# Breaking Seed Dormancy and Improving Seedling Growth of *Encephalartos altensteinii* Lehm. using Seed Hydration-dehydration Treatment and Acid Scarification

**Ntuthuko Mabuya, Muhali Olaide Jimoh, John October, and Charles Petrus Laubscher[1]***

## Abstract

Germination rate and percent germination are low for most cycads, including *Encephalartos altensteinii,* thereby affecting the production and conservation efforts for this endangered plant group. Although seed germination experiments for cycads have been done mainly on *Zamia* and *Cycas*, there is limited germination research on the African genus *Encephalartos*. The effects of hydration-dehydration treatment on seed longevity, embryo development, moisture content and viability were studied by hydrating seeds of *E. altensteinii* Lehm during storage for 24 hr and dehydrating for 96 hr every month for 8 months. Simultaneously, seeds were soaked in different concentrations (0%, 10%, 25%) of sulfuric acid ($H_2SO_4$, pH 0.3) and gibberellic acid ($GA_3$) between 0.5 and 48 hr to break dormancy. Thereafter, seeds were sown in silica-sand-filled-sowing beds in the greenhouse and monitored over eight months. Hydration-dehydration treatments had positive effects on the viability of *E. altensteinii* seeds stored at room temperature and that further decreases in moisture content were detrimental. Also, a combination of $H_2SO_4$ and $GA_3$ significantly improved germination percentage whereas no significant difference was recorded in seeds treated with only $H_2SO_4$. These results suggest that seed viability and germination of *E. altensteinii* can be improved with hydration-dehydration and acid scarification treatments.

**Species used in this study:** Eastern Cape giant cycad, *Encephalartos altensteinii* Lehm.

**Chemicals used in this study:** Sulfuric acid, gibberellic acid, captan, 2,3,5-triphenyltetrazolium chloride.

**Index words:** Cycad, desiccation tolerance, *Encephalartos altensteinii*, gibberellic acid, sulfuric acid, Zamiaceae.

### Significance to the Horticulture Industry

Breaking morpho-physiological seed coat dormancy of difficult to germinate seeds such as *Encephalartos altensteinii* due to low seed viability and complex dormancy conditions is of utmost importance to the horticulture industry. As documented in the literature, research on seed germination for cycads have focused mainly on *Zamia* and *Cycas*, and there is limited research done on the seed germination of the native African genus *Encephalartos*. Findings from this study have established suitable pre-treatments that could improve the germination percentage and reduce the germination period of *E. altensteinii*. This will assist in developing a propagation protocol for this vulnerable species. These results will benefit horticulturists, plant enthusiasts and researchers by increasing the propagation of this species or its vulnerable relatives, allowing for reintroduction into the wild or for commercial purposes.

### Introduction

South Africa has one of the most diverse cycad species worldwide, with 38 species, and of these, 29 species are found in South Africa (IUCN 2003, Swart et al. 2018). However, cycads remain the most threatened plant group in the country with 78% of their naturally occurring population endemic to South Africa threatened with extinction compared to the worldwide average of 62% of their population (Raimondo 2010, Bamigboye et al. 2018). According to the Red List of South African plants, 11 species are critically endangered, and three species are already extinct in the wild (Donaldson 2010, Woodenberg et al. 2014). One of the efforts to stop indiscriminate removal of cycads in their natural habitats, especially species in the genus *Encephalartos* are listed in Appendix 1 of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (CITES 2014, Russo 2015). Specifically, the families Zamiaceae, Stangeriaceae and Cycadaceae are included in Appendix II of CITES. The convention resolved that collecting and trading of any indigenous cycads is prohibited except for research or for plants that are propagated by horticulturists (CITES 2014). CITES aims to regulate all international trade of endangered species or species threatened by international trade (Konings 2016).

*Encephalartos altensteinii* is one of the largest African cycad species and mainly occurs in the Eastern Cape region of South Africa. Mature plants of *E. altensteinii* usually grow to 5 m (16 ft) tall and form clumps of 2 to 3 stems with basal suckers and only occurs in the Eastern Cape province and south coast of KZN province in South Africa (Cousins and Witkowski 2017). The wild population has declined over the years due to habitat destruction from development and collection. Currently, this species is listed as Vulnerable (VU) on the Red List of South African plants, with an estimate of only 10,000 plants left in the wild (Rousseau 2012, Forest et al. 2018). *Encephalartos altensteinii* has gained extensive popularity over the years in garden designs and cycad collections with the result that the species removal from the wild has placed a continued pressure on this threatened red list species (Donaldson 2010, Forest et al. 2018). *Encephalartos altensteinii* is mostly propagated from seed; however seed sources remain low

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[1]Department of Horticultural Sciences, Faculty of Applied Sciences, Cape Peninsula University of Technology, Bellville 7535, Symphony Way, City of Cape Town, South Africa.

*Corresponding author email: laubscherc@cput.ac.za.

  

with additional problems of low seed viability and complex dormancy conditions.

Research on seed germination for cycads have been done mainly on *Zamia* and *Cycas*, and there is limited research done on the germination of the native African genus *Encephalartos*. Seed hydration-dehydration (H-D) treatments during storage have been reported to have positive effects on desiccation tolerance, germination percentage and longevity (Lima and Meiado 2017). Broome (2001) reported earlier that H-D treatment prolonged the viability of cycad seeds during storage (Woodenberg et al. 2015). Also, several studies have suggested that pre-germination treatments with sulfuric acid ($H_2SO_4$) and gibberellic acid ($GA_3$) could assist in breaking seed dormancy (Dehgan 1997, Zarchini et al. 2011, Gogoi et al. 2017, Puttha et al. 2014), while morphological dormancy can be broken by removing the sarcotesta layer of seeds before they are stored and treated with $GA_3$ to enhance embryo development and germination (Gogoi et al. 2017). This was achieved successfully in some cycad seeds soaked in hot water at 100 C (212 F) for 1 hour and 25% $H_2SO_4$ for 2 hours (Zarchini et al. 2011). According to Frett (1987), *Cycas revoluta* seed treated with $GA_3$ at 500, 1,000 and 5,000 pm for 12 h resulted in low seed germination when compared with untreated (control) seeds, and Xaba (2014) reported that there was no significant difference in germination rates in seeds of *E. latifrons* and *E. altensteinii* pre-treated with $GA_3$. Thus, conflicting results on cycad seed germination raised a question about whether hydration-dehydration, acid scarification or a combination of scarification and growth regulator ($GA_3$) treatment could improve the germination of selected *Encephalartos* species.

