results showed improved germination on cracked (77%) seeds when compared to un-cracked (38%) seeds. In this study, cracked seeds only germinated on the first month with no further germination. The negative effected could be as a result of water penetration to fast through the cracks, minor damages on the endosperm as a result of cracking and fungal infection that lead to rotting of the seeds (Witte, 1977; Broome, 2001)

Table 4.1: Effects of mechanical scarification by sanding down the projecting coronula (MS1) and cracking the seed coat (MS2), no treatment control (MS0) on the germination of *E. altensteinii* (n=15).

Mechanical scarification	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8
MS0	0.00±0.00b	6.67±6.66b	13.33±9.09b	20.00±10.69b	26.67±11.82b	26.67±11.82b	26.67±11.82b	33.33±12.60ab
MS1	33.33±12.60a	46.67±13.33a	53.33±13.33a	60.00±13.09a	60.00±13.09a	60.00±13.09a	60.00±13.09a	60.00±13.09a
MS2	13.33±9.09ab	13.33±9.09b	13.33±9.09b	13.33±9.09b	13.33±9.09b	13.33±9.09b	13.33±9.09b	13.33±9.09b
One-Way ANOVA								
Mechanical scarification	3.50*	4.52*	4.67*	5.19**	4.40*	4.40*	4.40*	3.98*

Mean values ±SE are shown in columns. The mean values followed by different letters are significantly different at $P < 0.05$ (*) and $P < 0.01$ (**) as calculated by Fisher's least significant difference.

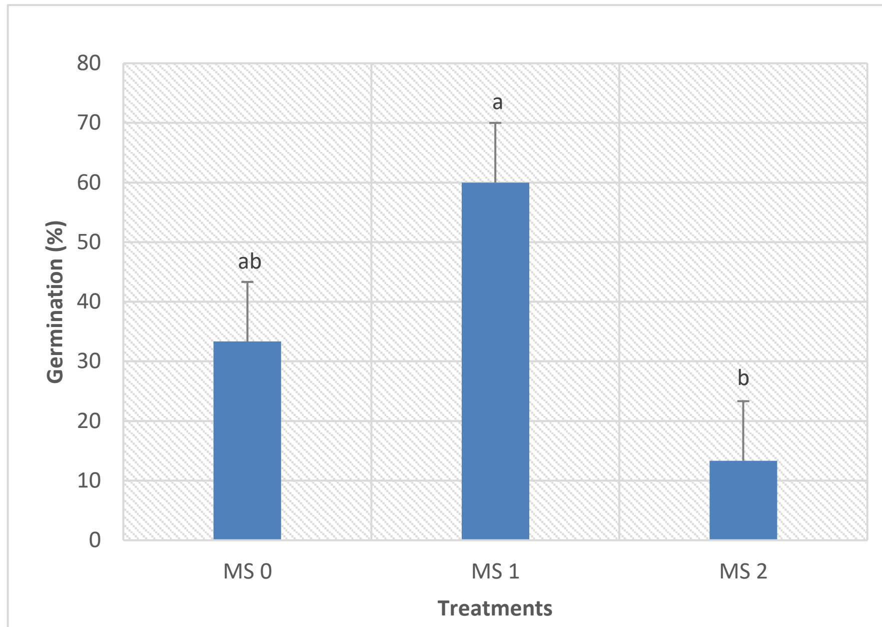

Figure 4.4: The final germination percentage of *E. altensteinii* seeds in response to mechanical scarification treatment. Vertical columns are means and the bars on each column are ± standard errors of mean. The mean values represented by different letters differ significantly at $P < 0.05$ as calculated by Fisher's least significant difference. Treatments= MS0 (control), MS1 (sanding down the projecting coronula, MS2 (cracking the seed coat), (n=15).

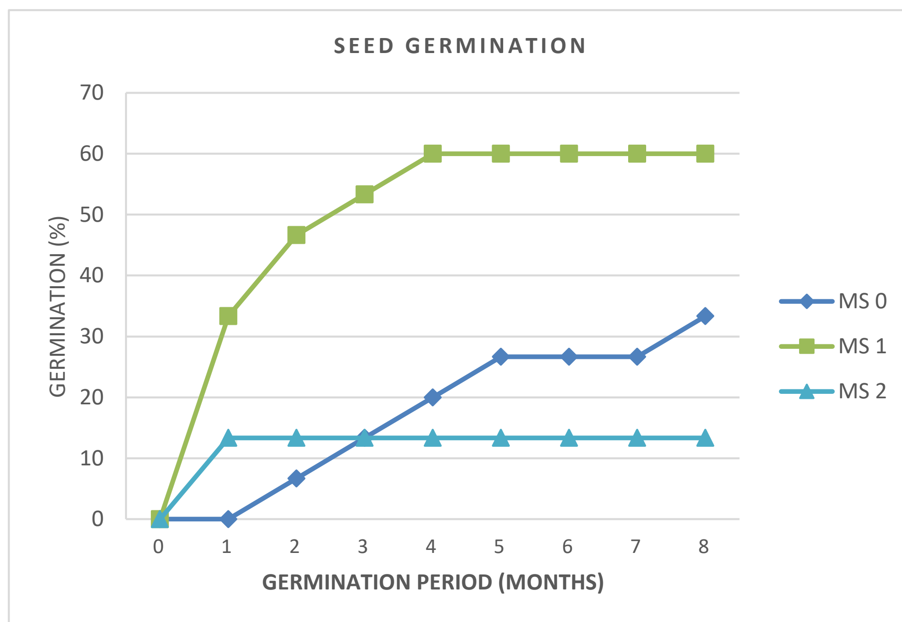

Figure 4.5: The effects of Mechanical scarification on Germination rate of *E. altensteinii* at 27 °C. Graph illustrates means of monthly germination over 8 months for all treatments. Treatments = MS0 (control), MS1 (sanding down the projecting coronula) and MS2 (cracking the seed coat), (n=15).

4.4.2. The effects of mechanical scarification on seedling growth

Leaf sprouting

The leaf sprouting was dependent on the seed germination and new leaves sprouting from the paired cotyledonary petiole of germinated seeds. A significant difference at P < 0.01 was recorded on leaf sprouting for this study among different treatments. The most rapid sprouting average (153 days) when compared with other treatments was obtained on treatment MS1, followed by MS0 (193 days) and no sprouting was recorded for treatment MS2, all cracked seeds started to rot after germination. Broadly leaves of untreated seeds sprouted more rapidly when compared with mechanical scarified seeds. (See Table 4.2). Mechanical scarification by sanding down the projected coronula did not reduce the period between germination and leaf sprouting when compared with MS0 (control) and MS1 were significantly different to MS2. Breaking physiological dormancy by mechanically scarifying seed coat did not significantly influence growth of seedling, perhaps early germination had negative effect on the leaf sprouting and seeds that germinated in winter took longer to sprout leaves because of low soil temperatures when o seeds that germinated in spring and summer (Marry *et al.,* 2018). *E. altensteinii* naturally flushes new leaves in summer while other *Encephalartos* species flush new leaves between spring and summer.

Number of leaves

There was significant difference ($P < 0.01$) on the number of leaves from the mechanical scarification treatment (MS1) and the control (MS0). However, there was no significant difference between MS1 and MS0 but significantly different when compared with MS2 when number of leaves means were separated using Tukey LSD test. An average leaf count of 0.67 for MS1, and 0.47 MS0 were recorded and no leaf was recorded on treatment MS2 (See Table 4.2). Based on these results, the number of leaves increasing following leaf sprouting. Sanding down the coronula advanced the leaf number while cracking the seed coat had a negative result on number of leaf development. Conflicting results were reported by Witte (1977) when cracked seed coats of *Zamia integrifolia* and *Z. floridana* had the highest number of leaves when compared with un-cracked seeds. However, soil medium and growing environmental conditions may have had an influence on the difference of these results (Dehgan *et al*., 2004, Malwane, 2019). There is also evidence that cycads species in generally respond differently on pre-treatments (Dehgan, 1983; Xaba, 2014), depending on seed morphology (Osborne, 1988), seed development and dormancy (Bewley & Black, 1994; Grobbelaar, 2002; Whitelock, 2002).

Table 4.2: Effects of mechanical scarification by sanding down the projecting coronula (MS 1), cracking the seed coat (MS 2), and no treatment as control (MS 0) on the leaf sprouting and number of leaves of *E. altensteinii* (n=15).

Treatments	Leaf Sprouting (days)		Number of Leaves (n)	
Ms0	193	±20.00a	0.47	±0.19a
Ms1	156	±19.95a	0.67	±0.16a
Ms2	245	±0.00b	0.00	±0.00b
One-way ANOVA				
Mechanical scarification	7.49**		5.64**	

Mean values ±SE are shown in columns. The mean values followed by different letters are significantly different at P < 0.01 (**) as calculated by Fisher's least significant difference

4.4.3. The effects of mechanical scarification in combination with gibberellic acid (GA_3) germination

Mechanical scarification

The results for this study showed that the mechanical scarification treatment MS1-$GA_3$1 (sanding without GA_3) was the most successful pre-germination treatment in promoting germination of *E. altensteinii* seed compared to the control. The significance level for this treatment from month 2 to 8 was consistently higher at $P < 0.001$ compared to month 1 at $P < 0.01$ within the same treatment. The MS1+GA_3 1 (sanding with GA_3/24hrs) treatment was highly significant from month 2 to 8 at the same level as the same treatment without GA_3 compared to the control. A further pre-germination treatment MS1+$GA_3$2 (sanding with GA_3/48hrs) became highly significant at month 7 to 8 of the germination period compared to the control. Being significant throughout the germination period the results show that these three treatments had a major significance in germination with mechanical scarification with the highest value compared to the control. All other treatments showed no significant levels for mechanical scarification and or an effect on GA_3 soaking. (See Table 4.3). This study agrees with Jones (2002) who reported that sanding the seed coat improved germination on *Encephalartos* species, while Perez-Farrera *et al.* (1999) earlier documented an increase in germination of *Cycas merolae*. Both Smith (1974) and Witte (1977) reported that the removal and cracking of the hard seed coat improved germination rate of *Zamia* seeds, while Broome (2001) documented that the cracking of the cycad seed coats are only effective when seeds are sown under aseptic conditions. While seed scarification has become common practice to improve germination on cycads, there are limited studies on the genus *Encephalartos.*

Scarifying seeds by sanding around the coronula were successful to break seed dormancy in *E. altensteinii.*

Gibberellic acid

The results showed that GA_3 had no effect on the treatment of seed from months 1 to 8 as it showed no significance compared to the control treatment. Treatment MS0+$GA_3$1 (GA_3/24hrs) had significantly low germination of 20% and no germination was recorded for MS0+$GA_3$2 (GA_3/48hrs) treatment (See Table 4.3).

Interaction of mechanical scarification and gibberellic acid

From the results it is clear that there was no significant interaction between the mechanical scarification and gibberellic acid in pre-treatments presented in this study for any of the treatments over the germination period. (See Table 4.3). The combination of mechanical scarification and soaking seeds in GA_3 did not further improve germination percent and germination rate. As noted earlier mechanical scarification was successful in which the germination period was reduced to four months and 60% germination were obtained in both sanding without GA_3 (MS1-$GA_3$1) and sanding in combination with soaking in GA_3 for 24hrs (MS1+$GA_3$1). It was also observed that soaking seeds for 48 hours (MS1+$GA_3$2) had prolonged the germination period when compared with 24 hours soaking and scarification without GA_3. These results revealed that mechanical scarification is sufficient for breaking seed dormancy of *E. altensteinii* (Frett,1987). Pérez-Farrera *et al.* (1999) reported similar results on the germination of *Dioon merolae* seeds, similarly Xaba (2014) reported low total germination on *Encephalartos latifrons* seeds that were treated with MS in combination with soaked in GA3 for 48 or 96 hours, and sanding in combination with soaking in GA_3 did not have positive effects on the germination of *E. altensteinii*. These results are in contrast with earlier studies that removed the sarcotestae and then soak seed with GA_3 to improve embryo development and germination (Dehgan, 1983; Dehgan, 1999), and also suggesting that exposing seeds to GA_3 for long period inhibits germination (Perez-Farerra *et al.*,1999). however other studies suggested that soaking periods for cycads differs on species (Dehgan,1983; Frett, 1987; Perez-Farerra *et al.*,1999; Xaba, 2014), perhaps soaking period or concentration was not suitable to further improve germination for *Encephalartos altensteinii*. (pers obs).

Table 4.3: Effects of pre-treating seeds with varying different mechanical scarification methods in combination with 1000 ppm of gibberellic acid (GA_3) on germination (mean ± standard error [SE]) of *E. altensteinii.* Treatments = MS0-$GA_3$1 (control), MS0+$GA_3$1 (24hrs), MS0+$GA_3$2 (48hrs), MS1- $GA_3$1 (sanded no GA_3), MS1+$GA_3$1 (sanded / 24hrs), MS1+$GA_3$2 (sanded / 48hrs), MS2+$GA_3$1 (cracked / 24hrs) and MS2+$GA_3$2 (cracked / 48hrs). (n=15).

Mechanical scarification	With GA_3	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8
MS0-GA_3 1	No	0±0.00b	6.67±6.67c	13.33±9.09bc	20±10.70bc	26.67±11.82bc	26.67±11.82bc	26.67±11.82b	33.33±12.60ab
MS0+GA_3 1	Yes	6.67±6.67b	6.67±6.67c	6.67±6.67c	13.33±9.09bc	20±10.70c	20±10.70c	20±10.70b	20±10.70bc
MS0+GA_3 2	Yes	0±0.00b	0±0.00c	0±0.00c	0±0.00c	0±0.00c	0±0.00c	0±0.00b	0±0.00c
MS1-GA_3 1	No	33.33±12.60a	46.67±13.33a	53.33±13.33a	60±13.09a	60±13.09a	60±13.09a	60±13.09a	60±13.09a
MS1+GA_3 1	Yes	20±10.70ab	46.67±13.33a	53.33±13.33a	60±13.09a	60±13.09a	60±13.09a	60±13.09a	60±13.09a
MS1+GA_3 2	Yes	13.33±9.09ab	33.33±12.60a b	40±13.09ab	40±13.09ab	53.33±13.33ab	53.33±13.33ab	60±13.09a	60±13.09a
MS2-GA_3 1	No	13.33±9.09ab	13.33±9.09bc	13.33±9.09bc	13.33±9.09bc	13.33±9.09c	13.33±9.09c	13.33±9.09b	13.33±9.09bc
MS2+GA_3 1	Yes	13.33±9.09ab	13.33±9.09bc	13.33±9.09bc	13.33±9.09bc	13.33±9.09c	13.33±9.09c	13.33±9.09b	13.33±9.09bc
MS2+GA_3 2	Yes	0±0.00b	0±0.00c	0±0.00c	0±0.00c	0±0.00c	0±0.00c	0±0.00b	0±0.00c
Two-way ANOVA									
Mechanical Scarification		4.96**	14.98***	18.60***	19.30***	20.20***	20.20 ***	22.33***	21.14***
Gibberellic Acid (GA_3)		1.65 ns	1.45	1.68 ns	2.89 ns	2.03 ns	2.03 ns	1.48 ns	1.82 ns
Mechanical Scarification*Gibberellic Acid (GA_3)		0.59 ns	0.06 ns	0.05ns	0.08 ns	0.26 ns	0.26 ns	0.48 ns	0.70 ns

Mean values ±SE are shown in columns. The mean values followed by different letters are significantly difference at $P < 0.01$ (**), $P < 0.001$ (***) and ns = not significant as calculated by Fisher's least significant difference.

