Overcoming desiccation in an African cycad genus: Adaptive approaches for improving recruitment in *Encephalartos*

by

Ngawethu Ngaka

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Supervisor: Prof. Sjirk Geerts

Co-supervisor: Dr John Donaldson

Co-supervisor: Mr Phakamani Xaba

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Declaration

I, Ngawethu Ngaka, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

N. Ngaka

06/ 12/ 2024

Signed **Date**

Abstract

Cycad populations in the wild are declining due to human pressures, and recovery efforts are constrained by the high mortality of seeds and seedlings, particularly as a result of desiccation. Desiccation reduces seed survival and thus seedling recruitment in the wild. This limits the viability of cycad populations in the wild. Therefore, this study involved testing different substrates designed to retain moisture and thus improve cycad seed survival and juvenile establishment in the wild. It was hypothesized that the addition of water-retaining compounds, such as Coir and Hydrogel, into the growing medium would improve moisture availability and increase seed and juvenile establishment and survival under drought conditions. To test the hypothesis, experimental treatments comprising four substrates and three watering regimes, representing no drought, moderate drought and prolonged drought, were tested in a greenhouse to evaluate their effects on cycad establishment and survival. Chapter two of this thesis investigates the effects of these treatments on growth from seeds for *Encephalartos altensteinii*, with an additional test to determine the effect of sowing depth on seedling emergence and survival. Chapter three tested the effectiveness of the treatments on translocated juvenile plants (8 years old) of *Encephalartos altensteinii*. The results proved that Coir and Hydrogel significantly enhanced substrates moisture content used for growing cycad seeds and juveniles. Seedling development and survival was also significantly influenced by substrates, watering regimes, and sowing depth, as seeds sown 3cm deep had higher seedling survival than at the surface within all the substrates except in Coir. However, for the cycad juveniles, neither substrates or watering treatments significantly influenced survival. Hydrogel and Coir proved to enhance moisture retention which subsequently improving seedling establishment and survival, thus provides key information for cycad restoration protocols. This study further impactfully contributes to the conservation of threatened cycads by providing new knowledge that could be essential to maximize moisture availability and thus improve the establishment and survival of cycad seeds and juveniles in the wild.

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Dedication

This thesis is dedicated to;

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My sisters,

Mikhulu Ngaka

&

Nikhanye Ngaka

My late parents,

Andile Kenneth Maxakato (1973 - 2008)

&

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This one is for you.

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Glossary

Acronyms/Abbreviations

IUCN	Internation Union for Conservation of Nature
KB	Kirstenbosch mix
KBC	Kirstenbosch Mix + Coir
KBH	Kirstenbosch Mix + Hydrogel
LCI	Lower Confidence Level
UPI	Upper Confidence Level
WHC	Water Holding Capacity

CHAPTER ONE: General introduction

1.1. Statement of the research problem

Cycads are a group of plants that are among the most threatened living organisms globally (IUCN , 2024; Fig. 1.1). Threats to cycads include illegal removal, habitat destruction, and damage to plants for traditional and medical use (Donaldson, 2003; Okubamichael *et al.*, 2016). Threatened cycad species, which may comprise fewer than 250 plants and often less, face further challenges compounded by low seedling recruitment in the wild (Raimondo and Donaldson, 2003). This may be due to numerous factors affecting seed set (e.g. low numbers of coning adults) (Cousins and Witkowski, 2017) as well as seed survival and seedling recruitment (e.g. fire, low seed viability, seed desiccation, predation by rodents) (Nadarajan *et al*., 2018). As a result, conservation actions typically involve some form of intervention to improve recruitment to increase population size.

Some of the interventions that have been implemented to recover or reintroduce cycad populations include the sowing of seeds and transplanting of nursery-grown or confiscated plants into wild populations (Vovides *et al.*, 2010). These interventions have had variable success because of the high mortality of seeds due to desiccation and the effects of post-transplant water stress on plant growth and survival. Therefore, testing different solutions for overcoming desiccation and water stress could improve recovery practices for threatened cycads *in situ*. Currently, there are limited published guidelines in place to guide the recovery of threatened cycad species. Developing protocols to reduce water stress and improve seed and seedling development could make an important contribution to cycad conservation. Furthermore, successful recovery of cycads species could improve population viability, thus contributing to cycad conservation.

The purpose of this study is to investigate effectiveness of horticultural techniques to increase the survival of cycad seeds and juveniles by reducing the effects of water stress and desiccation. To achieve this, methods to increase moisture retention of the media used to grow and transplant cycads into the wild were tested.