The Eastern Cape giant cycad is part of a plant group with complex seed dormancy, which may have resulted from morpho-physiological dormancy, a combination of morphological and physiological dormancy (Baskin and Baskin 2014, Baskin et al. 2001, Dehgan 1997). The continued threatened status of the species, as well as the high commercial value, necessitates the need to cultivate this slow-growing species. It is uncertain how *E. altensteinii* seeds will respond to varying concentrations and soaking periods in sulfuric acid and gibberellic acid as growth facilitators that can reduce germination time and could support future propagation of the species for commercial and conservation purposes. This study was conducted to investigate suitable treatments that will improve seed viability, germination percentage, germination period and to determine successful seedling growth of *E. altensteinii* to develop a suitable propagation protocol for this vulnerable species.

## Materials and Methods

*Artificial pollination*. Due to the unavailability of commercial seeds of *E. altensteinii*, CITES restrictions on wild seed collections, low seed viability, and cross-pollination in ex-situ collections, cones of stock plants were hand pollinated to produce seed for the experiments. Female cones open sporophylls for a few days for pollination and close again, and the ovulation period varies from species to species (Suinyuy et al. 2009, Terry et al. 2012, Brenner et al. 2003). Xaba (2014) reported that sporophylls on female cones of *E. altensteinii* open for 14 to 25 days. 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 May 2018 using a wet pollination method. Five grams (0.18 oz) of pollen was mixed with 500 mL (17 fl oz) of distilled water and this was used to pollinate receptive female cones three times every second day in the mid-morning and late evening when the micropylar droplets formed. The pollen solution was injected between the loose sporophylls using a syringe. Unused pollen was packaged in a paper envelope, sealed in a plastic container with silica gel and stored at -15 C (5 F) (Xaba 2014, Terry et al. 2012).

*Seed collection and storage*. Naturally, seeds of *E. alternsteinii* take at least 7 months from pollination to shedding, therefore seeds were collected at KNBG when they started disintegrating on the cone. Collected seeds were soaked in tap water for a week, followed by removing the fleshy layer (sarcotestae), and were washed and air-dried at room temperature. The seeds were then dusted with captan fungicide, placed in mesh bags and stored in a dry condition at a constant 15 C (59 F) and 15% relative humidity for 17 months (Robbertse et al. 2011, Xaba 2014). The seeds were transported to the Pretoria National Botanical Garden in mesh bags on a two-hour flight at room conditions in March 2019 and stored at room temperature for 5 days before the experimental work commenced.

*Seed hydration-dehydration experiment*. The experiment was conducted in the seed storeroom at the production nursery of the Pretoria National Botanical Garden, East of Pretoria, in Gauteng province, South Africa, −25°44′18.2″ S 28°16′19.8″ E 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 captan (Efekto Fungi-Nill 500 WP Captan) fungicide to prevent the development of fungi, placed in mesh bags, and stored under dry conditions at room temperature during the experiment (Lima and Meiado 2017, Xaba 2014).

*Seed viability tests*. For the hydration-dehydration study, 360 seeds of *E. altensteinii* obtained from ex-situ collection at KNBG in 2018 were cleaned as described above and were divided into 2 sets of 180 seeds each. The first set of 180 seeds were placed in mesh bags and stored at room temperature (without soaking) while the second group were placed in mesh bags, soaked in distilled water for 24 hr at 4-week intervals and stored in mesh bags at room temperature. A random sample of 15 seeds from the above 2 treatments was used to measure seed viability at 8-week intervals. The seed coat was cracked with a nutcracker and the seed coat was removed around the endosperm after which the endosperm was cut longitudinally with a scalpel blade to expose the embryo. Thereafter each seed was soaked in a 250 mL glass beaker containing a solution of 0.1% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, Johannesburg, South Africa) for 60 minutes to determine viability. Seed embryos that changed the color to purple were regarded as viable and


**Fig. 1. Seeds treated with 2,3,5-Triphenyltetrazolium chloride to test viability, (A) embryos with pink colour showing that seed is viable, (B) unchanged embryo indicating that seed is not viable (Pictures A & B: Mabuya).**

seeds that did not turn purple were regarded as as non-viable seeds (Jimoh et al. 2019, ISTA 2005).

For the seeds used for acid scarification, viability was tested using the water floating and triphenyl-tetrazolium methods (Fig. 1). In the water floating test, seeds were immersed in water. Seeds that floated in water were regarded as non-viable seeds and discarded and all sinking seeds accepted as potentially viable (Calonje et al. 2011, Broome 2001). Random samples of 15 seeds each with 4 replicates were selected for the triphenyl-tetrazolium test as described earlier.

*Seed hydration-dehydration treatment*. To assess the effects of seed hydration dehydration treatment on *E. altensteinii*, 360 seeds were used for this experiment. The seeds were divided into 2 groups and each group consisted of 180 seeds. The imbibition period was evaluated by weighing 15 seeds on an analytical balance and placed for imbibition in a 3-L (3.2 qt) plastic container with distilled water, which was maintained in the seed storeroom at room temperature. Seeds were imbibed in water and weighed at 4 hr intervals until the seed weight was stable. For each weight evaluation, the seeds were removed from the water, dried with filter paper and weighed again. Seeds of *E. altensteinii* were also subjected to H-D 1 (untreated) and H-D 2 (soaked in distilled water for 24 hr every month) treatments.