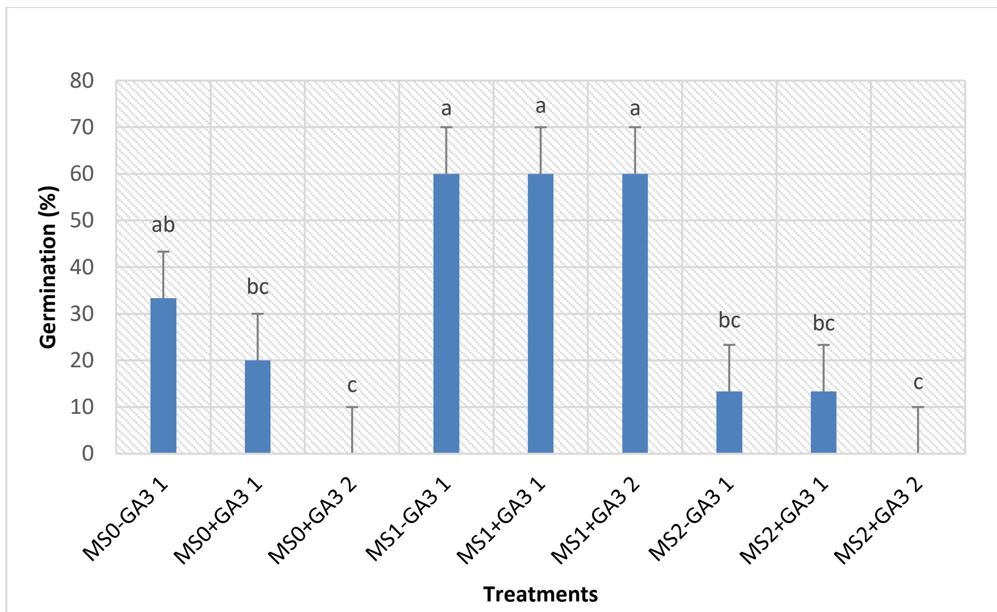

Figure 4.6: Effects of mechanical scarification in combination with gibberellic acid on seed germination of *E. altensteinii* measured at month eight, the final month of measurements. Vertical columns are means and the bars on each column are ± standard errors of mean. Treatments = MS0-$GA_3$1 (control), MS0+$GA_3$1 (GA_3/24hrs), MS0+$GA_3$2 (GA_3/48hrs), MS1-$GA_3$1 (sanded no GA_3), MS1+$GA_3$1 (sanded / 24hrs), MS1+$GA_3$2 (sanded / 48hrs), MS2+$GA_3$1 (cracked / 24hrs) and MS2+$GA_3$2 (cracked / 48hrs). (n=15)

Percentage germination

The highest final germination of 60% was obtained on MS1-GA_3 1 (sanding without GA_3), MS1+$GA_3$1 (sanding followed by 1000 ppm GA_3 for 24hrs), MS1+$GA_3$2 (sanding followed by 1000 ppm GA_3 for 48hrs) followed by MS0-$GA_3$1 (control) respectively compared to the control. Low germination of 0% (no germination) was recorded on MS0+$GA_3$2 (1000 ppm GA_3 for 48hrs) and MS2+$GA_3$2 (cracking followed by 1000 ppm GA_3 for 48hrs) respectively. The most rapid germination was found MS1-$GA_3$1 (33.33%) and MS1+$GA_3$1 (20%), with germination in month 1, both reached 53.33% in three months and last germination of 60% was recorded in month 4 of sowing, followed by MS1+$GA_3$2 reached 53.33% in month 5 and last germination recorded in month 8. (See Figure 4.7).

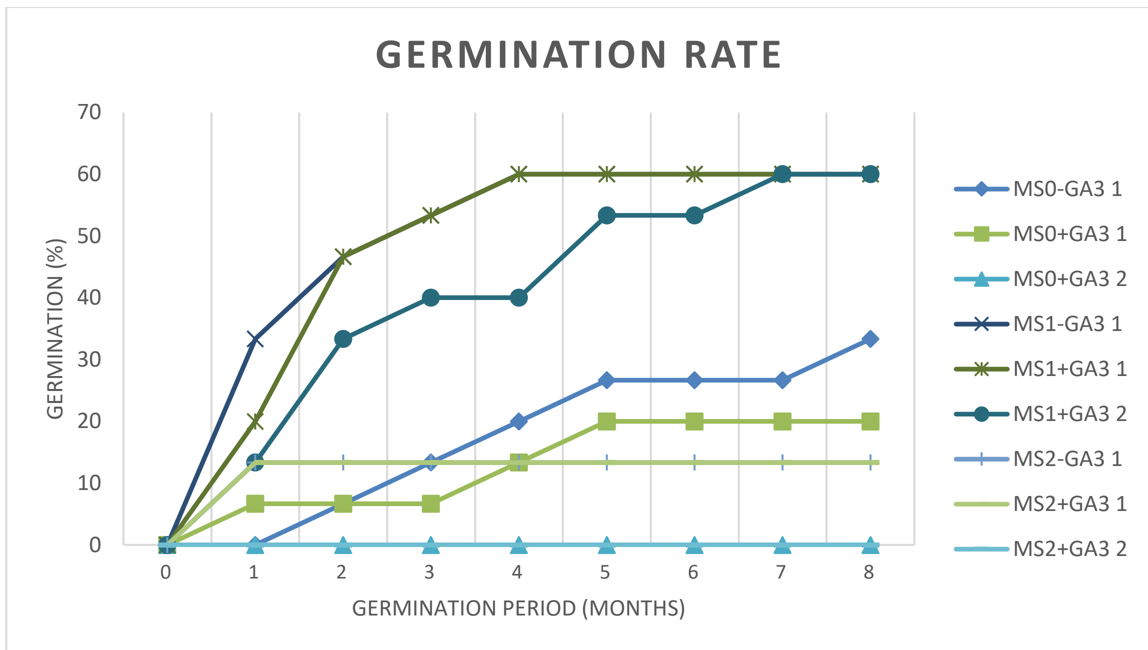

Figure 4.7: Effects of mechanical scarification in combination with gibberellic acid on seed germination rate of *E. altensteinii* measured Treatments = MS0-$GA_3$1 (control), MS0+$GA_3$1 (24hrs), MS0+$GA_3$2 (48hrs), MS1- $GA_3$1 (sanded no GA_3), MS1+$GA_3$1 (sanded / 24hrs), MS1+$GA_3$2 (sanded / 48hrs), MS2+$GA_3$1 (cracked / 24hrs) and MS2+$GA_3$2 (cracked / 48hrs) (n 15).

4.4.4. The effects of mechanical scarification in combination with gibberellic acid on seedling growth

Mechanical Scarification

Leaf sprouting

Two mechanical scarification treatments without GA_3 were used for this study, seeds treated with MS1-$GA_3$1 (sanding) recorded an average of 156 days and no leaf sprouting recorded in MS2-$GA_3$1 (cracking). The results indicate that mechanical scarification had rapid sprouting rate when compared with 193 days for untreated seeds and treatment MS1-$GA_3$1 reduced sprouting period on *E. altensteinii*.

Number of leaves

No significant difference observed on both mechanical scarification methods, suggesting that MS is not sufficient to stimulate vegetative growth of *E. altensteinii*. Conflicting results were reported by Witte (1977) when cracked seed coats of *Zamia integrifolia* and *Z. floridana* had highest number of leaves when compared with un-cracked seeds.

Gibberellic acid

Leaf sprouting

Most rapid leaf sprouting was recorded on treatment MS0-$GA_3$1 with 193 days (Control) compared to 210 for treatment MS0+$GA_3$1 (GA_3 for 24 hours) in the leaf sprouting of *E. altensteinii* seedlings. No leaf sprouting recorded in treatment MS0+$GA_3$2 (GA_3 for 48 hours) these results suggest that prolonged soaking in GA_3 had negative effects on germination (Perez-Farerra *et al.*,1999), consequently, leave sprouting of *E. altensteinii* seeds

Number of leaves.

Results showed that both treatment MS0+$GA_3$1 (GA_3 for 24 hours) and MS0+$GA_3$2 (GA_3 for 48 hours) had negative effect on the number of leaves, with an average of 0.33±0.72bc and 0±0.00c respectively when compared with 0.47±0.74ab for control.

Interaction of mechanical scarification and gibberellic acid

Leaf sprouting and number of leaves

The leaf sprouting was dependent on the seed germination and new leaves sprouting from the paired cotyledonary petiole of germinated seeds. Results showed no leaf sprouting was recorded on treatment MS2-$GA_3$1, MS2+$GA_3$1, MS2+$GA_3$2 and MS0+$GA_3$2 during the period of the experiment, these results suggest that cracking seed coat and prolonged soaking in GA_3 (Perez-Farerra *et al.*, 1999) had negative effects on germination, consequently leave sprouting. Conflicting results were reported by Witte (1977) when cracked seed coats of *Zamia integrifolia* and *Z. floridana* had highest number of leaves when compared with un-cracked seeds. The most rapid sprouting was observed on treatment MS1-$GA_3$1, MS1+$GA_3$2 and MS1+$GA_3$1 with an average of 156, 157 and 160 days, respectively. These results suggest that GA_3 and the combination of GA_3 and MS promote leaf sprouting when compared with untreated seeds (Table 4.4). The number of leaves counted were highly significant *(P < 0.05)* in the MS1+$GA_3$1 treatment compare to the control. The number of leaves were enhanced by the sprouting of leaves as indicated (Table 4.4). As seed dormancy in *Encephalartos* species is divided into two types, morphological dormancy and physical dormancy. Both these dormancies play a significant role in delaying germination with the result delay in leaf sprout and leave number. The positive relationship between germination rate and seedling growth performance suggests that, effect of pre-treatment on germination of *E. altensteinii* also translates into seedling leaf count. In the present study, scarification, GA_3 application had positive effect on seedling establishment, growth and plant height (Kouakou *et al.*, 2016) and scarification treatments promote germination and growth of seedling in many of the angiosperms and gymnosperms (Esen *et al.*, 2007). Therefore, the positive relationship between germination rate and growth parameters of *E. altensteinii*

seedlings implies that, benefits of pre-treatment does not end in germination but also contribute to the leaf count and establishment of seedlings.

Table 4.4: Effects of different mechanical scarification of sanding down the projecting coronula (MS1) and cracking in combination with 1000 ppm of gibberellic acid (GA_3) on the leaf sprouting and number of leaves. Treatments = MS0-$GA_3$1 (control), MS0+$GA_3$1 (24hrs), MS0+$GA_3$2 (48hrs), MS1- $GA_3$1 (sanded no GA_3), MS1+$GA_3$1 (sanded / 24hrs), MS1+$GA_3$2 (sanded / 48hrs), MS2+$GA_3$1 (cracked / 24hrs) and MS2+$GA_3$2 (cracked / 48hrs). (n=15).

Treatments	With GA_3	Leaf sprouting (days)		No of Leaves	
MS0-GA_3 1 (control)	No	193	±20.00ab	0.47	±0.74ab
MS0+GA_3 1	Yes	210	±18.43bc	0.33	±0.72bc
MS0+GA_3 2	Yes	245	±0.00c	0.00	±0.00c
MS1-GA_3 1	No	156	±19.95a	0.67	±0.62ab
MS1+GA_3 1	Yes	160	±19.14a	0.73	±0.71a
MS1+GA_3 2	Yes	157	±19.89a	0.60	±0.51ab
MS2-GA_3 1	No	245	±0.00c	0.00	±0.00c
MS2+GA_3 1	Yes	245	±0.00c	0.00	±0.00c
MS2+GA_3 2	Yes	245	±0.00c	0.00	±0.00c
Two-Way ANOVA					
Mechanical Scarification		28.11***		20.64***	
Gibberellic Acid (GA_3)		1.10ns		1.72ns	
Mechanical Scarification*Gibberellic Acid (GA_3)		1.10ns		1.04ns	

Mean values ±SE are shown in columns. The mean values followed by different letters are significantly different at $P < 0.001$ (***) and ns = not significant as calculated by Fisher's least significant difference.

4.5. Conclusions

This study has been conducted to investigate suitable pre-treatments that will improve germination percentage and the germination period of *E. altensteinii* in order to develop a propagation protocol for this vulnerable species. Overall results proved that mechanical scarification in sanding down the seed coat was highly successful in breaking seed dormancy of *E. altensteinii* seeds. Breaking physiological seed coat dormancy and morphological seed embryo dormancy are both complex processes which are controlled by various factors which inhibit germination to protect the seed in nature from premature germination and failure to succeed. Understanding the processes and document the results will greatly benefit the cultivation of species for reintroduction into the wild and to cultivation larger numbers for commercial purposes. Further germination studies could investigate other pre-germination treatments such as sulphuric acid, the role of light and temperature or combinations of these factors during germination.

4.6. Acknowledgement

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4.7. References

Baskin, C.C. & Baskin, J.M. 1988. Germination of ecophysiology of herbaceous plant species in a temperate region. *American Journal of Botany*, 75: 286-305.

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

Broome, T. 2001. Optimizing cycad seed germination. *The Cycad Newsletter*, 24(2): 5–7.

Burch, D. 1981. Propagation of Florida *Zamia* from Stem Pieces. SNA Research Conference, 221-223.

Calonje, M., Kay, J. & Griffith, M.P. 2011. Propagation of Cycad Collections from Seed. *Sibbaldia: The International Journal of Botanic Garden Horticulture*, 9: 79-96.

Dehgan, B. 1983. Propagation and growth of cycads – a conservation strategy. *Florida state Horticultural Society*, 96: 137-139.

Dehgan, B. & Johnson, C.R. 1983. Improved seed germination of *Zamia floridana* (sensu lnto) with H_2SO_4 and GA s. *Scientia Horticulturae,* 19: 357-361.

Dehgan, B. & B. Schutzman. 1983. Effect of H_2SO_4 and GA_3 on seed germination of Zamia furfuracea. *HortScience,* 18(3):371-372.

Dehgan, B & Almira, F.C. 1993. Horticultural practices and conservation of cycads. In: Stevenson, D.W & Norstog, K.J. (eds.), *The biology, structure, and systematics of the Cycadales: Proceedings of CYCAD 90, the Second International Conference on Cycad Biology*. Palm & Cycad Societies of Australia Cycads, Milton, Queensland, Australia. Pp. 332–338.

Dehgan, B. 1996. Permian permanence. *American Nurseryman,* 183(2): 66.

Dehgan, B., Almira, F.C, Dudeck, A.E. & Schutzman B. 2004. Effects of Varying Shade and Fertilizer on the Growth of *Zamia floridana* A. DC. *The Botanical Review*, 70(1): 79–85.

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. In: Biology and Conservation of Cycads. Proceedings of the 4th International Conference on Cycad Biology (ed. C-J. Chen). Panzhihua, China. Academic Publishers, Beijing.

Donaldson, J.S. 2003. Regional Overview: Africa. In: Donaldson, J. (ed.). *Cycads: status survey and conservation action plan.* Gland, Switzerland and Cambridge, UK: IUCN, 9–19.