Figure 1.1: The IUCN Redlist of Threatened species, version 2024-2. Species are grouped in classes with best estimates of percentage threatened species, Cycads being the most threatened with 71% (circled). EW - Extinct in the Wild, CR - Critically Endangered, EN - Endangered, VU - Vulnerable, NT - Near Threatened, DD – Data deficiency, and LC – Least Concerned. Extracted from IUCN 2024, https://www.iucnredlist.org/resources/summary-statistics.

1.2. Background of the research problem

Currently, there are 377 accepted cycad species (Calonje *et al.*, 2024), and over 70% of these species are listed as threatened (The IUCN 2024). Africa is home to three genera of cycads namely, *Encephalartos, Stangeria,* and *Cycas* (Donaldson, 2003). South Africa is a centre of cycad diversity in Africa, with 37 out of 65 species of *Encephalartos* occurring here (Donaldson, 2003, 2008) as well as the only species in the monotypic genus *Stangeria*. This study focuses on the genus *Encephalartos,* as it is the most threatened group in Africa (The IUCN Red List of Threatened Species, 2024). According to the IUCN (2024), *Encephalartos* species from South Africa are comprise of species are Extinct in the Wild (7.1%), Critically Endangered (27.1%), Endangered (11.4%), and Vulnerable (28.6%). It is clear that cycads are facing an extinction crisis in South Africa where the most common threat is the removal of mature plants from the wild (Okubamichael *et al.*, 2016). The prime conservation aim is to preserve plants in natural habitat, but due to the recent declines, extensive restoration action is required (Daly *et al*., 2006). However, there are limited seed and seedling desiccation studies published (Raimondo and Donaldson, 2003) to guide the restoration of cycad species, which could potentially play a role in the failure of cycad restoration projects. The several cycad recovery projects conducted have had variable success, due to high mortality (Boyd 1995; Bezuidenhout, 2020). Various methods have been tried for enhancing cycad populations, but most have been undertaken without good experimental protocols or sufficient monitoring and evaluation of the different treatments (pers comm, Donaldson). Therefore, the need for evidence-based restoration protocols is important (Maschinski and Haskins, 2012) to improve the success rate and survival chances of cycads in the wild.

Among abiotic stresses, water deficit is one of the most challenging factors for plant growth and productivity and this has been shown in cycads (Dehgan, 1983). Cycads require specific moisture conditions for survival (Whitelock, 2002), and these conditions are often not considered in traditional recovery efforts (Boyd, 1995), which can hinder the survival chances of cycad seeds and juveniles. Furthermore, the few documented cycad restoration guidelines (Boyd, 1995) are based on generalized methods for restoration, not species-specific, which limits their application. Therefore, research into moisture retaining additives could provide solutions to overcoming desiccation and high mortality in seeds and juveniles.

Maschinski and Haskins (2012) stated that reintroduction involves different sectors including horticulture, this study, therefore, seeks to address the gaps mentioned above by evaluating the effectiveness of different horticultural techniques to improve cycad seeds and juvenile establishment and survival in the wild.

1.3. Literature review

Cycads are classified as gymnosperms (Chamberlain, 1919; Nicholls & Norstog, 1997) with a total of 377 accepted species from 10 genera (Calonje *et al*., 2024) naturally occurring in the world's tropical and subtropical regions (Donaldson, 2003). Cycads occur in a variety of habitats including forest (*E. altensteinii)*, Grassy Fynbos (*E. latifrons*), savanna (E. *transvenosus*), and grassland (*E. laevifolius*), (Donaldson, 2008; Swart, 2019) from arid to moist tropical areas (Nicholls & Norstog, 1997; Donaldson, 2003). Moreover, cycads are slow growing (Dehgan, 1983) with different growth forms (Raimondo and Donaldson, 2003) varying from dwarf (e.g. *Encephalartos horridus*)**,** arborescent (e.g. *Lepidozamia hopei*), and subterranean (e.g. *Zamia pumila*) (Webb and Osborne, 1989).