*Embryo growth*. The effects of seed hydration dehydration treatment on embryo growth were monitored by dividing 360 seeds of *E. altensteinii* into 2 replications of 180 seeds each and treated as described above. Embryo growth was measured at 8-week intervals. Hard seed coats were cracked with a nutcracker, the seed coats around the endosperm were removed and the endosperms were cut open longitudinally using a scalpel blade to expose the embryo (Fig. 2). Thereafter the length of the embryo growth for 15 seeds from the above 2 treatments was measured using an electronic microscope and ZEN microscope software obtained from ZEISS (Olszewski et al. 2009).

*Seed moisture content*. Fifteen seeds from the above 2 treatments were used to measure seed moisture content at 8-week intervals. Each seed was individually weighed, and the fresh weight was recorded, followed by cracking the seed coat with a nutcracker, removal of the seed coat around the endosperm and cutting the endosperm longitudinally using a scalpel blade. Thereafter the seed was dried in an oven at 103 C (217 F) for 17 hours and weighed again to record dry weight (Fig. 3). Moisture content was expressed as a percentage of the wet weight of the sample, calculated to two decimal places using the following formula (ISTA 2005).

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

*Chemical scarification*. The experiment consisted of chemical scarification methods which were applied to seeds of *E. altensteinii* in combination with gibberellic acid ($GA_3$). In addition to untreated seeds, 2 germination experiments were conducted. A randomized complete 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 (Fig. 4). All seeds used were soaked for 24 hr in distilled water before the treatment. Germination treatments were divided into 2 Experiments: *Experiment 1*:

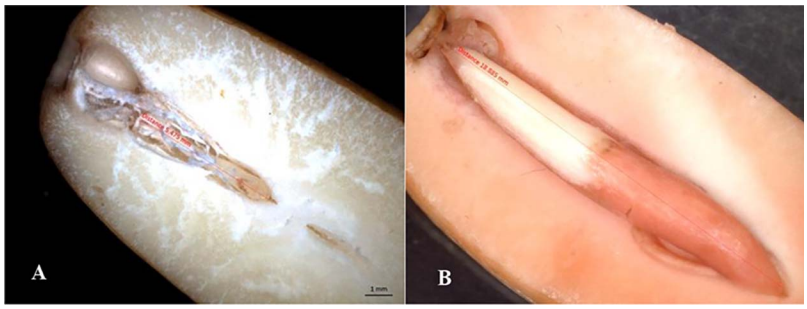


**Fig. 2. Measuring embryo growth, (A) underdeveloped embryo after 6 months of storage, (B) fully developed embryo after 14 months of storage at room temperature (Pictures: Mabuya).**

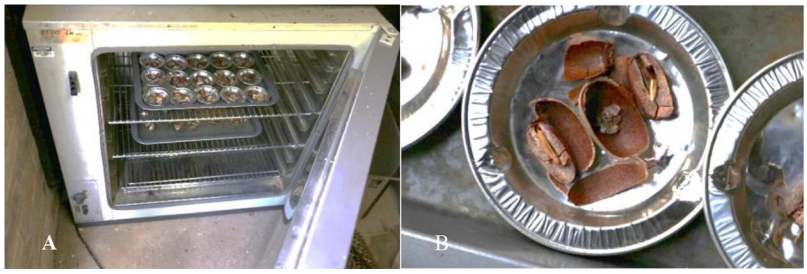

Fig. 3. **Drying seeds to measure dry weight, (A) preparing to dry seeds in an oven at 103 C, (B) dry seed after 17 hours at 103 C (B) (Picture A & B: by Mabuya).**

the seeds were soaked in 10% or 25% of sulfuric acid ($H_2SO_4$) for 0.5 hr or 1 hr. *Experiment 2*: seeds were chemically scarified with a combination of 10% or 25% of $H_2SO_4$ for 0.5 hr or 1 hr, washed in running water for 5 minutes and then soaked in 1,000 ppm of $GA_3$ for 24 hr or 48 hr (Zarchini et al. 2011, Dehgan 1997).

*Greenhouse experiment*. The greenhouse experiment was conducted in the production nursery of the Pretoria National Botanical Garden. The greenhouse environmental conditions were maximum day temperatures which ranged between 21-28 C (70-82 F) and night temperatures between 15-21 C (15-70 F) with an average of 43% relative humidity. The roof was covered with a 30% shade net which provided a cooler air temperature. A seed sowing bench of 11 × 5 m (36 × 16 ft) with heating cables provided bottom heating during seed germination.

*Germination study and data collection*. After the H-D treatment, embryo growth, moisture content and viability were measured at 8-week intervals. Measurements for embryo length were done in millimetres using an electronic microscope and ZEN microscope software obtained from ZEISS (ZEISS, St. Louis, MO). Moisture contents and viability were recorded as a percentage of the sample.

All the seeds treated with acids ($H_2SO_4$ and $GA_3$) were sown in a heated germination bench at 27 C (81 F) by pushing them halfway in the silica sand used as a sowing medium. The germination bench was covered with 100% shade cloth and the seeds 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 was recorded as germinated when the growth of the seed radicle elongated to 3 mm or greater. 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 × 233 mm (0.5 × 9 in) plastic planting bags in a soil medium consisting of a ratio of 1:2:1 river sand, compost, and fine bark. Potted seedlings were placed in a greenhouse with an average temperature of 24 C (75 F) and night temperature ranging between 15 - 21 C (59-70 F). Leaf emergence 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 by 4 months because of slow leaf sprouting from the paired cotyledonary petiole (Jimoh et al. 2019, Robbertse et al. 2011, Xaba 2014).