Esen, D., Yildiz, O., Sarginci, M. & Isik, K. 2007. Effects of different pretreatments on germination of *Prunus serotina* seed sources. *Journal of Environmental Biology*, 28: 99-104.

Fenner, M. 1985. *Seed Ecology.* Chapman and Hall: London.

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

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

Grobbelaar, N. 2004. *Cycads: With special reference to the southern African species.* Published by the author. Pretoria.

Giddy, C. 1974. *Cycads of South Africa*. Purnell and Sons Ltd, Cape Town, South Africa.

Giddy, C. 1993. Cycad conservation legislation – does it work in South Africa? *In: Proceedings of Cycad 90, the 2nd International Conference on Cycad Biology* (eds. D.W. Stevenson and K.J. Norstog) Palm and Cycad Societies of Australia, Milton, Queensland

Gorski, T. & Gorska, K. 1979. Inhibitory effects of full daylight on the germination of *Lactuca sativa* L. *Planta,* 144: 121-124.

Hendricks, I.G. 1980. Germination of cycad seed. *The Cycad Newsletter,* 3: 3-8.

Hooft, J. 1970. *Zamia* from seed. *Carolina Tips*, 33: 21--22.

ISTA. 2003. ISTA Working Sheets on Tetrazolium Testing. Vol I Agricultural, Vegetable & Horticultural Species. International Seed Testing Association (ISTA), Bassersdorf, CH Switzerland.

IUCN. 2016. The IUCN red list of threatened species. Version 2016–3. http://www.iucnredlist.org.

Jones, D.L. 1993. *Cycads of the world*. Reed, Canberra.

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

Kouakou, L.K., Camille Kouakou, C., Koffi, K.K., Dao, J., Beugré, M.M., Baudoin, J.P. & Zoro Bi, I.A. 2016. Effect of mechanical scarification and gibberellins (GA_3) on seed germination and growth of Garcinia kola (Heckel). *Journal of Applied Biosciences*, 103:9811 – 9818.

Malwane, T.S.D. 2019. *In vitro* tissue culture: Towards conservation of threatened desiccation sensitive *Encephalartos* cycads seeds. MSc Dissertation, University of Cape Town.

Meerow, A.W. & Broschat, T.K. 1991. *Palm seed germination.* University of Florida IFAS Cooperative Extension BUL274, Gainesville, FL.

Nicolaeva, M.G. 1977. Factors controlling the seed dormancy pattern. In: Khan AA (Ed) *The Physiology and Biochemistry of Seed Dormancy and Germination*. North Holland, Amsterdam, Netherlands, 51–74.

LaRue, C.D. 1948. Regeneration in the megagametophyte of *Zamia floridana.* Bull. *Torrey Botany Club,* 75: 597--603.

Lima, A.T. & Meiado, M.V. 2017. Discontinuous hydration alters seed germination under stress of two populations of cactus that occur in different ecosystems in Northeast Brazil. *Seed Science Research,* 27: 292-302.

Osborne, R., Robbertse, P.J. & Claassen, M.I. 1991. The longevity of cycad pollen – Results of some preliminary experiments. *Journal of Cycad Society of South Africa*, 28: 10–13.

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

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

Pons, T. 1992. The role of temperature in the regulation of dormancy and germination. In: M. Fenner, ed. *Seeds: The ecology of regeneration in plant communities.* Wallingford: CABI Publishing, 261-292.

Smith, G.S. 1974. Seed treatments to speed germination of *Zamia integrifolia*. *Florida state Horticultural Society,* 436–438.

Steel, R.G.D. & Torrie, J.H. 1980. *Principle and procedures of statistics: a biometrical approach.* 2nd edition. McGraw-Hill, New York, NY.

Tang, W. 1993. Nectar-like secretions in female cones of cycads. *The Cycad Newsletter,* 16(2): 10–13.

Tommasi, F., Paciolla, C., Pinto, M.C. & De Gara, L. 2006. Effects of storage temperature on viability, germination and antioxidant metabolism in *Ginkgo biloba* L. seeds. *Plant Physiology and Biochemistry,* 44: 359–368.

Umair, S. 2011. Cycad Seed Biology and Embryo Cryopreservation: Especially *Cycas siamensis*, *Dioon edule* and *Zamia floridana*. MSc Thesis, University of Bedfordshire and Royal Botanic Gardens Kew, UK.

Whitelock, L.M. 1995. Cycad conservation in the past and the need for improvements in the future. *In: Proceedings of the 3rd International Conference on Cycad Biology* (ed. P. Vorster). The Cycad Society of South Africa, Matieland, South Africa.

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

Winter, J. 2004. *Encephalartos altensteinii* Lehm. South African National Diversity Institute. (Online). Available on the internet at: http://pza.sanbi.org/encephalartos-altensteinii (Accessed 20 July 2018).

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

Woodenberg, W.R., Erdey, D., Pammenter, N. & Berjak, P. 2007. Post-shedding seed behaviour of selected *Encephalartos* species. Abstracts from the 5th International Workshop on Desiccation Tolerance and Sensitivity of Seeds and Vegetative Plant Tissues. *South African Journal of Botany,* 73(3): 496.

Xaba, P.M.A. 2014. Pollination and Germination as Limiting Factors in the Propagation of Threatened Cycads, *Encephalartos* (*Zamiaceae*). MSc Thesis, University of Western Cape, South Africa.

Zarchini, M., Hashemabadi, D., Kaviani, B., Fallahabadi, P.R. & Negahdar, N. 2011. Improved germination conditions in *Cycas revoluta* L. by using sulphuric acid and hot water. *Plant Omics Journal*, 4 (7): 350-353.

CHAPTER 5:

THE EFFECTS OF CHEMICAL SCARIFICATION ON BREAKING SEED DORMANCY AND IMPROVING SEEDLING GROWTH OF THE *ENCEPHALARTOS ALTENSTEINII*, THE EASTERN CAPE GIANT CYCAD

The effects of chemical scarification on breaking seed dormancy and improving seedling growth of the *Encephalartos altensteinii*, the Eastern Cape giant cycad

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5.1 Abstract

Germination percentage and germination rate are generally low in most cycad species, including *E. altensteinii* and this has a negative effect on the production and conservation efforts for this most endangered plant group. Research on seed germination for cycads have been done mainly on *Zamia* and *Cycas*, and there is a limited research done on the germination of the African genus *Encephalartos.* In an effort to break seed dormancy of *E. altensteinii*, different concentrations of sulphuric acid (H_2SO_4) (0%, 10% & 25%) and gibberellic acid (GA_3) (1000ppm) for different soaking period were tested. Seeds were sown in silica sand over an eight-month period using 20 treatments and 15 replicates. Results of H_2SO_4 in combination with GA_3 tests showed statistically significant variance at $P < 0.05$ in germination percent and no significant difference recorded on H_2SO_4 (without GA_3). Germination percent among treatments ranged from 73.33% to 0.00%, highest germination (73.33%) was recorded on $H_2SO_4$2a+$GA_3$1 (25% of H_2SO_4 for 0.5hr followed by 1000ppm GA_3 for 24hrs). Furthermore, there was a significant difference ($P < 0.05$) among the treatments on leaf sprouting on seeds treated with combination of H_2SO_4 and GA_3. Also, on number of leaves results showed that there was a significant difference ($P < 0.001$). These results suggest that seed germination for *E. altensteinii* can be improved by using different scarification methods, the treatments also had positive effects on leaf sprouting, and leaf count. Soaking seeds in GA_3 for 48hrs has a negative effect on both germination percent and germination rate of *E. altensteinii* seeds.

Key words: germination rate, germination percentage, gibberellic acid (GA_3), sulphuric acid (H_2SO_4), mechanical scarification

5.2. Introduction

South African is a diversity hotspot for cycads worldwide, with 38 species and of these, 29 species are only found in South Africa (Donaldson, 2003), however this plant group remains the most threatened species with 78% threatened with extinction compare to the worldwide average of 62% (Raimondo *et al.*, 2009). According to the Red List of South African plants 11 species are critically endangered and three species are already extinct in the wild (Donaldson, 2008; Bosenberg & Donaldson, 2009; Donaldson, 2009a Donaldson, 2009b). One of the efforts to stop removal of cycads in their natural habitats, all species under genus *Encephalartos* are listed in Appendix 1 of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), meaning that collecting and trading of any plant material of indigenous cycads is prohibited except for research or that plants are artificially propagated. The aim of CITES is to regulate all international trade of endangered species, or species threatened by international trade.

Encephalartos altensteinii is the one of the largest African cycad species which mainly occurs in the eastern Cape region of South Africa. *E. altensteinii* is a large cycad which grows to 5 meters tall. Mature plants usually form clumps of 2 to 3 stems with basal suckers, only occur in the Eastern Cape province and south coast of KZN province in South Africa (Jones, 1993). The wild population has declined over the years due to habitat destruction for development and collection, currently this species in listed as Vulnerable (VU) on the Red List of South African plants, with an estimate of only 10 000 plants left in the wild (Winter, 2004; Donaldson, 2009c). *E. altensteinii* have gain extensive popularity over the years in garden designs and cycad collections with the result that the species removal from the wild has placed a continues pressure on the threatened red list species (Giddy, 1993; Whitelock, 1995; Donaldson & Bösenberg, 1999; Donaldson, 2003; IUCN, 2016). *E. altensteinii* is mostly propagated from seed, however seed sources remain low with additional problems of low seed viability and complex dormancy conditions.

Breaking physiological seed dormancy of cycad species has been achieved by chemical scarification with H_2SO_4 and morphological dormancy by removing sarcotestae layer of seeds before they are stored and pre-treated with GA_3 to enhance embryo development and germination (Frett, 1987; Dehgan, 1983). The Eastern Cape giant cycad is part of a plant group with complex seed dormancies, the species may have morpho-physiological dormancy (MPD), a combination of morphological and physiological dormancy (Witte, 1977; Dehgan, 1996). It remains unclear how-to successful break the seed dormancies of many African cycads.

Several studies have suggested that pre-germination treatments of sulphuric acid (H_2SO_4) and gibberellic acid (GA_3) could enhance seed germination in breaking seed dormancy conditions (Dehgan & Johnson, 1983; Dehgan & Schutzman, 1983; Frett, 1987; Dehgan, 1999; Perez-Farerra *et al.*, 1999; Zarchiniand *et al.*, 2011). Breaking physical seed dormancy of cycad species has been achieved by chemical scarification with H_2SO_4 (Frett, 1987; Zarchini *et al.,* 2011), and also soaking seeds in hot water at 100 ˚C for 1 hour and 25% of H_2SO_4 for 2 hours (Zarchini *et al.*, 2011). Pre-treatment of cycad seeds with GA_3 has been reported to stimulate rapid embryo growth and seed germination (Dehgan, 1983). Different soaking periods in GA_3 solutions (24 h or 48 h) in *Encephalartos* species are reported to improve the germination period and uniform germination (Dehgan, 1999). Combinations of chemical scarification with H_2SO_4 and soaking in GA_3 have resulted to 100% germination in four weeks in *Zamia floridana* (Dehgan & Johnson, 1983). *Cycas revoluta* showed improved germination when seeds treated with H_2SO_4 (Zarchini *et al.*, 2011). According to Frett (1987) *Cycas revoluta* seed treated with GA_3 at 500, 1000 and 5000 pm for 12 h resulted to low seed germination when compared with untreated (control) seeds, and Xaba (2014) reported that there was no significant difference in seed pre-treatment with GA3 on *E. latifrons* and *E. altensteinii* respectively, and these results disagree with the other studies on cycad seed germination and this raise a question about whether scarification or combination of scarification and growth regulator (GA_3) treatment could improve the germination of selected *Encephalartos* species. It is uncertain how *E. altensteinii* seeds will respond to varying concentration and soaking periods in sulphuric acid (H_2SO_4) and gibberellic acid (GA_3), as growth regulators showed an improvement on speeding up germination could support future propagation of the species for commercial and conservation purposes.

The continued threatened status of the species as well as the high commercial value necessitate the need to cultivate this slow growing species. This study has been conducted to investigate suitable pre-treatments that will improve germination percent and germination period of *E. altensteinii* in order to develop propagation protocols for this vulnerable species. This study was conducted to determine the effects of seed pre-treatments on breaking seed dormancy and to determine successful seedling growth of *E. altensteinii* to develop a propagation protocol for this species.

5.3. Materials and methods

Varying chemical seed scarification methods were used to test germination and seedling growth of the Eastern Cape giant cycad.

5.3.1. Artificial pollination

Receptive female cones of *E. altensteinii* were hand pollinated at Kirstenbosch National Botanical Garden (KNBG), Cape Town, in the Western Cape Province (-25°59'22.24″ S 18°25'44.2″ E) in April 2017 using a wet pollination method (Grobberlaar, 2004). Five grams of pollen were mixed with 500 ml of distilled water and used to pollinate receptive female cones three times every second day during the mid-morning and early evening when the micropylar droplets have formed (Tang, 1993; Jones, 2002; Xaba, 2014). The pollen solution was injected between the loose sporophylls using a syringe. Pollen used was collected and stored in a paper envelope and sealed in a plastic container with silica gel and stored at -15°C (Osborne *et al*. 1991, 1992).

5.3.2. Seed collection and storage

Naturally seeds take at least 7 months from pollination to shedding, therefore seeds were collected at KNBG when they started disintegrated on the cone. Collected seeds were soaked in tap water for a week, followed by removing the fleshy layer (sarcotestae), washed and air dried at room temperature (Burch, 1981; Frett, 1987; Meerow & Broschat, 1991). Seed were then dusted with Efekto Fungi-Nill 500 WP Captab fungicide, placed in mesh bags and stored in dry condition at a constant 15 °C and 15% relative humidity for 17 months (Hendricks, 1980). The seeds were transported to Pretoria in mesh bags on a two-hour flight at room conditions during in March 2019 and stored at room conditions for 5 days before the experimental work commenced.

5.3.3. Seed viability testing

Seed viability was tested using two methods 1) the water floating test where seeds were immersed in water. Seed that floated in water were regarded as non-viable seeds, discarded and all sinking seeds accepted as potentially viable (Burch, 1981; Perez-Farrera et al, 1999; Grobbelaar, 2002; Broome, 2001). 2) By soaking seeds in a solution of 2,3,5-Triphenyltetrazolium chloride (TTC). Random samples of 15 seeds each with 4 replicates were selected, the hard seed coat cracked with a nutcracker, the seed coat removed around the endosperm and the endosperm cut open longitudinally using scalpel blade to expose the embryo. Each seed was soaked in a 250 ml glass container in a solution of 0.5 g of TTC and 500 ml of distilled water for 60 minutes to determine viability percentage. Seed embryos that changed to a purple colour were regarded as viable and seeds that remained the same colour discarded as non-viable seeds (ISTA 2003; Tommasia, *et al.*, 2006).