According to the IUCN (2024), 71% of the world’s cycads are threatened with extinction. The threats are a result of the illegal removal of cycads in the wild (Donaldson, 2003; Okubamichael *et al.*, 2016). *Encephalartos* is the largest African genus with 65 accepted species along with two other genera, *Stangeria* and *Cycas* , with one species each (Calonje *et al.*, 2024). Out of the 65 accepted species of *Encephalartos*, 37 species are found in South Africa, making it a hotspot for *Encephalartos* (Donaldson, 2003, 2008). Despite having a greater diversity of *Encephalartos* species, South Africa is regarded as a country with highly threatened cycads in Africa (IUCN, 2024). The threats are leading to the decline of cycads in the wild, and that may lead to the extinction of some species of *Encephalartos* (Golding and Hurter, 2003). This is a result of human activities including illegal removal from private, communal and conservation lands (Donaldson, 2003; Okubamichael *et al*., 2016).

Moreover, cycads are facing numerous challenges, which include lack of natural recruitment in some populations in the wild (*E. latifrons*) due to the absence of pollinators and too great a distance between the plants for self-pollination (Daly *et al*., 2006), as well as low seedling recruitment (Raimondo & Donaldson, 2003). As a result, *ex situ* and *in situ* conservation are required to prevent extinction in some species, as recommended by numerous conservation structures (IUCN, 2003; South Africa, 2017).

Cycads face further challenges in *ex situ* conservation, one of which is that seeds are recalcitrant and thus cannot be included in seed banks for storage (Woodenberg *et al.*, 2014; Wyse, Dickie and Willis, 2018; Marques *et al.*, 2019). Recalcitrant species desiccate faster compared to orthodox seeds (Baskin & Baskin 2001), and this is often due to rapid water loss in recalcitrant species (Kermonde, and Finch-Savage, 2002). According to Swart *et al*., (2018), species reintroduction for cycads in a suitable habitat is preferred, however, a protected area is most preferred in order to limit the chances of illegal poaching and harvesting (Volis, 2019; Daly *et al.*, 2006; Minterr and Collins, 2010; Rohr *et al.*, 2018). The Society for Ecological Restoration (SER) (2022) defines restoration as the process whereby an ecosystem that has been degraded, damaged, and disturbed is restored to its natural habitat, and the process involves planning as well as measurable goals (Bischoff *et al.*, 2010; Perring *et al.*, 2015; Nilsson *et al.*, 2016). The term restoration typically refers to habitat restoration (Zedler, 2005; Vaughn *et al*., 2010), whereas this study focused on the recovery of declining *Encephalartos* species Some of the lessons and principles from restoration are also relevant to species recovery, such as the need for appropriate methods and strategies (Oliveira *et al*., 2011). Therefore, employing different restoration conservation efforts, which involve different practical-based methods for cycads recovery could be essential. However, the species recovery can be a success (Akçakaya *et al*., 2018; Volis, 2019), or a failure due to numerous limiting factors such as desiccation (Godefroid *et al.*, 2011; 2020).

Godefroid *et al*, (2020) identified desiccation as one of the main reasons for the failure of reintroductions, However, the failure of the restoration project is sometimes due to the use of plants of poor quality to withstand environmental conditions in the wild (Godefroid *et al.*, 2011; Thomas *et al.*, 2015). Limited documentation and publication to guide future projects is also one of the factors limiting successful restoration (Godefroid *et al*., 2011). Thus, formal documentation of the results is important to pass on the knowledge to assist in future restoration projects (Ren *et al.*, 2014; Nilsson *et al.*, 2016). Lesage *et al.* (2020) stated that reintroduction project based on thorough and practical-based research are less likely to fail. Furthermore, poor planning and implementation in restoration negatively impact on project efficiency and effectiveness (UCN, 2013; Maschinski and Haskins, 2012). Although cycads are a threatened group of plants, not much research on ecological restoration has been undertaken and published (Bezuidenhout, 2020), therefore, there is a need to develop restoration protocols (Donaldson, 2003; Maschinski and Haskins, 2012) and conservation action to address the decline and extinction of this group (Swart, 2019). Henceforth, the end goal of this study is to improve the survival and establishment of seeds and juveniles during recovery projects, thereby contributing to the development of restoration protocols.

Recovery efforts for cycads, undertaken in different parts of the world, include the reintroduction of *Cycas debaoensis, Dioon edule, and Encephalartos spp* (Boyd, 1995; Bezuidenhout, 2020; Jian *et al.*, 2020). The projects were a success, however, mortality was experienced, as out of 300 juvenile cycads were planted to restore the Near Threatened (NT) *Dioon edule* in Mexico (Octavio-Aguilar *et al.*, 2008), 60 suffered mortality (Vovides *et al*., 2010). The experiment included plants from three age classes and showed 20% mortality in the first year. Furthermore, Boyd (1995) reported 92% survival for translocated juvenile plants of *Encephalartos cupidus* and 80% for *E. dyerianus,* although the long-term survival of these plants has not been determined. In contrast, herbivory was seen in the reintroduction of 20 *E. middelburgensis* seedlings under 10 years old, where the majority of exposed seedlings (those planted away from rocks in areas more accessible to herbivores) were uprooted and did not survive the attempted recovery (Rousseau and Rousseau, 2011).