Fig. 4. **The experimental arrangement on a heated bench showing seeds planted in rows in silica sand using a randomized complete block design (Picture A & B: Mabuya).**

*Statistical analysis*. Data obtained from H-D analysis were analysed using one-way analysis of variance (ANOVA) on the TIBCO STATISTICA 13.5 computing software program (Tibco, Palo Alto, CA). Also, a one-way ANOVA was used for $H_2SO_4$ treatments only while data obtained from a combination of $H_2SO_4$ and $GA_3$ treatments were analysed using two-way ANOVA and computed on STATISTICA. The occurrence of statistical difference was determined by using the Fisher's Least Significance Difference (LSD) at values of $P < 0.05$; $P < 0.01$ and $P < 0.001$ levels of significance (Faber et al. 2020).

## Results and Discussion

*Effects of hydration dehydration treatments on embryo growth*. Embryo development response of *E. altensteinii* seeds on hydrated dehydrated (H-D) treatment was measured over 14 months. 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. 5). The H-D 2 treatment caused no significant difference in embryo length compared to H-D 1 (control) seeds. These results confirm findings from similar studies that most cycads species shed seeds with underdeveloped embryos and require storage for 1 to 12 months for the embryo to develop (Robbertse et al. 2011, Woodenberg et al. 2015), and similarly, Xaba (2014) reported that 6 to 12 months of storage for *E. latifrons* and *E. altensteinii* improved the germination rate. The findings showed that embryo growth of *E. altensteinii* was sustained over a 12-month period towards maturity, and embryo

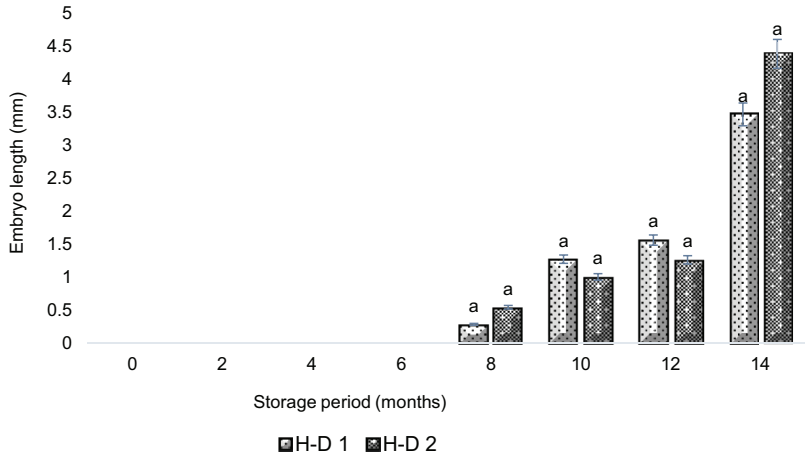


**Fig. 5. Embryo growth response of *E. altensteinii* seeds to monthly (0 to 14 months) hydration dehydration (H-D) treatments, stored at room temperature. H-D-1 = Control treatment: seeds were not hydrated and H-D 2: seeds were hydrated in distilled water for 24 hours (n=15). Mean embryo length was ranked on a monthly basis and compared between HD-1 and HD-2 treatments.**

length continuing to increase even after the embryos had germinated. This constant embryo development characterizes recalcitrant seeds, which are constantly metabolically active, continuing from seed abscission through to germination (Woodenberg et al. 2014). As the embryo length was sustained in the H-D 1 treatment until month 10, the seed may contain adequate moisture for the embryo to develop to this point. Whereas in the H-D 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 seed treated with the H-D 1 treatment lacked further moisture for embryo development to proceed. Even though the study showed comparative measurements, there was no significant difference in the moisture content measured between the H-D 1 and H-D 2 treatments, which explains that seed development requires both oxygen and moisture to develop (Baskin et al. 2014).

*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 (Fig. 6). The H-D 2 treatment caused seed moisture content to remain higher compared to the H-D 1 treatment (control) during a storage period of 8 months. The control showed a sharper decline in moisture content from the 6th to the 8th month. Results showed a significant difference ($p < 0.001$) in the seed moisture content for the H-D 2 treatment compared to the H-D 1 treatment in the 2nd and 8th months of storage. Although between the 4th and 6th month, no significant difference occurred, the H-D treatment caused a greater moisture content compared to the control at month 8. Seed moisture content for the control declined sharply in the H-D 1 to 22.60% compared to the H-D 2 treatment (29.40%) at the 8th month. This study revealed that seed moisture content of *Encephalartos altensteinii* declined for 8 months when stored at room temperature. It has also been reported that maintaining moisture storage conditions can regulate seed viability and extend seed longevity (Broome et al. 2011).

*Effect of hydration-dehydration treatments on seed viability*. The H-D 2 treatment improved seed viability compared to the control at 8 months Fig. 7). Seed viability for the control declined rapidly (53.34%) between 0 to 8 months, while no significant decline was noted for the H-D 2 treatment. This study showed that the H-D-treated seeds had greater viability over a long period, up to 8 months during storage, whereas in the non-H-D-treated seeds, viability declined sharply. Hydrated recalcitrant seeds are metabolically active and experience germination-associated changes in storage which suggests a requirement for supplementary water to be present in the seed on shedding (Cousins and Witkowski 2017, Pammenter et al. 1994). The availability of moisture over a long period determines the longevity of the seed and could advance seed viability (Berjak and Pammenter 1997, Lima and Meiado 2017) suggesting that *E. altensteinii* is sensitive to desiccation. According to Broome 2001), H-D treatments improve longevity of cycad seeds. This current study concurs with these results as the findings showed significant comparisons where moisture was added in the H-D 2 treatment. The study showed that where moisture content decreased below 25%, viability of seed deteriorated. Similar results were reported in De Andrade (2001), where the viability of *Euterpe edulis* seeds decreased below 30% moisture content compared to moisture- treated seeds which remained at 80% moisture content for four months. Results also showed that moisture content was also maintained during low temperatures in winter months, indicating that a low storage temperature could maintain viability, hence extending storage of seeds.