5.3.4. Seed treatments and experimental design

The experiment consisted of chemical scarification methods which were applied on seeds of *E. altensteinii* in combination with gibberellic acid (GA_3). In addition to untreated seeds, there were 2 germination experiments that were tested. A randomized block design, with a total number of 300 seeds of *E. altensteinii,* made up of 20 treatments each with 15 replicates was used to investigate the effects of seed pre-treatment on germination of the Eastern Cape giant cycad. All seeds used were soaked for 24hrs in distilled water before the treatment. Germination treatments were divided into 2 Experiments: *Experiment 1*: the seeds were soaked in 10% or 25% of sulphuric acid (H_2SO_4) for 0.5hr or 1hr (Zarchini *et al.*, 2011). *Experiment 2*: seeds were chemical scarified with combination of 10% or 25% of H_2SO_4 for 0.5hr or 1hr followed by washing the seeds in running water for 5 minutes and soaked in 1000 ppm of GA_3 for 24hrs or 48hrs (Dehgan & Schutzman,1983; Dehgan and Johnson, 1983; Xaba, 2014).

Figure 5.1: The experimental arrangement (setup) on a heated bench showing seeds planted in rows in silica sand using a randomized block design.

Experiment 1: Sulphuric acid (H_2SO_4) treatments

1) $H_2SO_4$0 (Control)
2) $H_2SO_4$1a (seeds soaked in 10% of H_2SO_4 for 0.5hr)
3) $H_2SO_4$1b (seeds soaked in 10% of H_2SO_4 for 1hr)
4) $H_2SO_4$2a (seeds soaked in 25% of H_2SO_4 for 0.5hr)
5) $H_2SO_4$2b (seeds soaked in 25% of H_2SO_4 for 1hr)

Experiment 2: Sulphuric acid (H_2SO_4) and gibberellic acid (GA_3) treatments

1) $H_2SO_4$0-GA_3 1 (control)
2) $H_2SO_4$0+$GA_3$1 (soaking seeds in 1000ppm of GA_3 /24hrs)
3) $H_2SO_4$0+$GA_3$2 (soaking seeds in 1000ppm of GA_3 /48hrs)
4) $H_2SO_4$1a-$GA_3$1 (seeds soaked in 10% of H_2SO_4 for 0.5hr without GA_3)
5) $H_2SO_4$1a+$GA_3$1 (seeds soaked in 10% of H_2SO_4 for 0.5hr followed by soaking in 1000ppm of GA_3 /24hrs)
6) $H_2SO_4$1a+$GA_3$2 (seeds soaked in 10% of H_2SO_4 for 0.5hr followed by soaking in 1000ppm of GA3/48hrs)
7) $H_2SO_4$1b-$GA_3$1 (seeds soaked in 10% of H_2SO_4 for 1hr without GA_3)
8) $H_2SO_4$1b+$GA_3$1 (seeds soaked in 10% of H_2SO_4 for 1hr followed by soaking in 1000ppm of GA_3 /24hrs)
9) $H_2SO_4$1b+$GA_3$2 (seeds soaked in 10% of H_2SO_4 for 1hr followed by soaking in 1000ppm of GA_3 /48hrs)
10) $H_2SO_4$2a-$GA_3$1 (seeds soaked in 25% of H_2SO_4 for 0.5hr without GA_3)
11) $H_2SO_4$2a+$GA_3$1(seeds soaked in 25% of H_2SO_4 for 0.5hr followed by soaking in 1000 ppm GA_3 / 24hrs)
12) $H_2SO_4$2a+$GA_3$2 (seeds soaked in 25% of H_2SO_4 for 0.5hr followed by soaking in 1000ppm of GA_3 /48hrs)
13) $H_2SO_4$2b-$GA_3$1 (seeds soaked in 25% of H_2SO_4 for 1hr without GA_3)
14) $H_2SO_4$2b+$GA_3$1 (seeds soaked in 25% of H_2SO_4 for 1hr followed by soaking in 1000ppm of GA_3 / 24hrs)
15) $H_2SO_4$2b+$GA_3$2 (seeds soaked in 25% of H_2SO_4 for 1hr followed by soaking in 1000ppm of GA_3 /48hrs)

5.2.5. Greenhouse experiment

The experiment was conducted in the greenhouse of the Pretoria National Botanical Garden, East Pretoria, Gauteng province, South Africa, (-25°44’18.2″ S 28°16’19.8″ E) in the production nursery from April 2019 to December 2019. The greenhouse environmental conditions were controlled using maximum day temperatures which ranged between 21-28 °C and night temperatures between 15-21 °C, with an average of 43% relative humidity (Umair, 2011). The roof was covered with 30% shade net which provided a cooler controlled temperature. A seed sowing bench of 11 x 5 m with heating cables provided bottom heating during seed germination.

5.2.6. Germination study and data collection

After the pre-germination treatments, all seeds were sown in a germination bench at 27 °C by pushing them halfway in the silica sand used as sowing medium (Burch, 1981; Xaba, 2014). The germination bench was covered with 100% shade cloth and the seed were exposed to light only during watering and data collection. The soil medium was kept moist by watering once daily. Data recording for the experiment were done weekly. Seed germination was recorded when the growth of the seed radicle elongated to 3 mm or greater it was regarded as geminated (Tommasia, *et al.*, 2006). The germination percentage was determined by the number of seeds germinated at the end of the experiment for each treatment. The germination rate was indicated by the number of months it took for 50% of germinated seeds to germinate. Once germinated, seeds were removed from the bench and transplanted into 12 mm X 233 mm plastic planting bags in a soil media consisting of a ratio 1:2:1, river sand, compost and fine bark. Potted seedlings were placed in a greenhouse with an average temperature of 24 °C and night temperature ranging between 15 - 21 °C. Leaf sprouting was recorded daily for the first new leaf after germination and leaf count was recorded at the end of the experiment. The leaf count and leaf sprouting rate were dependent on the seed germination and new leaves sprouting from the paired cotyledonary petiole of germinated seeds. Leaf count data collection was delayed after the germination experiment by 4 months because of slow leaf sprouting from paired cotyledonary petiole.

5.2.7. Statistical analysis

Data for experiments 1: sulphuric acid (H_2SO_4) treatments was analysed using one-way and experiment 2: sulphuric acid (H_2SO_4) and gibberellic acid (GA_3) treatments were analysed using two-way analysis of variance (ANOVA) and computed software program TIBC STATISTICA Version 13.5. Occurrence of statistical difference was determined by using the Fisher Protected Least Significance Difference (L.S.D.) at values of $P < 0.05$; $P < 0.01$ and $P < 0.001$ levels of significance (Steel & Torrie, 1980).

5.4. Results and discussion

5.4.1. The effects sulphuric acid on germination

The results on seed scarification with sulphuric acid showed that there was no significant difference ($P > 0.05$) on the final seed germination percent of *E. altensteinii* among treatments (Table 4.2). The highest germination (33.33%) was recorded on $H_2SO_4$0 (control) followed by $H_2SO_4$1b (10% H_2SO_4 for 1hr) and $H_2SO_4$2a (25% H_2SO_4 for 0.5hr) with 13.33% germination respectively and low germination was recorded on $H_2SO_4$2b (25% H_2SO_4 for 1hr) with 0%

germination (Table 5.1). The most rapid germination was also obtained on $H2_SO_40$, started germination in month 2 with significant difference of $P < 0.05$ in month 5 when compared with treated seeds and slow germination was recorded on H_2SO_42a, started germination in month 6 (Figure 5.2). The results on this study indicated that pre-treating seeds by soaking in 10% or 25% of H_2SO_4 for 0.5hr or 1hr has a negative effect on the germination of *E. altensteinii* (Figure 5.1). Bareke (2018) described four types of seed dormancy, a) hard seed coat, b) embryo dormancy, c) immature embryo and d) chemical inhibitor, and cycad seeds may have morpho-physiological dormancy (MPD) (Witte, 1977; Dehgan, 1996). Perhaps the concentration or soaking period in only H_2SO_4 as described by Dehgan and Johnson (1983), and later by Frett (1987) are not suitable to break MPD of E. *altensteinii.* However, the responses are species specific and no one treatment has been reported to be effective universally (Clemens *et al*., 1977). High concentrations and long soaking period had no germination, similarly Baatuuwie *et al*. (2019) reported that high concentrations of H_2SO_4 with a long soaking period had low germination in *Detarium microcarpum* seeds, possibly H_2SO_4 penetrated into the endosperm and injured the seeds (Dehgan,1983; Zarchini *et al*. 2011).

Table 5.1: Effects of pre-treating seeds with varying concentrations of sulphuric acid on germination (mean ± standard error [SE]) of *E. altensteinii* (n=15). Treatments= H_2SO_40 (Control), H_2SO_41a (seeds soaked in 10% of H_2SO_4 for 0.5hr), H_2SO_41b (seeds soaked in 10% of H_2SO_4 for 1hr), H_2SO_42a (seeds soaked in 25% of H_2SO_4 for 0.5hr), H_2SO_42b (seeds soaked in 25% of H_2SO_4 for 1hr), (n=15).

Sulphuric acid	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8
H_2SO_40	0±0.00a	6.67±6.67a	13.33±9.09a	20±10.70a	26.67±11.82a	26.67±11.82a	26.67±11.82a	33.33±12.60a
H_2SO_41a	0±0.00a	0±0.00a	0±0.00a	6.67±6.67ab	6.67±6.67b	6.67±6.67ab	6.67±6.67ab	6.67±6.67b
H_2SO_41b	0±0.00a	0±0.00a	6.67±6.67a	6.67±6.67ab	6.67±6.67b	13.33±9.09ab	13.33±9.09ab	13.33±9.09ab
H_2SO42a	0±0.00a	0±0.00a	0±0.00a	0±0.00b	0±0.00b	6.67±6.67ab	13.33±9.09ab	13.33±9.09ab
H_2SO_42b	0±0.00a	0±0.00a	0±0.00a	0±0.00b	0±0.00b	0±0.00b	0±0.00b	0±0.00b
One-Way ANOVA								
Sulphuric acid	0.00 ns	1.00 ns	1.40 ns	1.60 ns	2.60*	1.60 ns	1.40 ns	2.10 ns

Mean values ±SE are shown in columns. The mean values followed by different letters are significantly different at $P < 0.05$ (*) and ns = not significant as calculated by Fisher's least significant difference.

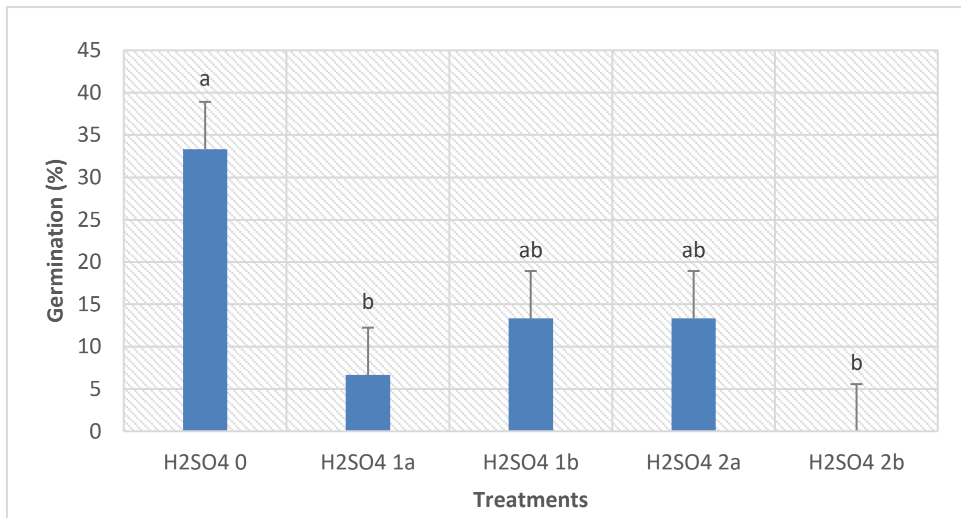

Figure 5.2: Effects of sulphuric acid on seed germination of *E. altensteinii*. Vertical columns are means and the bars on each column are ± standard errors of mean. The mean values represented by different letters differ significantly at $P < 0.05$ as calculated by Fisher's least significant difference. Treatments= $H_2SO_4$0 (Control), $H_2SO_4$1a (seeds soaked in 10% of H_2SO_4 for 0.5hr), $H_2SO_4$1b (seeds soaked in 10% of H_2SO_4 for 1hr), $H_2SO_4$2a (seeds soaked in 25% of H_2SO_4 for 0.5hr), $H_2SO_4$2b (seeds soaked in 25% of H_2SO_4 for 1hr) (n=15).

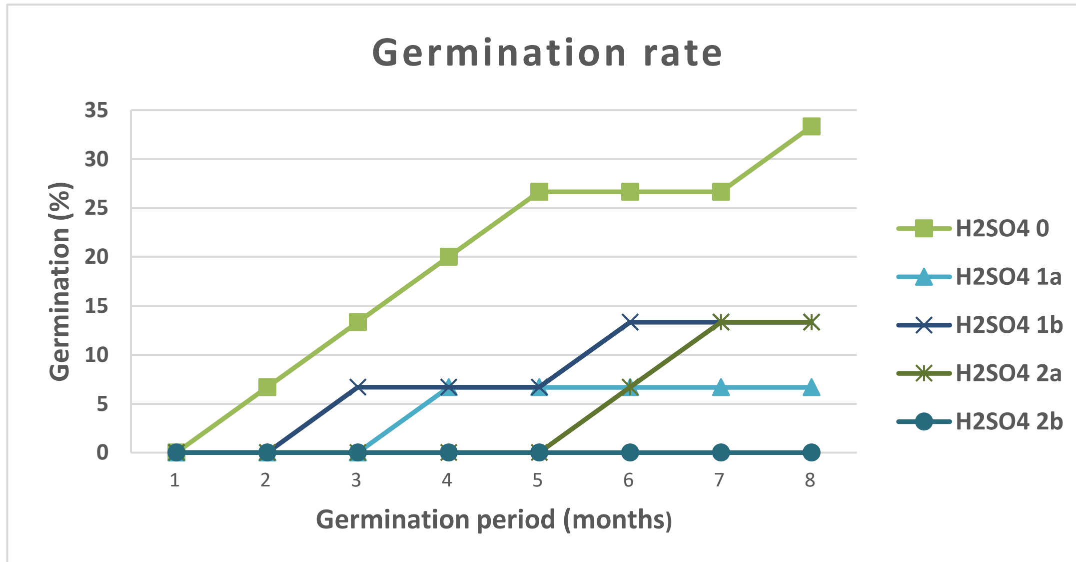

Figure 5.3: Effects of sulphuric acid on seed germination of *E. altensteinii* at 27 °C. Graph illustrates means of monthly germination over 8 months for all treatments. Treatments= $H_2SO_4$0 (Control), $H_2SO_4$1a (seeds soaked in 10% of H_2SO_4 for 0.5hr), $H_2SO_4$1b (seeds soaked in 10% of H_2SO_4 for 1hr), $H_2SO_4$2a (seeds soaked in 25% of H_2SO_4 for 0.5hr), $H_2SO_4$2b (seeds soaked in 25% of H_2SO_4 for 1hr), (n=15).