*The effects of sulfuric acid on germination percentage*. The results on seed scarification with sulfuric acid showed that there was no significant difference ($P > 0.05$) on the final seed germination percentage of *E. altensteinii* compared to non-treated seeds (Table 1). The highest germination (33.33%) was recorded in the control (no acid treatment) followed by seeds soaked in 10% $H_2SO_4$ for 1 hr or 25%

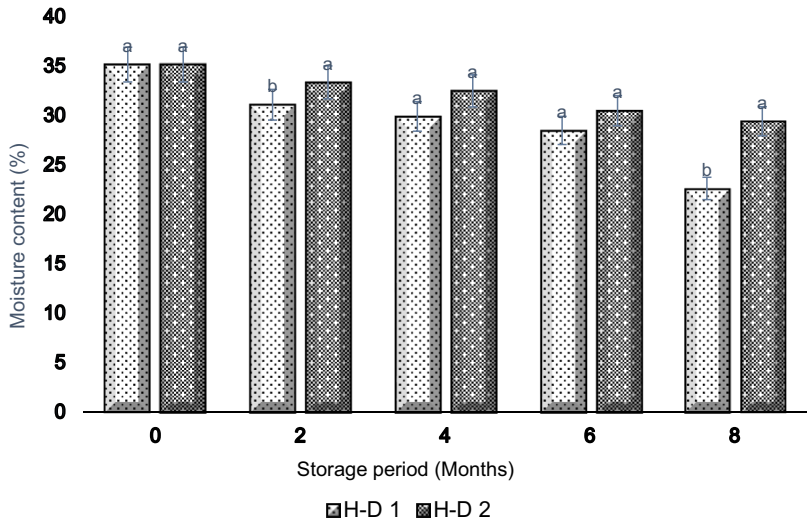


**Fig. 6. Effects of monthly hydration dehydration treatments on *E. altensteinii* seed moisture content (MC) stored at room temperature for 8 months. Treatments = H-D 1 (control) and H-D 2 (seeds hydrated in distilled water for 24 hours (n=15). Bars having different letters are significantly different at P < 0.01 as calculated by Fisher's least significant difference.**

$H_2SO_4$ for 0.5 hr, each of which resulted in 13.33% germination. However, no germination was recorded in seeds treated with 25% $H_2SO_4$ for 1 hr. The most rapid germination was also seen in the control, as germination started in month 2, with a significant difference of $P < 0.05$ in month 5 when compared with treated seeds. Slow germination was recorded in seeds soaked in 25% $H_2SO_4$ for 0.5 hr, as germination was not noted until the 6th month (Table 1). The results of this study indicated that pre-treating seeds by soaking in 10% or 25% of $H_2SO_4$ for 0.5 hr or 1 hr has a negative effect on the germination of *E. altensteinii*. The four types of seed dormancy namely, hard seed coat, embryo dormancy, immature embryo and chemical inhibitor as described in Bareke (2018) may have caused morpho-physiological dormancy in cycad seeds. Perhaps, the concentration or soaking period in only $H_2SO_4$ as stated by (Dehgan 1997) is not suitable to break the morpho-physiological dormancy of *E. altensteinii*. However, the observed germination responses are species-specific and no single treatment has been reported to be effective universally as acid treatment proved to be effective in breaking dormancy in some cycad species, though with a low germination percentage at high acid concentrations (Zarchini et al. 2011, Xaba 2014). Findings from this study agree with Baatuuwie et al. (2019), who reported that high concentrations of $H_2SO_4$ with a long soaking period had a low germination rate in *Detarium microcarpum* Guill. & Perr. seeds. Hence, $H_2SO_4$ may have penetrated the endosperm of *E. altensteinii* and injured the seed embryo.

*Effects of sulfuric acid on leaf sprouting and number of leaves*. There was no significant difference in leaf emergence and number of leaves for seeds of *E. altensteinii* soaked in varying concentrations of $H_2SO_4$ (Table 2). Low number of leaves, less than one per seed, was noted. These results agree with Missanjo et al. (2014), where no significant difference was reported in the number of leaves and seedling height of *Acacia polyacantha* Willd from similar treatments compared with the control. Leaf sprouting and

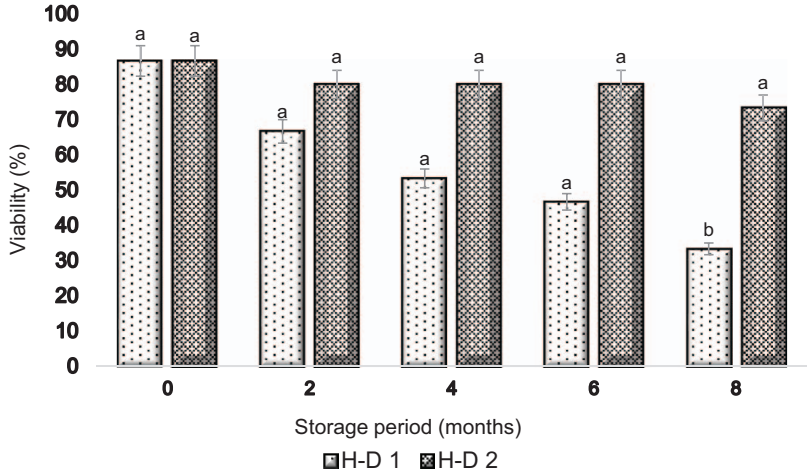


**Fig. 7. Effect of hydration dehydration treatments on *E. altensteinii* seed viability after 8 months, stored at room temperature. Treatments = H-D 1 (control) and H-D 2 (seeds hydrated in distilled water for 24 hours (n=15). Each bar represents mean values ±SE of percentage viability. The mean values followed by different letters are significantly different at P < 0.05 (*) and ns = not significant as calculated by Fisher's least significant difference.**