5.4.2. The effects sulphuric acid on seedling growth

Leaf sprouting

Germination and seedling establishment are important stages which influence quality and quantity of crop yields (Subedi & Ma, 2005). There was no significant difference recorded on leaf sprouting for seeds soaked in varying concentrations of H_2SO_4. It was however observed that the most rapid sprouting average (193 days) was obtained on $H_2SO_4$0 (control) when compared with other treatments followed by $H_2SO_4$1b, $H_2SO_4$2a and $H_2SO_4$1a (222, 222 & 233 days) respectively and no leaf sprouting was recorded for treatment $H_2SO_4$2b during the 245 days of the experiment (Table 5.2). As reported earlier that H_2SO_4 had a negative effect on the seed germination and was not sufficient to break morpho-physiological dormancy (MPD) which now also had a negative effect on the seedling growth of *E. altensteinii.*

Number of leaves

The different treatments of H_2SO_4 showed that there was no significant difference on the number of leaves. These results agree with Missanjo *et al.* (2014) reported no significant difference on the number of leaves and seedling height when compared with control. The highest average number of leaves was recorded on $H_2SO_4$0 (control) followed by $H_2SO_4$1b (seeds treated with 10% H_2SO_4 for 1hr) (0.47 and 0.40 leaves) respectively and no leaves were recorded on treatment $H_2SO_4$2b (Table 5.2). These results are largely dependent on the seed germination, and as reported earlier that improving water uptake was not sufficient to improve germination, which suggest that E. altensteinii may have morpho-physiological dormancy (MPD) as described to be a common phenomenon for cycads (Witte, 1977; Dehgan, 1996). and this had indirect negative effects on the vegetative growth of *E. altensteinii.*

Table 5.2: Effects of pre-treating seeds with varying concentrations and soaking periods in sulphuric acid (H_2SO_4), on seedling growth (mean ± standard error [SE]) of *E. altensteinii.* Treatments= $H_2SO_4$0 (Control), $H_2SO_4$1a (seeds soaked in 10% of H_2SO_4 for 0.5hr), $H_2SO_4$1b (seeds soaked in 10% of H_2SO_4 for 1hr), $H_2SO_4$2a (seeds soaked in 25% of H_2SO_4 for 0.5hr), $H_2SO_4$2b (seeds soaked in 25% of H_2SO_4 for 1hr) (n=15).

Treatments	Leaf Sprouting rate (days)		Number of Leaves	
$H_2SO_4$0 (control)	193	±20.00a	0.47	±0.19a
$H_2SO_4$1a	233	±11.00b	0.20	±0.20a
$H_2SO_4$1b	222	±15.63ab	0.40	±0.27a
$H_2SO_4$2a	223	±15.13ab	0.13	±0.09a
$H_2SO_4$2b	245	±0.00b	0.00	±0.00a
One-Way ANOVA				
Sulphuric acid (H_2SO_4)	1.84 ns		1.16 ns	

Mean values ±SE are shown in columns. The mean values followed by different letters are significantly different and ns = not significant as calculated by Fisher's least significant difference.

5.4.3. The effects of sulphuric acid in combination with gibberellic acid on germination.

Sulphuric acid

The results showed that scarification with sulphuric acid had no positive effect from month 1 to 8 as it showed no significant difference. Treatment $H_2SO_4$1b-$GA_3$1 (seeds soaked in 10% of H_2SO_4 for 1hr without GA_3) and $H_2SO_4$2a-$GA_3$1 (seeds soaked in 25% of H_2SO_4 for 0.5hr without GA_3) had significant low final germination of 13.33%, $H_2SO_4$1a-$GA_3$1 (seeds soaked in 10% of H_2SO_4 for 0.5hr without GA_3) had 6.67% and no germination was recorded for $H_2SO_4$2b-$GA_3$1 (seeds soaked in 25% of H_2SO_4 for 1hr without GA_3) (Table 5.3).

Gibberellic acid

The results showed that GA_3 had no effect on the treatment of seed from months 1 to 8 as it showed no significance compared to the control treatment. Treatment $H_2SO_4$0+$GA_3$1 (GA_3/24hrs) had significantly low germination of 20% and no germination was recorded for $H_2SO_4$0+$GA_3$2 (GA_3/48hrs) treatment (Table 5.3).

Interaction of sulphuric acid and gibberellic acid

From the results it is clear that there was significant interaction between the sulphuric acid and gibberellic acid in pre-treatments presented in this study The different treatments of H_2SO_4 in combination with GA_3 showed that there was a significant difference ($P < 0.05$) on the final seed germination of *E. altensteinii* (Table 5.3). As reported earlier, pre-treating seeds with only H_2SO_4 or GA_3 had low final germination. These results suggest that *E. altensteinii* may have morpho-physiological dormancy (MPD) as described to be a common phenomenon for cycads (Witte, 1977; Dehgan, 1996). The results showed that seeds soaked in 25% of H_2SO_4 for 0.5hr, followed by soaking in 1000 ppm of GA_3 for 24hrs ($H_2SO_4$2a+$GA_3$1) showed a significant difference ($P < 0.05$) in germination of *E. altensteinii* seed from months 2 to 8 compared to the control with the highest final germination of 73.33%, followed by $H_2SO_4$0-$GA_3$1 (control) with 33.33% germination. The results showed that pre-treating seeds with H_2SO_4 without addition of GA_3 generally had negative effect on the germination percent (13.33%) when compared with untreated seeds (33.33%). However, combination of soaking seeds in 25% of H_2SO_4 for 0.5hr followed by 1000ppm of GA_3 for 24hrs had highest germination percent (73.33%) when compared with all other treatments (Table 5.3). These findings are in agreement with results reported on similar study by Dehgan and Johnson (1983), and Dehgan and Schutzman (1983) which showed pre-treating seeds with the combination of H_2SO_4 and GA_3, improved seed germination of *Zamia floridana,* and Zarchini *et al.* (2011) also reported that 25% of H_2SO_4 increased germination of *Cycas revoluta*. Dehgan and Johnson (1983) reported a combination of soaking seeds in sulphuric acid followed by GA_3 treatments were effective in improving germination of *Zamia floridana* and *Zamia furfuracea,* while Frett (1987) reported that conflicting results with GA_3 had no significant difference on *Cycas revoluta* when compared with untreated seeds. However, seeds soaked in 25% of H_2SO_4 for 0.5hr followed by soaking in 1000 ppm of GA_3 for 48hrs had 26.67% final germination, compare to 73.33 for seeds soaked in GA_3 for 24 hours. These results suggesting that exposing seeds to GA_3 for long period inhibits germination (Perez-Farerra *et al.*,1999), however, soaking periods for cycads differs on species (Dehgan,1983; Frett, 1987; Perez-Farerra *et al.*,1999; Xaba, 2014). Low germination of 0.00% was recorded on $H_2SO_4$0+$GA_3$2 (1000ppm of GA_3 for 48hrs) and $H_2SO_4$2b-GA_3 1 (25% of H_2SO_4 for 1hr, without GA_3) respectively.

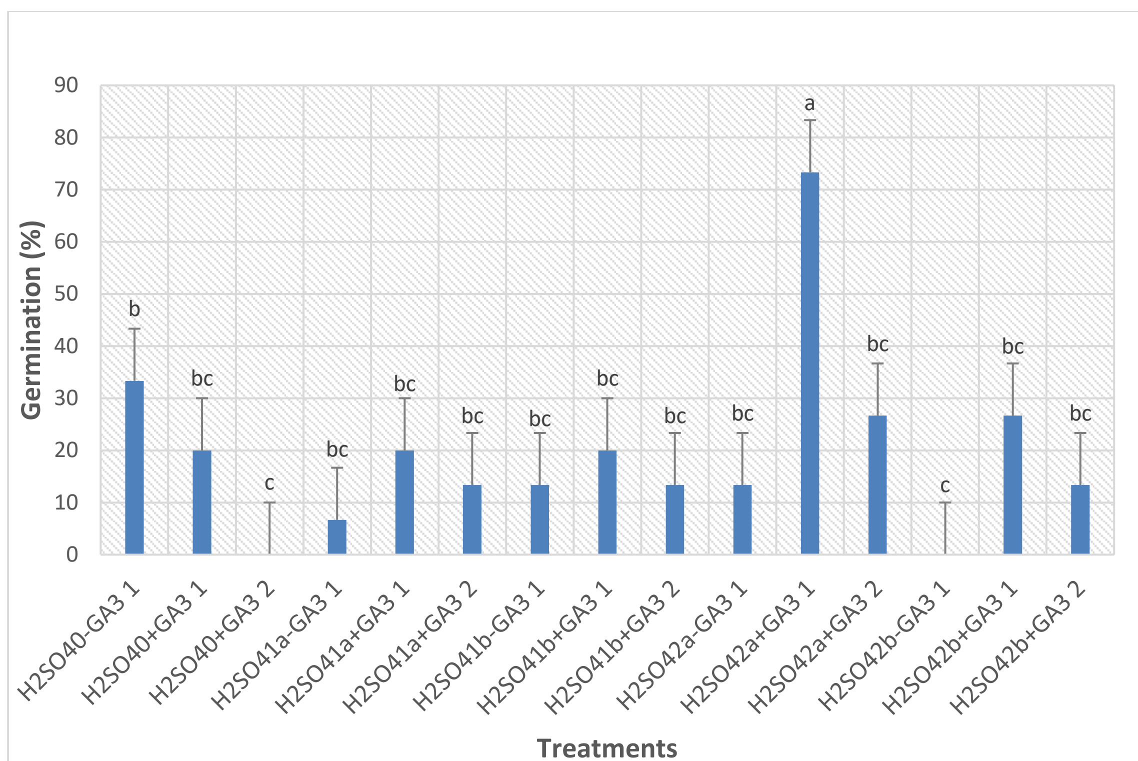

Figure 5.4: Effects of sulphuric acid in combination with gibberellic acid on seed germination of *E. altensteinii*. Vertical columns are means and the bars on each column are ± standard errors of mean. The mean values represented by different letters differ significantly at $P < 0.05$ as calculated by Fisher's least significant difference. Treatments= $H_2SO_4$0-$GA_3$1 (control).

Table 5.3: Effects of pre-treating seeds with varying concentrations and soaking periods in sulphuric acid (H_2SO_4), in combination with 1000 ppm of gibberellic acid (GA_3) on germination (mean ± standard error [SE]) of *E. altensteinii* (n=15).

Sulphuric acid	With GA_3	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8
$H_2SO_4$0-GA_3 1	No	0±0.00a	6.67±6.67ab	13.33±9.09ab	20±10.70ab	26.67±11.82ab	26.67±11.82b	26.67±11.82b	33.33±12.60b
$H_2SO_4$0+GA_3 1	Yes	6.67±6.67a	6.67±6.67ab	6.67±6.67b	13.33±9.09b	20±10.70bc	20±10.70bc	20±10.70bc	20±10.70bc
$H_2SO_4$0+GA_3 2	Yes	0±0.00a	0±0.00b	0±0.00b	0±0.00b	0±0.00c	0±00c	0±0.00c	0±0.00c
$H_2SO_4$1a-GA_3 1	No	0±0.00a	0±0.00b	0±0.00b	6.67±6.67b	6.67±6.67bc	6.67±6.67bc	6.67±6.67bc	6.67±6.67bc
$H_2SO_4$1a+GA_3 1	Yes	0±0.00a	0±0.00b	6.67±6.67b	6.67±6.67b	20±10.70bc	20±10.70bc	20±10.70bc	20±10.70bc
$H_2SO_4$1a+GA_3 2	Yes	0±0.00a	6.67±6.67ab	6.67±6.67b	6.67±6.67b	13.33±9.09bc	13.33±9.09bc	13.33±9.09bc	13.33±9.09bc
$H_2SO_4$1b-GA_3 1	No	0±0.00a	0±0.00b	6.67±6.67b	6.67±6.67b	6.67±6.67bc	13.33±9.09bc	13.33±9.09bc	13.33±9.09bc
$H_2SO_4$1b+GA_3 1	Yes	0±0.00a	0±0.00b	13.33±9.09ab	13.33±9.09b	20±10.70bc	20±10.70bc	20±10.70bc	20±10.70bc
$H_2SO_4$1b+GA_3 2	Yes	0±0.00a	0±0.00b	6.67±6.67b	6.67±6.67b	6.67±6.67bc	13.33±9.09bc	13.33±9.09bc	13.33±9.09bc
$H_2SO_4$2a-GA_3 1	No	0±0.00a	0±0.00b	0±0.00b	0±0.00b	0±0.00c	6.67±6.67bc	13.33±9.09bc	13.33±9.09bc
$H_2SO_4$2a+GA_3 1	Yes	6.67±6.67a	13.33±9.09a	26.67±11.82a	40±13.09a	46.67±13.33a	60±13.09a	73.33±11.82a	73.33±11.82a
$H_2SO_4$2a+GA_3 2	Yes	0±0.00a	6.67±6.67ab	13.33±9.09ab	13.33±9.09b	20±10.70bc	26.67±11.82b	26.67±11.82b	26.67±11.82bc
$H_2SO_4$2b-GA_3 1	No	0±0.00a	0±0.00b	0±0.00b	0±0.00b	0±0.00c	0±0.00c	0±0.00c	0±0.00c
$H_2SO_4$2b+GA_3 1	Yes	6.67±6.67a	13.33±9.09a	13.33±9.09ab	20±10.70ab	20±10.70bc	20±10.70bc	20±10.70bc	26.67±11.82bc
$H_2SO_4$2b+GA_3 2	Yes	0±0.00a	0±0.00b	0±0.00b	6.67±6.67b	13.33±9.09bc	13.33±9.09bc	13.33±9.09bc	13.33±9.09bc
Two-Way ANOVA									
Sulphuric acid		0.50 ns	0.84 ns	0.90 ns	0.90 ns	0.80 ns	2.13 ns	3.97**	3.50**
Gibberellic Acid		3.00 ns	1.69 ns	2.76 ns	3.94*	5.50**	4.92 **	6.10**	6.32**
Sulphuric acid *Gibberellic Acid		0.50 ns	1.04 ns	0.99 ns	1.63 ns	1.62 ns	1.82 ns	2.27*	2.53*

Mean values ±SE are shown in columns. The mean values followed by different letters are significantly different at $P < 0.05$ (*), $P < 0.01$ (**) and ns = not significant as calculated by Fisher's least significant difference (n=15).

5.4.4. The effects of sulphuric acid in combination with gibberellic acid on seedling growth

Sulphuric Acid

Leaf sprouting

In addition to untreated seeds, two concentrations and two soaking periods without GA_3 were used for this study. There was no significant difference recorded on leaf sprouting for seeds soaked in varying concentrations of H_2SO_4. Germination and seedling establishment are important stages which influence plant growth (Subedi & Ma, 2005), perhaps, poor germination indirectly influence leaf sprouting. It was observed that the most rapid sprouting average (193 days) was obtained on H_2SO_40-GA_31 (control), followed by H_2SO_41b-GA_31, H_2SO_42a-GA_31 and H_2SO_41a-GA_31 (222, 222 & 233 days) respectively and no leaf sprouting was recorded for treatment H_2SO_42b-GA_31 throughout the 245 days of the experiment (Table 5.4). As reported earlier that H_2SO_4 had negative effect on the seed germination, not sufficient to break morpho-physiological dormancy (MPD) (Witte, 1977; Dehgan, 1996) which also had a negative effect on the seedling growth of *E. altensteinii*

Number of leaves

The different treatments of H_2SO_4 showed that there was no significant difference on the number of leaves. These results agree with Missanjo *et al.* (2014) reported no significant difference on the number of leaves and seedling height when compared with control. The highest average number of leaves was recorded on H_2SO_40-GA_31 (control) followed by H_2SO_41b-GA_31 (seeds treated with 10% H_2SO_4 for 1hr) (0.47 and 0.40 leaves) respectively and no leaves were recorded on treatment H_2SO_42b (Table 5.4). These results are largely dependent on the seed germination, and as reported earlier that improving water uptake was not sufficient to improve germination, which suggest that *E. altensteinii* may have morpho-physiological dormancy (MPD) as described to be a common phenomenon for cycads (Witte, 1977; Dehgan, 1996). and this had indirect negative effects on the vegetative growth of *E. altensteinii*

Gibberellic acid

Leaf sprouting

In addition to control, seeds were soaked in 1000ppm of GA_3 for 24 or 48 hours to test the effect of GA_3 on leaf sprouting of *E. altensteinii*. Most rapid leaf sprouting was recorded on treatment H_2SO_40-GA_31 (control) followed by H_2SO_40+GA_31 (GA_3 for 24 hours). No leaf sprouting recorded in treatment H_2SO_40+GA_32 (GA_3 for 48 hours) (Table 5.4). These results suggest that GA_3 prolonged soaking in GA_3 had negative effects on germination, consequently, leaf sprouting of *E. altensteinii* seeds (Perez-Farerra *et al.*,1999). The responses are species specific and no one treatment has been reported to be effective

universally (Clemens *et al.*, 1977; Dehgan, 1983; Xaba, 2014), possibly GA_3 only is not sufficient or soaking periods are not suitable to stimulate seedling growth of *E. altensteinii*. Alternatively, soaking periods should be longer to be more effective.

Number of leaves

Results showed that both treatment $H_2SO_4$0+$GA_3$1 (GA_3 for 24 hours) and $H_2SO_4$0+$GA_3$2 (GA_3 for 48 hours) had a negative effect on the number of leaves, with an average of 0.33±0.72 and 0±0.00 respectively when compared with 0.47±0.74 for control (Table 5.4). These results differ with results reported that 1000 ppm of GA_3 improved number of leaves on *Cycas revoluta* (Ullah *et al.*, 2019) Perhaps the concentration or soaking periods used are not suitable to induce vegetative growth of *E. altensteinii* as pre-treatments differ from species (Clemens *et al.*, 1977; Dehgan, 1983; Xaba, 2014).

Interaction of sulphuric acid and gibberellic acid

Leaf sprouting and number of leaves

According to Witte (1977) and Dehgan (1996) cycads may have morpho-physiological dormancy (MPD) which is a combination of morphological and physiological dormancy (Nikolaeva, 1977). Gibberellins acid plays an important role in the stimulating vegetative growth, weakening endosperm around the embryo (Taiz & Zeiger, 2000), and sulphuric acid is effective in breaking morphological (hard seed coat) to improve water update and oxygen (Miranda *et* al., 2011; Olatunji *et al.*, 2013). The leaf sprouting and number of leaves on this study were dependent on the seed germination and new leaves sprouting from the paired cotyledonary petiole of germinated seeds. From the results it is clear that there was significant interaction between the sulphuric acid and gibberellic acid in pre-treatments presented in this study on both the leaf sprouting and number of leaves. (Table 5.4). The combination of sulphuric acid and soaking seeds in GA_3 improved seedling growth. The results showed that treatment $H_2SO_4$2a+$GA_3$1 (seeds soaked in 25% of H_2SO_4 for 0.5hr followed by soaking in 1000 ppm GA_3 / 24hrs) showed a significant difference ($P < 0.05$), reduced average leaf sprouting period to 118±20.66 compare to 193±20.00 for treatment $H_2SO_4$0-$GA_3$1 (control). The same treatment $H_2SO_4$2a+$GA_3$1 was highly significant ($P < 0.001$) with an average leaf count of 1.93±0.34a, followed by $H_2SO_4$2b+$GA_3$1 (seeds soaked in 25% of H_2SO_4 for 1hr followed by soaking in 1000 ppm of GA_3 / 24hrs) with an average leaf count of 0.73±0.30 compare with 0.47±0.19 for treatment $H_2SO_4$0-$GA_3$1 (control). It was reported earlier that pre-treating seeds with only H_2SO_4 or GA_3 had a negative effect on the seedling growth. No leaf sprouting of leaf count recorded for treatment $H_2SO_4$0+$GA_3$2 (soaking seeds in 1000 ppm of GA_3 /48hrs) and $H_2SO_4$2b-$GA_3$1 (seeds soaked in 25% of H_2SO_4 for 1hr without GA_3). These results confirm that cycad seed may have MPD (Witte, 1977; Dehgan & Schutzman, 1983; Dehgan, 1996; Xaba, 2014) and also suggest that long exposures to GA_3 or H_2SO_4 have a

negative effect on germination, consequently, seedling growth of *E. altensteinii* seeds (Perez-Farerra *et al.*,1999; Asl *et al.*, 2011; Azad *at al.*, 2012).

Germination and seedling establishment are important stages which influence plant growth (Subedi & Ma, 2005). As noted earlier that treatment $H_2SO_4$2a+$GA_3$1 (seeds soaked in 25% of H_2SO_4 for 0.5hr followed by soaking in 1000 ppm GA_3 / 24hrs) had the highest germination percent of 73.33%, and also significantly on both leaf sprouting ($P < 0.05$) and number of leaves ($P < 0.001$). The positive association between germination and seedling growth proposes that, result of pre-treatment on germination furthermore translates into vegetative growth. These results reveal that there is indirect influence of pre-treatment on growth performance of seedlings. Therefore, the positive relationship between germination and growth parameters of *E. altensteinii* seedlings indicates that, benefits of pre-treatment does not conclude in germination but also contribute to the establishment of seedlings.

Table 5.4: Effects of pre-treating seeds with varying concentrations and soaking periods in sulphuric acid (H_2SO_4), in combination with 1000 ppm of gibberellic acid (GA_3) on seedling growth (mean ± standard error [SE) of *E. altensteinii.* (n=15).

Treatments	With GA_3	Leaf Sprouting rate (days)		No of Leaves	
$H_2SO_4$0-GA_3 1	No	193	±20.00b	0.47	±0.19bcd
$H_2SO_4$0+GA_3 1	Yes	210	±18.44bc	0.33	±0.19bcd
$H_2SO_4$0+GA_3 2	Yes	245	±0.00c	0.00	0.00d
$H_2SO_4$1a-GA_3 1	No	233	±11.40bc	0.20	±0.20bcd
$H_2SO_4$1a+GA_3 1	Yes	209	±19.42bc	0.40	±0.21bcd
$H_2SO_4$1a+GA_3 2	Yes	220	±17.37bc	0.27	±0.18bcd
$H_2SO_4$1b-GA_3 1	No	222	±15.63bc	0.40	±0.27bcd
$H_2SO_4$1b+GA_3 1	Yes	213	±17.26bc	0.27	±0.15bcd
$H_2SO_4$1b+GA_3 2	Yes	225	±13.87bc	0.20	±0.14bcd
$H_2SO_4$2a-GA_3 1	No	223	±15.13bc	0.13	±0.09cd
$H_2SO_4$2a+GA_3 1	Yes	118	±20.66a	1.93	±0.34a
$H_2SO_4$2a+GA3 2	Yes	204	±18.37bc	0.6	±0.27bc
$H_2SO_4$2b-GA_3 1	No	245	±0.00c	0.00	0.00d
$H_2SO_4$2b+GA_3 1	Yes	194	±19.96b	0.73	±0.30b
$H_2SO_4$2b+GA_3 2	Yes	218	±18.31bc	0.27	±0.18bcd
Two-Way ANOVA					
Sulphuric acid (H_2SO_4)		3.10 *		5.06 ***	
Gibberellic Acid (GA_3)		7.12**		9.11 ***	
Sulphuric acid (H_2SO_4)*Gibberellic Acid (GA_3)		2.54*		4.17 ***	

Mean values ±SE are shown in columns. The mean values followed by different letters are significantly different at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) as calculated by Fisher's least significant difference.

5.5. Conclusion

This study has been conducted to investigate suitable pre-treatments that will improve germination percentage and the germination period of *E. altensteinii* in order to develop a propagation protocol for this vulnerable species. Overall succes proved that chemical scarification by seeds soaked in 25% of H_2SO_4 for 0.5hr followed by soaking in 1000 ppm GA_3 / 24hrs ($H_2SO_4$2a+$GA_3$1) was highly successful in breaking seed dormancy of *E. altensteinii* seeds. Breaking physiological seed coat dormancy and morphological seed embryo dormancy are both complex processes which are controlled by various factors which inhibit germination

to protect the seed in nature from premature germination and failure to succeed. Understanding the processes and document the results will greatly benefit the cultivation of species for reintroduction into the wild and to cultivation larger numbers for commercial purposes. Further germination studies could investigate other pre-germination treatments such as sulphuric acid, the role of light and temperature or combinations of these factors during germination.

5.6. Acknowledgement

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References

Asl, M.B., Sharivivash, R. & Rahbari, A. 2011. Effect of Different Treatments on Seed Germination of Honey Locust (*Gleditschia triacanthos*). *Modern Applied Science*, 5(1): 200-204.

Azad, M.S., Manik, M.R., Hasan, M.S. & Matin, M.A. 2011. Effect of different pre-sowing treatments on seed germination percentage and growth performance of *Acacia auriculiformis*. *Journal of Forestry Research,* 22(2): 183-188.

Baatuuwie, B.N., Nasare, L.I., Smaila, A., Issifu, H. & Asante, W.J. 2019. Effect of seed pre-treatment and its duration on germination of *Detarium microcarpum* (Guill. and Perr.) *African Journal of Environmental Science and Technology*, 13 (8): 317 323.

Bareke T. 2018. Biology of seed development and germination physiology. *Advances in Plants & Agriculture Research*, 8 (4): 336–346.

Bosenberg, J.D. & Donaldson, J.S. 2009. *Encephalartos brevifoliolatus* Vorster. National Assessment: Red List of South African Plants version 2017.1. http://redlist.sanbi.org/species.php?species=823-44. [Accessed on 2018/07/05].

Broome, T. 2001. Optimizing cycad seed germination. *The Cycad Newsletter,* 24(2): 5–7.

Burch, D. 1981. Propagation of Florida Zamia from Stem Pieces. SNA Research Conference, 221-223.

Clemens, J., Jones, P.G. & Gilbert, N.H. 1977. Effect of seed treatments on germination in *Acacia*. *Australian Journal of Botany*, 25: 269–276.

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

Dehgan, B. & Johnson, C.R. 1983. Improved seed germination of *Zamia floridana* (sensu Into) with H_2SO_4 and GA_3. *Scientia Horticulturae,* 19: 357-361.

Dehgan, B. & B. Schutzman. 1983. Effect of H_2SO_4 and GA_3 on seed germination of *Zamia furfuracea. HortScience*, 18: 371-372.

Dehgan, B. 1996. Permian permanence. *American Nurseryman,* 183(2): 66.

Dehgan, B.1999. Propagation and culture of cycads: A practical approach. In; Ruano, M.C. (ed.), Proceedings of the Second International Symposium on Ornamental Palms and other Monocots from the Tropics. *Acta Horticulture,* 486: 123-131.

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. In: Biology and Conservation of Cycads. Proceedings of the 4th International Conference on Cycad Biology (ed. C-J. Chen). Panzhihua, China. Academic Publishers, Beijing.

Donaldson, J.S. 2003. Regional Overview: Africa. In: Donaldson, J. (ed.). *Cycads: status survey and conservation action plan.* Gland, Switzerland and Cambridge, UK: IUCN, 9–19.

Donaldson, J.S. 2009. *Encephalartos altensteinii* Lehm. National Assessment: Red List of South African Plants version 2017.1. http://redlist.sanbi.org/species.php?species=823-2. [Accessed on 2018/06/19].

Donaldson, J. S. 2008. South African *Encephalartos* species. NDF workshop case studies: Case study 4: *Encephalartos*. Mexico.

Donaldson, J.S. 2009a. *Encephalartos woodii* Sander. National Assessment: Red List of South African Plants version 2017.1. http://redlist.sanbi.org/species.php?species=823-42. [Accessed on 2018/07/05].

Donaldson, J.S. 2009b. *Encephalartos nubimontanus* P.J.H.Hurter. National Assessment: Red List of South African Plants version 2017.1. http://redlist.sanbi.org/species.php?species=823-49. [Accessed on 2018/07/05].

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

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

Grobbelaar, N. 2004. *Cycads: With special reference to the southern African species.* Published by the author. Pretoria.

Giddy, C. 1993. Cycad conservation legislation – does it work in South Africa? *In: Proceedings of Cycad 90, the 2nd International Conference on Cycad Biology* (eds. D.W. Stevenson and K.J. Norstog) Palm and Cycad Societies of Australia, Milton, Queensland

Hendricks, I.G. 1980. Germination of cycad seed. *The Cycad Newsletter*, 3: 3-8.

Jones, D.L. 1993. *Cycads of the world.* Reed, Canberra. 180-181 & 216-217.

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

ISTA, 2003. ISTA Working Sheets on Tetrazolium Testing. Vol I Agricultural, Vegetable & Horticultural Species. International Seed Testing Association (ISTA), Bassersdorf, CH Switzerland.

IUCN, 2016. The IUCN red list of threatened species. Version 2016–3. http://www.iucnredlist.org.

Meerow, A.W. & Broschat, T.K. 1991. Palm seed germination. University of Florida IFAS Cooperative Extension BUL274, Gainesville, FL.

Miranda, R.Q., Oliveira, M.T.P., Correia, R.M., Almeida-Cortez J.S. & Pompelli, M.F. 2011. Germination of *Prosopis juliflora* (Sw) DC seeds after scarification treatments. *Plant Species Biology*, 26: 186-192.

Missanjo, E., Chioza, A. & Kulapani, C. 2014. Effects of Different Pretreatments to the Seed on Seedling Emergence and Growth of *Acacia polyacantha*. *International Journal of Forestry Research*, 6: 1-4.

Nicolaeva, M.G. 1977. Factors controlling the seed dormancy pattern. In: Khan AA (Ed) *The Physiology and Biochemistry of Seed Dormancy and Germination*. North Holland, Amsterdam, Netherlands, 51–74.

Olatunji, D., Maku, J.O. & Odumefun, O.P. 2013. The effect of pretreatments on the germination and early seedlings growth of *Acacia auriculiformis* Cunn. Ex. Benth. *African Journal of Plant Science*, 7: 325-330.

Osborne, R., Robbertse, P.J. & M.I. Claassen, M.I. 1991. The longevity of cycad pollen – Results of some preliminary experiments. *Journal of Cycad Society of South Africa*, 28: 10–13.

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

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

Raimondo, D., Von Staden, L., Foden, W., Victor, J.E., Helme, N.A., Turner, R.C., Kamundi, D.A. & Manyama, M.A (eds). 2009. *Red list of South African Plants*. Strelitzia 25. South African National Biodiversity Instituted. Pretoria

Steel, R.G.D. & Torrie, J.H. 1980. *Principle and procedures of statistics: a biometrical approach*, 2nd edition. McGraw-Hill, New York, NY.

Subedi, K.D. & Ma, B.L. 2005. Seed priming does not improve corn yield in a humid temperate environment, *Agronomy Journal*, 97(1): 211–218.

Taiz, L. & Zeiger, E. 2000 *Plant Physiology*. 3rd edition, Sinauer Associates, Sunderland.

Tang, W. 1993. Nectar-like secretions in female cones of cycads. *The Cycad Newsletter,* 16(2): 10–13.