**Table 1. Effects of pre-treating seeds with varying concentrations of sulfuric acid on percentage germination of *E. altensteinii*[z].**

| Treatments | $H_2SO_4$ Concentration | $H_2SO_4$ Soaking time | Month 1 | Month 2 | Month 3 | Month 4 | Month 5 | Month 6 | Month 7 | Month 8 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Control | Control | 0±0 | 6.67±6.67 | 13.33±9.09 | 20±10.70 | 26.67±11.82a | 26.67±11.82 | 26.67±11.82 | 33.33±12.6 |
| 2 | 10% $H_2SO_4$ | 0.5 hr | 0±0 | 0±0 | 0±0.00 | 6.67±6.67 | 6.67±6.67b | 6.67±6.67 | 6.67±6.67 | 6.67±6.67 |
| 3 | 10% $H_2SO_4$ | 1 hr | 0±0 | 0±0 | 6.67±6.67 | 6.67±6.67 | 6.67±6.67b | 13.33±9.09 | 13.33±9.09 | 13.33±9.09 |
| 4 | 25% $H_2SO_4$ | 0.5 hr | 0±0 | 0±0 | 0±0.00 | 0±0.00 | 0±0b | 6.67±6.67 | 13.33±9.09 | 13.33±9.09 |
| 5 | 25% $H_2SO_4$ | 1 hr | 0±0 | 0±0 | 0±0.00 | 0±0.00 | 0±0.00b | 0±0.00 | 0±0.00 | 0±0.00 |
| **One-Way ANOVA** | | | | | | | | | | |
| F-Statistics | | | 0 ns | 1.0 ns | 1.4 ns | 1.6 ns | 2.6* | 1.6 ns | 1.4 ns | 2.1 ns |

[z]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 (n=15).

**Table 2. Effects of pre-treating seeds with varying concentrations of $H_2SO_4$ and soaking periods on days to leaf sprouting and number of leaves of *E. altensteinii*[z].**

| S/N | $H_2SO_4$ Concentration | $H_2SO_4$ Soaking time | Leaf sprouting rate (days) | Number of leaves |
|---|---|---|---|---|
| 1 | Control | Control | 193.00±20.00 | 0.47±0.19 |
| 2 | 10% $H_2SO_4$ | 0.5 hr | 233.00±11.00 | 0.20±0.20 |
| 3 | 10% $H_2SO_4$ | 1 hr | 222.00±15.63 | 0.40±0.27 |
| 4 | 25% $H_2SO_4$ | 0.5 hr | 223.00±15.13 | 0.13±0.09 |
| 5 | 25% $H_2SO_4$ | 1 hr | 245.00±0.00 | 0.00±0.00 |
| **One-Way ANOVA** | | | | |
| F-Statistics | | | ns | ns |

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

number of leaves are largely dependent on seed germination, and as reported earlier, improving water uptake was not sufficient to improve germination. This suggests that *E. altensteinii* may have morpho-physiological dormancy, known to be a common phenomenon for cycads (Dehgan 1997, IUCN 2003) and this had impacted negatively, the vegetative growth of *E. altensteinii*.

*Interactive effect of sulfuric acid and gibberellic acid.* There was a significant difference ($P < 0.05$) in the final seed germination of *E. altensteinii* at the 7th and 8th months among the different treatments of $H_2SO_4$ in combination with $GA_3$. (Table 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 described to be a common phenomenon for cycads (Dehgan 1997, IUCN 2003). The results showed that seeds soaked in 25% $H_2SO_4$ for 0.5 hr, followed by soaking in 1,000 ppm of $GA_3$ for 24 hr had the highest germination success of 73.33% in *E. altensteinii* at the 7th and 8th months (Table 3).The results showed that pre-treating seeds with $H_2SO_4$ without the addition of $GA_3$ generally had a negative effect on germination percentage (13.33%) when compared with untreated seeds (33.33%). These findings are in agreement with results reported on a similar study by Dehgan and Johnson (1983) and Schutzman (2016), which showed pre-treating seeds with a combination of $H_2SO_4$ and $GA_3$ improved seed germination of *Zamia floridana* A. DeCandolle. Zarchini et al. (2011) also reported that 25% $H_2SO_4$ increased germination of *Cycas revoluta* Thunb while Frett (1987) reported conflicting results that $GA_3$ did not increase seed germination of *C. revoluta* when compared with untreated seeds. No germination was observed for treatment with 1,000 ppm of $GA_3$ for 48 hr and 25% $H_2SO_4$ for 1 hr, suggesting that exposing seeds to $GA_3$ for long periods inhibits germination (Rehman and Park 2000), although soaking periods may differ among cycads species (Xaba 2014, Dehgan and Johnson 1983, Zarchini et al. 2011).

*Synergistic effects of $H_2SO_4$ and $GA_3$ on leaf sprouting rate and number of leaves.* The leaf sprouting and the number of leaves in this study was dependent on new leaves

Table 3. **Interactive effects of soaking periods and different concentrations of $H_2SO_4$ and $GA_3$ on percent germination of *E. altensteinii*[z].**

| | $H_2SO_4$ treatment | | $GA_3$ treatment | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S/N | Concentration | Soaking time | Concentration | Soaking time | Month 1 | Month 2 | Month 3 | Month 4 | Month 5 | Month 6 | Month 7 | Month 8 |
| 1 | 0% $H_2SO_4$ | No soaking | 0% $GA_3$ | No soaking | 0±0.00 | 6.67±6.67 | 13.33±9.09 | 20±10.70 | 26.67±11.82 | 26.67±11.82 | 26.67±11. 82b | 33.33±12.6b |
| 2 | 0% $H_2SO_4$ | No soaking | 1000 ppm GA3 | 24 hr | 6.67±6.67 | 6.67±6.67 | 6.67±6.67 | 13.33±9.09 | 20±10.7 | 20±10.7 | 20±10.70bc | 20±10.70bc |
| 3 | 0% $H_2SO_4$ | No soaking | 1000 ppm GA3 | 48 hr | 0±0.00 | 0±0.00 | 0±0.00 | 0±0.00 | 0±0.00 | 0±0 | 0±0.00c | 0±0.00c |
| 4 | 10% $H_2SO_4$ | 0.5 hr | 0% $GA_3$ | No soaking | 0±0.00 | 0±0.00 | 0±0.00 | 6.67±6.67 | 6.67±6.67 | 6.67±6.67 | 6.67±6.67bc | 6.67±6.67bc |
| 5 | 10% $H_2SO_4$ | 0.5 hr | 1000 ppm GA3 | 24 hr | 0±0.00 | 0±0.00 | 6.67±6.67 | 6.67±6.67 | 20±10.7 | 20±10.7 | 20±10.7bc | 20±10.7bc |
| 6 | 10% $H_2SO_4$ | 0.5 hr | 1000 ppm GA3 | 48 hr | 0±0.00 | 6.67±6.67 | 6.67±6.67 | 6.67±6.67 | 13.33±9.09 | 13.33±9.09 | 13.33±9.09bc | 13.33±9.09bc |
| 7 | 10% $H_2SO_4$ | 1 hr | No | No soaking | 0±0.00 | 0±0.00 | 6.67±6.67 | 6.67±6.67 | 6.67±6.67 | 13.33±9.09 | 13.33±9.0 9bc | 13.33±9.09bc |
| 8 | 10% $H_2SO_4$ | 1 hr | 1000 ppm GA3 | 24 hr | 0±0.00 | 0±0.00 | 13.33±9.09 | 13.33±9.09 | 20±10.7 | 20±10.7 | 20±10.70bc | 20±10.7bc |
| 9 | 10% $H_2SO_4$ | 1 hr | 1000 ppm GA3 | 48 hr | 0±0.00 | 0±0.00 | 6.67±6.67 | 6.67±6.67 | 6.67±6.67 | 13.33±9.09 | 13.33±9.09bc | 13.33±9.09bc |
| 10 | 25% $H_2SO_4$ | 0.5 hr | No | No soaking | 0±0.00 | 0±0.00 | 0±0.00 | 0±0.00 | 0±0.00 | 6.67±6.67 | 13.33±9.09bc | 13.33±9.09bc |
| 11 | 25% $H_2SO_4$ | 0.5 hr | 1000 ppm GA3 | 24 hr | 6.67±6.67 | 13.33±9.09 | 26.67±11.82 | 40±13.09 | 46.67±13.33 | 60±13.09 | 73.33±11.82a | 73.33±11.82a |
| 12 | 25% $H_2SO_4$ | 0.5 hr | 1000 ppm GA3 | 48 hr | 0±0.00 | 6.67±6.67 | 13.33±9.09 | 13.33±9.09 | 20±10.70 | 26.67±11.82 | 26.67±11.82b | 26.67±11.82bc |
| 13 | 25% $H_2SO_4$ | 1 hr | No | No soaking | 0±0.00 | 0±0.00 | 0±0.00 | 0±0.00 | 0±0.00 | 0±0.00 | 0±0.00c | 0±0.00c |
| 14 | 25% $H_2SO_4$ | 1 hr | 1000 ppm GA3 | 24 hr | 6.67±6.67 | 13.33±9.09 | 13.33±9.09 | 20±10.70 | 20±10.7 | 20±10.70 | 20±10.7bc | 26.67±11.82bc |
| 15 | 25% $H_2SO_4$ | 1 hr | 1000 ppm GA3 | 48 hr | 0±0.00 | 0±0.00 | 0±0.00 | 6.67±6.67 | 13.33±9.09 | 13.33±9.09 | 13.33±9.09bc | 13.33±9.09bc |
| | | | | | **F-Statistics** | | | | | | | |
| $H_2SO_4$ | | | | | 0.5 ns | 0.84 ns | 0.90 ns | 0.90 ns | 0.80 ns | 2.13 ns | 3.97** | 3.50** |
| $GA_3$ | | | | | 3.0 ns | 1.69 ns | 2.76 ns | 3.94* | 5.50** | 4.92 ** | 6.1** | 6.32** |
| $H_2SO_4$ * $GA_3$ | | | | | 0.5 ns | 1.04 ns | 0.99 ns | 1.63 ns | 1.62 ns | 1.82 ns | 2.27* | 2.53* |

[z]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). $H_2SO_4$ = Sulfuric acid, $GA_3$ = Gibberellic acid.

Table 4. Synergistic effects of pre-treating seeds with varying concentrations $H_2SO_4$ and 1000 ppm $GA_3$ in different soaking periods on seedling growth of *E. altensteinii*[z].