Tommasi, F., Paciolla, C., Pinto, M.C. & De Gara, L. 2006. Effects of storage temperature on viability, germination and antioxidant metabolism in *Ginkgo biloba* L. seeds. *Plant Physiology and Biochemistry,* 44: 359–368.

Ullah, Z., Hassan, I., Hafiz, I.A. & Abbasi, N.A. 2019. Effect of different priming treatments on seed germination of sago palm (*Cycas revoluta* L.). *World Journal of Biology and Biotechnology*, 5(1): 1-3.

Umair, S. 2011. Cycad Seed Biology and Embryo Cryopreservation: Especially *Cycas siamensis*, *Dioon edule* and *Zamia floridana*. MSc Thesis, University of Bedfordshire and Royal Botanic Gardens Kew, UK.

Whitelock, L.M. 1995. Cycad conservation in the past and the need for improvements in the future. *In: Proceedings of the 3rd International Conference on Cycad Biology* (ed. P. Vorster). The Cycad Society of South Africa, Matieland, South Africa.

Winter, J. 2004. *Encephalartos altensteinii* Lehm. South African National Diversity Institute. (Online). Available on the internet at: http://pza.sanbi.org/encephalartos-altensteinii (Accessed 20 July 2018).

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

Xaba, P.M.A. 2014. Pollination and Germination as Limiting Factors in the Propagation of Threatened Cycads, *Encephalartos* (*Zamiaceae*). MSc Thesis, University of Western Cape, South Africa.

Zarchini, M., Hashemabadi, D., Kaviani, B., Fallahabadi, P.R. & Negahdar, N. 2011. Improved germination conditions in *Cycas revoluta* L. by using sulphuric acid and hot water. *Plant Omics Journal*, 4 (7): 350-353.

CHAPTER 6:

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

General discussion, conclusion and recommendations

6.1. Effect of hydration dehydration on moisture content, embryo growth and seed viability.

The principal aim of this study was to investigate whether the loss of seed viability is influenced by moisture content and storage conditions. The objective for this study was to determine the effects of seed HD on the moisture content, embryo development and viability of *E. altensteinii* seeds. The results showed that embryos were only more visible after 6 months of maturation and HD treatment slowed down moisture content loss consequently with improved seed viability. The results also showed that there was a significant decrease in viability when moisture content was below 25% and there was no significant difference on the embryo growth of HD seeds when compared with untreated seeds. In conclusion, HD has the potential to slow down viability loss and thus the potential to increase seed longevity of *E. altensteinii*. Based these conclusions the study recommends that there is a further need to test the effects different HD treatments on seeds stored under different storage conditions. It is also recommended that a be expanded to test the effects of HD treatments using GA_3 on embryo growth. Furthermore, it will be interesting to evaluate seed longevity and moisture content on seeds stored with the sarcotesta, outer fleshy layer of *E. altensteinii* seeds.

6.2. The effects of seed pre-treatment on germination and seedling growth

This study investigated whether seed germination of *E. altensteinii* was affected by different pre-germination treatments prior to sowing the seeds. The study measured different scarification methods on germination percentage, germination rate and seedling establishment of *E. altensteinii*. The significant findings on this study suggested that *E. altensteinii* seeds germination can be enhanced by pre-treating seeds by soaking seeds in 25% of H_2SO_4 for 0.5hr followed by soaking in 1000 ppm of GA_3 for 24hrs, and also by sanding down projecting coronula. The findings showed that sanding down the projecting coronula had rapid germination which reduced germination period to 4 months with a positive relationship between germination and seedling growth. Long soaking periods in both GA_3 and H_2SO_4 proved to be negative on the seed germination while cracking seed coat did not advance germination of *E. altensteinii* either. The findings on this study support future cultivation of *E. altensteinii* with improved germination success to assist growers in cultivate the species to support the ornamental landscape demand which could reduce illegal harvesting on wild populations and also increase plants to through conservation cultivation. Based on the findings of the seed germination of *E. altensteinii* the following recommendations for future

studies are suggested. 1) to test different methods of cracking seeds that will not damage endosperm and grown in vitro; 2) to investigate other growth regulators that are likely to improve leaf sprouting rate on germinated seeds and to reduce the period between germination and first leaf sprouting; 3) to investigate growth regulators to enhance root growth and desiccation tolerance on young seedlings.

CHAPTER 7:

LIST OF REFERENCES

7.1. List of references

Asl, M.B., Sharivivash, R. & Rahbari, A. 2011. Effect of Different Treatments on Seed Germination of Honey Locust (*Gleditschia triacanthos*). *Modern Applied Science,* 5 (1): 200-204.

Azad, M.S., Manik, M.R., Hasan, M.S. & Matin, M.A. 2011. Effect of different pre-sowing treatments on seed germination percentage and growth performance of *Acacia auriculiformis*. *Journal of Forestry Research,* 22(2):183-188.

Baatuuwie, B.N., Nasare, L.I., Smaila, A., Issifu, H. & Asante, W.J. 2019. Effect of seed pre-treatment and its duration on germination of *Detarium microcarpum* (Guill. and Perr.) *African Journal of Environmental Science and Technology*, 13 (8): 317 323.

Bareke T. 2018. Biology of seed development and germination physiology. *Advances in Plants & Agriculture Research,* 8 (4): 336–346.

Baskin, C.C. & Baskin, J.M. 1988. Germination of ecophysiology of herbaceous plant species in a temperate region. *American Journal of Botany*, 75: 286-305.

Baskin, C.C.; Baskin, J.M. 2014. *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination.* San Diego: Academic Press, 1586.

Berjak, P., Dini, M. & Pammenter, N.W. 1984. Possible mechanisms underlying the differing dehydration responses in recalcitrant and orthodox seeds: desiccation-associated subcellular changes in propagules of *Avicennia marina*. *Seed Science and Technology,* 12: 365-384.

Berjak, P., Farrant, J.M. & Pammenter, N.W. 1989. The basis of recalcitrant seed behaviour. In: Taylorson, R.B. (ed.), *Recent Advances in the Development and Germination of Seeds*. Plenum Publishing Corporation Inc., New York and London. 89-108.

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

Bosenberg, J.D. & Donaldson, J.S. 2009. *Encephalartos brevifoliolatus* Vorster. National Assessment: Red List of South African Plants version 2017.1. http://redlist.sanbi.org/species.php?species=823-44. [Accessed on 2018/07/05].

Broome, T. 2001. Optimizing cycad seed germination. *The Cycad Newsletter*, 24(2): 5–7.

Buitink, J., Vu, B.L., Satour, P. & Leprince, O. 2003. The re-establishment of desiccation tolerance in germinated radicles of *Medicago truncatula* Gaertn. seeds. *Seed Science Research*, 13: 273–286.

Burch, D. 1981. Propagation of Florida *Zamia* from Stem Pieces. SNA Research Conference, 221-223.

Calonje, M., Kay, J. & Griffith, M. P. 2011. Propagation of Cycad Collections from Seed. Sibbaldia: *The International Journal of Botanic Garden Horticulture*, 9: 79-96.

Chamberlain, C.J. 1935. *Gymnosperms: Structure and Evolution*. University of Chicago Press. Chicago.

Chien, C.T., Chen, S.Y., Chang, S.H. & Chung, J.D. 2012. Seed Germination and Storage of *Cycas taitungensis* (*Cycadaceae*). *Taiwan Journal for Science*, 27(1): 1-11.

Chin, H.F. & Roberts, E.H. 1980. Recalcitrant Crop Seeds. Tropical Press. SDN. BHD. Kuala Lumpur, Malaysia.

Clemens, J., Jones, P.G. & Gilbert, N.H. 1977. Effect of seed treatments on germination in Acacia. *Australian Journal of Botany,* 25: 269–276.

Conservation action plan. Gland, Switzerland and Cambridge, UK: IUCN, 48–57.

Convention on International Trade in Endangered Species. 2019. The CITES Trade Database. https://trade.cites.org/en/cites_trade/download/view_results? [Accessed on 2020/06/19].

Dadugula, S.R. 2011. Assessment of desiccation and freezing sensitivity towards development of cryopreservation protocols for three cycad species*: Cycas revoluta, Dioon*

edule and *Zamia furfuracea*. MSc. Thesis, University and Bedfordshire and Royal Botanic Gardens Kew, UK.

De Andrade, A.C.S. 2001. The effect of MC and temperature on the longevity of heart of palm seeds (*Euterpe edulis*). *Seed Science and Technology,* 29: 171-182.

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

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

Dehgan, B. & Johnson, C.R. 1983. Improved seed germination of *Zamia floridana* (sensu Into) with H2SO4 and GA s. *Scientia Horticulturae*, 19: 357-361.

Dehgan, B. & Schutzman, B. 1989. Embryo development and germination of Cycas seeds. *Journal of the American Society for Horticultural Science,* 114(1): 125–129.

Dehgan, B & Almira, F.C. 1993. Horticultural practices and conservation of cycads. In: Stevenson, D.W & Norstog, K.J. (eds.), The biology, structure, and systematics of the Cycadales: Proceedings of CYCAD 90, the Second International Conference on Cycad Biology. Palm & Cycad Societies of Australia Cycads, Milton, Queensland, Australia. Pp. 332–338

Dehgan, B. 1996. Permian permanence. *American Nurseryman*, 183(2): 66.

Dehgan, B. 1999. Propagation of and culture of cycads: a practical approach. In: Ruano, M.C. (ed.), Proceedings of the Second International Symposium on Ornamental Palms and other Monocots from the Tropics. *Acta Horticulture*, 486: 123-131.

Dehgan, B., Almira, F.C, Dudeck, A.E. & Schutzman B. 2004. Effects of Varying Shade and Fertilizer on the Growth of *Zamia floridana* A. DC. *The Botanical Review*, 70(1): 79–85.

Demir, I. & Mavi, K. 2003. Effect of controlled hydration treatments on storage longevity of *aubergine* seeds during development. *Israel Journal of Plant Sciences,* 51(4): 291-295.

Department of Environmental Affairs. 2017. Biodiversity Management Plan for 11 critically endangered and 4 endangered *Encephalartos* species. https://cer.org.za/wp-content/uploads/2004/09/National-Environmental-Management-Biodiversity-Act-10-2004-Biodiversity-Management-20170428-GGN-40815-00315.pdf. [Accessed on 2020/07/22].

Dinakar, C. & Bartels, D. 2013. Desiccation tolerance in resurrection plants: new insights from transcriptome, proteome, and metabolome analysis. *Frontiers in Plant Science*, 4: 482.

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

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. In: Biology and Conservation of Cycads. Proceedings of the 4th International Conference on Cycad Biology (ed. C-J. Chen). Panzhihua, China. Academic Publishers, Beijing.

Donaldson, J.S., Dehgan, B., Vovides, A.P. & Tang, W. 2003. Cycads in trade and sustainable use of cycad populations. In: Donaldson, J. (ed.). *Cycads: status survey and conservation action plan.* Gland, Switzerland and Cambridge, UK: IUCN, 48–57.

Donaldson, J.S., Hill, K.D. & Stevenson, D.W. 2003. Cycads of the World: An Overview. In: Donaldson, J. (ed.). Cycads: status survey and conservation action plan. Gland, Switzerland and Cambridge, UK: IUCN, 3–8.

Donaldson, J.S. 2003. Regional Overview: Africa. In: Donaldson, J. (ed.). Cycads: status survey and conservation action plan. Gland, Switzerland and Cambridge, UK: IUCN, 9–19.

Donaldson, J.S. 2008. South African *Encephalartos* species. NDF workshop case studies: Case study 4: *Encephalartos*. Mexico.

Donaldson, J.S. 2009a. *Encephalartos woodii* Sander. National Assessment: Red List of South African Plants version 2017.1. http://redlist.sanbi.org/species.php?species=823-42. [Accessed on 2018/07/05].

Donaldson, J.S. 2009b. *Encephalartos nubimontanus* P.J.H.Hurter. National Assessment: Red List of South African Plants version 2017.1. http://redlist.sanbi.org/species.php?species=823-49. [Accessed on 2018/07/05].

Donaldson, J.S. 2009c. *Encephalartos altensteinii* Lehm. National Assessment: Red List of South African Plants version 2017.1. http://redlist.sanbi.org/species.php?species=823-2. [Accessed on 2018/06/19].

Dyer, R. A. 1965. The cycads of Southern Africa. *Bothalia* 8: 405- 515.

El-Keblawy, A. & Al-Ansari, F. 2000. Effect of site of origin, time of seed maturation and seed age on germination behavior of *Portulaca oleracea* L. from old and new world. Canadian Journal of Botany, 78: 279-287.

El-Keblawy, A. & Al-Rawai, A. 2006. Effects of seed maturation time and dry storage on light and temperature requirements during germination in invasive *Prosopis juliflora*. *Flora*, 201: 135-143.

Esen, D., Yildiz, O., Sarginci, M. & Isik, K. 2007. Effects of different pretreatments on germination of *Prunus serotina* seed sources. *Journal of Environmental Biology*, 28:99-104.

Farrant, J.M., Pammenter, N.W. & Berjak, P. 1992. Development of recalcitrant (homoiohydrous) seeds of *Avicennia marina*: Anatomical, ultrastructural and biochemical events associated with development from histodifferentiation to maturation. *Annals of Botany,* 70:75-86.

Fenner, M. 1985. *Seed Ecology*. Chapman and Hall: London.

Finch-Savage, W.E. & Blake, P.S. 1994. Indeterminate development in desiccation sensitive seeds of *Quercus robur* L. *Seed Science Research,* 4:127-133.

Foley, M.E. & Chao, W.S. 2008. Growth Regulators and Chemicals Stimulate Germination of Leafy Spurge (*Euphorbia esula*) Seeds. *Weed Science*, 56(4): 516-522.

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

Fragniere, Y., Betrisey, S., Cardinaux, L., Stoffel, M. & Kozlowski, G. 2015.Fighting their last stand? A global analysis of the distribution and conservation status of gymnosperms. *Journal of Biogeography,* 42: 809– 820.

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

Fu, J.R., Jin, J.P., Peng, Y.F. & Xia, Q.H. 1994. Desiccation tolerance in two species with recalcitrant seeds: *Clausena lansium* L. and *Litchi chinensis* S. *Seed Science Research,* 4:257-261.

Galloway, L.F. 2002. The effect of maternal phenology on offspring life history in the herbaceous plant *Campanula americana. Journal of Ecology,* 90: 851-858.

Giddy, C. 1974. *Cycads of South Africa.* Purnell and Sons Ltd, Cape Town, South Africa.

Giddy, C. 1993. Cycad conservation legislation – does it work in South Africa? In: Proceedings of Cycad 90, the 2nd International Conference on Cycad Biology (eds. D.W. Stevenson and K.J. Norstog) Palm and Cycad Societies of Australia, Milton, Queensland

Giddy, C. 1996. Cycad specialist group: 6th CITES committee meeting, Tenerife, Canary Island, June 1995. Species, 26-27: 106-107.

Giddy, C. 1996. Cycad Specialist Group: Cycad action plan. Species 26–27: 106–107.

Goode, D. G. 2001. *Cycads of Africa Volume* I. D & E Cycads of Africa Publishers, Sandton.

Gorski, T. & Gorska, K. 1979. Inhibitory effects of full daylight on the germination of *Lactuca sativa* L. *Planta*, 144: 121-124.