| S/N | $H_2SO_4$ treatment | | $GA_3$ treatment | | Leaf sprouting rate | No of leaves |
|---|---|---|---|---|---|---|
| | Concentration | Soaking time | Concentration | Soaking time | | |
| 1 | 0% $H_2SO_4$ | No soaking | 0% $GA_3$ | No soaking | 193±20.00bc | 0.47±0.19bcd |
| 2 | 0% $H_2SO_4$ | No soaking | 1000 ppm $GA_3$ | 24 hr | 210±18.44b | 0.33±0.19bcd |
| 3 | 0% $H_2SO_4$ | No soaking | 1000 ppm $GA_3$ | 48 hr | 245±0.00a | 0±0.00d |
| 4 | 10% $H_2SO_4$ | 0.5 hr | 0% $GA_3$ | No soaking | 233±11.40b | 0.2±0.20bcd |
| 5 | 10% $H_2SO_4$ | 0.5 hr | 1000 ppm $GA_3$ | 24 hr | 209±19.42b | 0.4±0.21bcd |
| 6 | 10% $H_2SO_4$ | 0.5 hr | 1000 ppm $GA_3$ | 48 hr | 220±17.37b | 0.27±0.18bcd |
| 7 | 10% $H_2SO_4$ | 1 hr | 0% $GA_3$ | No soaking | 222±15.63b | 0.4±0.27bcd |
| 8 | 10% $H_2SO_4$ | 1 hr | 1000 ppm $GA_3$ | 24 hr | 213±17.26b | 0.27±0.15bcd |
| 9 | 10% $H_2SO_4$ | 1 hr | 1000 ppm $GA_3$ | 48 hr | 225±13.87b | 0.2±0.14bcd |
| 10 | 25% $H_2SO_4$ | 0.5 hr | 0% $GA_3$ | No soaking | 223±15.13b | 0.13±0.09cd |
| 11 | 25% $H_2SO_4$ | 0.5 hr | 1000 ppm $GA_3$ | 24 hr | 118±20.66c | 1.93±0.34a |
| 12 | 25% $H_2SO_4$ | 0.5 hr | 1000 ppm $GA_3$ | 48 hr | 204±18.37b | 0.6±0.27bc |
| 13 | 25% $H_2SO_4$ | 1 hr | 0% $GA_3$ | No soaking | 245±0.00a | 0±0.00d |
| 14 | 25% $H_2SO_4$ | 1 hr | 1000 ppm $GA_3$ | 24 hr | 194±19.96bc | 0.73±0.30b |
| 15 | 25% $H_2SO_4$ | 1 hr | 1000 ppm $GA_3$ | 48 hr | 218±18.31b | 0.27±0.18bcd |
| | | | F-Statistics | | | |
| $H_2SO_4$ | | | | | 3.10 * | 5.06*** |
| $GA_3$ | | | | | 7.12** | 9.11*** |
| $H_2SO_4$ * $GA_3$ | | | | | 2.54* | 4.17*** |

[z]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 (n=15).

sprouting from the paired cotyledonary petiole of germinated seeds. From the results, the combination of $H_2SO_4$ and $GA_3$ had a significant effect on leaf sprouting and the number of leaves that emerged from pre-treated seeds (Table 4). The results showed that seeds soaked in 25% $H_2SO_4$ for 0.5 hr and soaking in 1,000 ppm $GA_3$ for 24 hr had the least effect on leaf sprouting rate (118 ± 20.66) compared to the control, which had a leaf sprouting rate of 193 ± 20.00. However, this treatment caused a significant effect ($P < 0.001$) on leaf count as it resulted in the highest value of 1.93 ± 0.34, followed by seeds soaked in 25% $H_2SO_4$ for 1 hr, and soaking in 1,000 ppm of $GA_3$ for 24 hr with a mean leaf count of 0.73 ± 0.30 compared with the control's (0.47 ± 0.19) leaf count.

It was observed that pre-treating seeds with only $H_2SO_4$ or $GA_3$ for 1 hr and 48 hr respectively, had a negative effect on seedling growth. For instance, no leaves were seen for seeds soaked in 1,000 ppm of $GA_3$ only for 48 hr, nor for seed soaked in 25% $H_2SO_4$ for 1hr without $GA_3$ (Table 4). These results confirm that long exposure to $GA_3$ or $H_2SO_4$ has a negative effect on germination, and thus seedling growth of *E. altensteinii* seeds. According to Dehgan (1997) and Puttha et al. (2014), seed dormancy in cycads may have been induced by morphological and physiological factors and to overcome this, different species-specific techniques have been proposed. Gibberellins acid plays an important role in stimulating vegetative growth, by affecting endosperm around the embryo (Taiz et al. 2015), while sulfuric acid is effective in breaking morphological dormancy to improve water update and oxygen level (Miranda et al. 2011, Olatunji et al. 2012).

Germination and seedling establishment are important complex stages that influence plant growth (Rasmussen et al. 2015, Viljoen et al. 2021). As noted earlier, seeds soaked in 25% $H_2SO_4$ for 0.5 hr followed by soaking in 1,000 ppm $GA_3$ for 24 hr had the highest germination percentage (73.33%), and this treatment also significantly increased both leaf sprouting ($P < 0.05$) and the number of leaves ($P < 0.001$). The positive association between germination and seedling growth proposes that the result of pre-treatment on germination furthermore translates into vegetative growth. These results reveal that there is an indirect influence of pretreatment on the growth performance of seedlings. Therefore, the positive relationship between germination and growth parameters of *E. altensteinii* seedlings indicates that the benefits of pre-treatment does not only translate to germination but also contributes to seedling establishment.

In conclusion, breaking physiological seed coat dormancy and morphological seed embryo dormancy are both complex processes that are controlled by various factors which inhibit germination to protect the seed in nature from premature germination and failure to develop. The availability of moisture over a long period advances the viability of *E. altensteinii* seeds. This suggests that seeds of *E. altensteinii* are sensitive to desiccation. Findings from this study have also established suitable pre-treatments that could improve germination percentage and the germination period of *E. altensteinii*. This will assist in developing a propagation protocol for this vulnerable species. Chemical scarification of the seeds when soaked in 25% $H_2SO_4$ for 0.5 hr, followed by 1,000 ppm $GA_3$ for 24 hr was highly successful in breaking seed dormancy of the plant. These results will benefit horticulturists, plant enthusiasts and researchers to cultivate a larger number of the species or its vulnerable relatives for reintroduction into the wild and commercial purposes.

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