Grobbelaar, N. 2002. *Cycads – with special reference to the Southern African species.* Published by the author, Pretoria, South Africa, 331.

Grobbelaar, N. 2004. *Cycads: With special reference to the southern African species.* Published by the author. Pretoria.

Hendricks, I.G. 1980. Germination of cycad seed. *The Cycad Newsletter,* 3: 3-8.

Hong, T.D. & Ellis, R.H. 1990. A comparison of maturation drying, germination, and desiccation tolerance between developing seeds of *Acer pseudoplatanus* L. and *Acer platenoides* L. *New Phytologist,* 116: 589-596.

Hooft, J. 1970. *Zamia* from seed. *Carolina Tips*, 33: 21--22.

Hubbuch, C. 1987. Cycads: Propagation and container culture*. Fairchild Tropical Garden Bulletin,* 42: 5–8.

Ibrahim, A.E., Roberts, E.H. & Murdoch A.J. 1983. Viability of lettuce seeds. II Survival and oxygen uptake in osmotically controlled storage. *Annals of Botany*, 34: 631-640.

Ingram, J. and Bartels, D. 1996. The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 47: 377–403.

ISTA. 2003. ISTA Working Sheets on Tetrazolium Testing. Vol I Agricultural, Vegetable & Horticultural Species. International Seed Testing Association (ISTA), Bassersdorf, CH Switzerland.

ISTA. 2008. International Rules for Seed Testing, Edition 2008. International Seed Testing Association (ISTA), Bassersdorf, CH-Switzerland.

IUCN 2016. The IUCN red list of threatened species. Version 2016.3. http://www.iucnredlist.org. [Accessed 05 July 2020]

Jones, D.L. 1993. *Cycads of the world*. Reed, Canberra, 180-181 &216-217.

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

Khan, M.M., Thompson, K., Usman, M. & Fatima, B. 2001. Role of MC and controlled atmosphere in Citrus seed storage. *International Journal of Agriculture & Biology*, 04(2): 259–266.

Kouakou, L.K., Camille Kouakou, C., Koffi, K.K., Dao, J., Beugré, M.M., Baudoin, J.P. & Zoro Bi, I.A. 2016. Effect of mechanical scarification and gibberellins (GA3) on seed germination and growth of Garcinia kola (Heckel). *Journal of Applied Biosciences*, 103:9811 – 9818.

LaRue, C.D. 1948. Regeneration in the megagametophyte of *Zamia floridana.* Bull. *Torrey Botany Club,* 75: 597--603.

Leprince, O., Hendry, G.A.F. & McKersie, B.D. 1993. The mechanisms of desiccation tolerance in developing seeds. *Seed Science Research*, 3: 231–246.

Lima, A.T. & Meiado, M.V. 2017. Discontinuous hydration alters seed germination under stress of two populations of cactus that occur in different ecosystems in Northeast Brazil. Seed Science Research, 27: 292-302.

Lima, A.T., Cunha, P.H.J., Dantas, B.F. & Meiado, M.V. 2018a. Does discontinuous hydration of *Senna spectabilis* (DC.) H.S. Irwin & Barneby var. *excelsa* (Schrad.) H.S. Irwin & Barneby (Fabaceae) seeds confer tolerance to water stress during seed germination? *Journal of Seed Science*, 40(1): 036-043.

Lima, A.T., Oloveira, D.M. & Meiado, M.V. 2018b. Effect of hydration and dehydration treatments on *Macroptilium atropurpureum* seeds germination under water deficit conditions. *Communications in plant sciences*, 08: 55-61.

Lin, T.P. & Chen, M.H. 1995. Biochemical characteristics associated with the development of the desiccation-sensitive seeds of *Machilus thunbergii. Annals of Botany,* 76: 381-387.

Maia, J., Dekkers, B.J.W., Provart, N.J., Ligterink, W. & Hilhorst, H.W.M. 2011. The Re-Establishment of Desiccation Tolerance in Germinated *Arabidopsis thaliana.* Seeds and Its Associated Transcriptome. PLoS One, 6(12): e29123.

Maia, J., Dekkers, B.J.W., Dolle, M.J., Ligterink W. & Hilhorst H.W.M. 2014. Abscisic acid (ABA) sensitivity regulates desiccation tolerance in germinated Arabidopsis seeds. *New Phytologist,* 203: 81–93.

Malwane, T.S.D. 2019. In vitro tissue culture: Towards conservation of threatened desiccation sensitive *Encephalartos* cycads seeds. MSc Dissertation, University of Cape Town.

Marler, P. N., & T.E. Marler. 2015. An assessment of red list data for the Cycadales. *Tropical Conservation Science,* 8: 1114–1125. www.tropicalconservationscience.org. [Accessed on 2018/06/19].

McCarty, D.R. 1995. Genetic control and integration of maturation and germination pathways in seed development. *Annual Review Plant Physiology and Plant Molecular Biology*, 46: 71–93.

Meerow, A.W. & Broschat, T.K. 1991. *Palm seed germination*. University of Florida IFAS Cooperative Extension BUL274, Gainesville, FL.

Meyer, S.E & Monsen, S.B. 1991. Habitat-correlated variation in mountain big sagebrush (*Artemisia tridentate ssp. vaseyana*) seed germination patterns. *Ecology,* 72: 739-724

Meyer, S.E. & Allen, P.S. 1999. Ecological genetics of seed germination regulation in *Bromus tectorum* L. II. Reaction norms in response to a water stress gradient imposed during seed maturation. *Oecologia*, 120: 35-43.

Miranda, R.Q., Oliveira, M.T.P., Correia, R.M., Almeida-Cortez J.S. & Pompelli, M.F. 2011. Germination of *Prosopis juliflora* (Sw) DC seeds after scarification treatments. *Plant Species Biology*, 26: 186-192.

Missanjo, E., Chioza, A. & Kulapani, C. 2014. Effects of Different Pretreatments to the Seed on Seedling Emergence and Growth of *Acacia polyacantha. International Journal of Forestry Research*, 6: 1-6.

Mostert, C. 2000. Aspekte van die Keimingsgedrag en Fynstruktuur van *Encephalartos* stuimeel. MSc Thesis. University of Pretoria, South Africa.

Mucina, L. & Rutherford, M.C. (eds) 2006. The vegetation of South Africa, Lesotho and Swaziland. *Strelitzia 19*. South African National Biodiversity Institute, Pretoria. *Newsletter*, 22(1): 10–12.

Nicolaeva, M.G. 1977. Factors controlling the seed dormancy pattern. In: Khan AA (Ed) *The Physiology and Biochemistry of Seed Dormancy and Germination*. North Holland, Amsterdam, Netherlands, 51–74.

Ntuli, T.M., 2012. Drought and Desiccation-Tolerance and Sensitivity in Plants. *Botany*, 67: 435-441.

Olatunji, D., Maku JO & Odumefun OP (2013). The effect of pretreatments on the germination and early seedlings growth of *Acacia auriculiformis* Cunn. Ex. Benth. *African Journal of Plant Science*, 7: 325-330.

Osborne, R., Robbertse, P.J. & Claassen, M.I. 1991. The longevity of cycad pollen – Results of some preliminary experiments. *Journal of Cycad Society of South Africa*, 28: 10–13.

Osborne, R., Robbertse, P.J. & Claassen, M.I. 1992. The longevity of cycad pollen in storage. *South African Journal of Botany*, 58(4): 250–254.

Palmbob. 2004. Encephalartos Species, Altenstein's Cycad, Bread Tree, Eastern Cape Giant Cycad, Prickly Cycad (*Encephalartos altensteinii*). Dave's Garden. https://davesgarden.com/guides/pf/showimage/57616/. [Accessed 20 July 2020]

Pammenter, N.W., Farrant, J.M. & Berjak, P. 1984. Recalcitrant seeds: short-term storage effects in *Avicennia marina* (Forsk.) Vierh. may be germination-associated. *Annals of Botany* 54: 843-846.

Pammenter, N.W., Berjak, P., Farrant, J., Smith, M., & Ross, G. 1994. Why do stored hydrated recalcitrant seeds die? *Seed Science Research*, 4(2): 187-191.

Pammenter, N.W. & Berjak, P. 1999 A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms, *Seed Science Research,* 9: 13–37.

Parihar, S.S., Dadlani, M., Lal, S.K., Tonapi, V.A., Nautiyal, P.C. & Basu, S. 2014. Effect of seed MC and storage temperature on seed longevity of hemp (*Cannabis sativa*). *Indian Journal of Agricultural Sciences*, 84 (11): 1303–1309.

Pellmyr, O., Tang, W., Groth, I., Bergstrom, G. & Thien, L.B. 1991. Cycad cone and angiosperm floral volatiles: inferences for the evolution of insect pollination. *Biochemical Systematics and Ecology,* 19: 623– 627.

Pérez-Farrera, M.A., Vovides, A.P. & Alvarez-Moctezuma, J.G. 1999. A study on seed germination of the cycad, *Dioon merolae* (*Zamiaceae*). *The New Plantsman*, 6: 214–218.

Pons, T. 1992. The role of temperature in the regulation of dormancy and germination. In: M. Fenner, ed. Seeds: The ecology of regeneration in plant communities. Wallingford: CABI Publishing, 261-292.

Raimondo, D., Von Staden, L., Foden, W., Victor, J.E., Helme, N.A., Turner, R.C., Kamundi, D.A. & Manyama, M.A (eds). 2009. Red list of South African Plants. *Strelitzia 25.* South African National Biodiversity Instituted. Pretoria

Retief, K., West, A.G. & Pfab, M.F. 2014. Can stable isotopes and radiocarbon dating provide a forensic solution for curbing illegal harvesting of threatened cycads? *Journal Forensic Science*, 59(6): 1541-1551.

Roach, D.A. & Wulff, R. 1987. Maternal effects in plants. *Annual Review of Ecology, Evolution, and Systematics,* 18: 209-235.

Roberts, E.H. 1972. *Storage environment and control of viability. Viability of Seeds.* In Roberts E H (Ed.). Chapman and Hall, London, 14–58.

Roberts, E.H. 1973. Predicting the storage life of seeds. *Seed Science and Technology*, 1: 499–514.

Rodriguez, M.C.S., Edsgard, D., Hussain, S.S., Alquezar, D., Rasmussen, M., Gilbert, T., Nielsen B.H., Bartels, D. & Mundy, J. 2010. Transcriptomes of the desiccation-tolerant resurrection plant *Craterostigma plantagineum*, *The Plant Journal*, 63: 212–228.

Santini, B.A., Rojas-Arechiga, M. & Morales, E.G. 2017. Priming effect on seed germination: Is it always positive for cacti species? *Journal of Arid Environments*, 1-4.

Saxton, W.T. 1910. The Development of the Embryo of *Encephalartos*. *Botanical Gazette*, 49(1): 13-18.

Smith, G.S. 1974. Seed treatments to speed germination of *Zamia integrifolia*. *Florida state Horticultural Society*, 436–438.

Steel, R.G.D. & Torrie, J.H. 1980. *Principle and procedures of statistics: a biometrical approach.* 2nd edition. McGraw-Hill, New York, NY.

Stevenson, D.W., Norstog, K. & Fawcett, P. 1998. Pollination biology of cycads. In: Owen, S. & Rudall, P. (ed) *Reproductive biology: In systematics, conservation, and economic botany.* Royal Botanic Gardens, Kew, UK.

Subedi K.D. & Ma, B.L. 2005. Seed priming does not improve corn yield in a humid temperate environment, *Agronomy Journal*, 97(1): 211–218.

Taiz, L. & Zeiger, E. 2000 *Plant Physiology*. 3rd edition, Sinauer Associates, Sunderland.

Tang, W. 1986. Pollinating cycads. *Encephalartos*, 8: 16–19.

Tang, W. 1987. Heat production in cycad cones. *Botanical Gazette*, 148: 165–174.

Tang, W. 1993. Nectar-like secretions in female cones of cycads. *The Cycad Newsletter,* 16(2): 10–13.

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

Tommasi, F., Paciolla, C., Pinto, M.C. & De Gara, L. 2006. Effects of storage temperature on viability, germination and antioxidant metabolism in *Ginkgo biloba* L. seeds. *Plant Physiology and Biochemistry*, 44: 359–368.

Tompsett, P.B. & Pritchard, H.W. 1993. Water status changes during development in relation to the germination and desiccation tolerance of *Aesculus hippocastanum* L. seeds. *Annals of Botany*, 71: 107-116.

Tompsett, P.B. & Pritchard, H.W. 1998. The effect of chilling and moisture status on the germination, desiccation tolerance and longevity of *Aesculus hippocastanum* L. seed. *Annals of Botany,* 82: 249-261.

Tompsett, P.B. 1983. The influence of gaseous environment on the storage life of *Araucaria hunsteinii* seed. *Annals of Botany,* 52: 229-237.

Ullah, Z., Hassan, I., Hafiz, I.A. & Abbasi, N.A. 2019. Effect of different priming treatments on seed germination of sago palm (*Cycas revoluta* L.). *World Journal of Biology and Biotechnology*, 5(1): 1-3.

Umair, S. 2011. Cycad Seed Biology and Embryo Cryopreservation: Especially *Cycas siamensis, Dioon edule* and *Zamia floridana*. MSc Thesis, University of Bedfordshire and Royal Botanic Gardens Kew, UK.

Vargas–Luna, I., Ortiz–Montiel, G., Chávez, V.M., Litz, R.E. & Moon, P.A. 2004. Biochemical characterization of developmental stages of cycad somatic embryos. *The Botanical Review,* 70(1):54-62.

Vorster, P. 1995. Aspects of reproduction of cycads. An annotated review of known information. in: Vorster, P. (ed.). *Proceedings of the Third International Conference of Cycad Biology*. Stellenbosch, South Africa: cycad Society of South Africa, 379–387.

Walters, T.W. 1999. Off-site cycad preservation: I. what is off-site preservation? *The Cycad Newsletter*, 22(1): 10–12.

Whitelock, L.M. 1995. Cycad conservation in the past and the need for improvements in the future. In: *Proceedings of the 3rd International Conference on Cycad Biology* (ed. P. Vorster). The Cycad Society of South Africa, Matieland, South Africa.

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

Winter, J. 2004. *Encephalartos altensteinii* Lehm. South African National Diversity Institute. (Online). http://pza.sanbi.org/encephalartos-altensteinii. [Accessed 20 July 2018].

Witte, T.W. 1977. Storage and germination of *Zamia* seed. *Proceedings of Florida state horticultural society,* 90: 89–91.

Woodenberg, W.R., Erdey, D., Pammenter, N. & Berjak, P. 2007. Post-shedding seed behaviour of selected *Encephalartos* species. Abstracts from the 5th International Workshop on Desiccation Tolerance and Sensitivity of Seeds and Vegetative Plant Tissues. *South African Journal of Botany,* 73(3): 496.

Xaba, P.M.A. 2014. Pollination and Germination as Limiting Factors in the Propagation of Threatened Cycads, *Encephalartos* (*Zamiaceae*). MSc Thesis, University of Western Cape, South Africa.

Zarchini, M., Hashemabadi, D., Kaviani, B., Fallahabadi, P.R. & Negahdar, N. 2011. Improved germination conditions in *Cycas revoluta* L. by using sulphuric acid and hot water. *Plant Omics Journal*, 4 (7): 350-353.