In China, *in situ* reintroductions were carried out to conserve four *Cycas* species (*Cycas debaoensis, C. diannanensis, C. panzhihuaensis, and C. fairylakea*) (Zheng *et al*., 2017). In these cases, it was discovered that all 500 reintroduced seedlings in *C. debaoensis* were developing well with completely developed roots and leaves. Seedling survival was additionally observed in *C. diannanensis* and *C. panzhihuaensis*, as well as improved seed germination and survival in C. *fairylakea* (Jian *et al.,* 2020).

The application of horticultural techniques to increase water availability in soils is one possible way to overcome desiccation in cycad seeds and seedlings. This includes the use of hydrogels, which are hydrophilic polymers that absorb large quantities of water (Peppas *et al.*, 2012; Ullah *et al.*, 2015). The purpose of the polymers is to keep water available around the roots of the plant for later use, particularly during dry periods (Abdallah, 2019; Prakash *et al.,* 2021). Hydrogels have been used in different projects all over the world, including reintroduction projects. In a project focusing on pine juveniles for reforestation, hydrogel was used effectively to improve seedling growth and survival (Sarvaš *et al.*, 2007). The use of hydrogel has been proven to reduce drought stress, thereby improving seedling survival (Crous, 2017), but this has never been tested in cycads. The use of organic and inorganic growth substrates, such as coir, peat, and vermiculite, can result in more efficient and effective water and fertilizer use, essential for plant growth and development (Wilkinson *et al.*, 2014, Carlie *et al.,* 2019). Growth substrates are made from different organic and inorganic materials (Wilkinson *et al.*, 2014), and an ideal substrate must have the ability to retain water without waterlogging, as excessive water kills roots, more especially in container-grown plants (Daniels *et al.*, 2012). A decrease in soil moisture negatively impacts plant functionality, thus negatively affecting plant growth (Dodd and Ryan, 2016; Parkash and Singh, 2020), and according to Evans (2010), the optimal water-holding capacity of a substrate varies based on species of plants and growing conditions.

Most cycads require substrate conditions that include significant amounts of organic materials to improve water retention (Calonje *et al.,* 2010). Normal soil in the wild can impede plant growth due to constraints such as inappropriate soil pH, poor drainage, and poor soil sterility (Asaduzzaman, *et al.*, 2015; Fussy and Papenbrock, 2022). As an alternative, soilless substrates can be used as a substitute to avoid the use of normal soil. It is reported that, soilless substrates improve plant growth and quality compared to soil (Fussy and Papenbrock, 2022), and that could be essential for restoration and recovery programs in the wild. There are different types of soilless substrates, including rockwool, sawdust, and pumice (Asaduzzaman, 2015; Farhan *et al.*, 2018), with the more common and commercially available ones including coir, peat moss, and vermiculite (Asaduzzaman, 2015). These substrates vary in physical properties (see Table 1.1 below), and according to Calonje *et al*., (2010), a substrate must keep its physical properties over time, particularly in hot regions.

Coconut coir is produced from fibre extracted from coconut shells (Mariotti *et al.*, 2020) and has many desirable qualities which include, high water-holding capacity, excellent drainage, absence of weeds and pathogens (Handreck and Black, 2002; Prasad, 2021). The presence of the small fibres within coir aid with water and nutrients absorption (Maher *et al.*, 2008; Carlile, Raviv, and Prasad, 2021), and it is reported that coir takes at least four years to completely decompose (Vishnudas *et al.*, 2006; Delarue, 2017). When used as a substrate, coir holds a large amount of water and thus serves the primary purpose of storing water and nutrients (Withers, 2014). Furthermore, increased plant available water can be achieved when coir is augmented with other soilless substrates like pine bark (Jahromi *et al.*, 2020). Coir has been efficiently used in different conditions including green-roof gardening (De-ville *et al.*, 2017) and growing some forest species (Mariotti *et al.*, 2020).

Peat moss is a soilless substrate made from partially decomposed dead plant remains in bog areas which is dried after harvesting (Papadopoulos *et al.*, 2008; Cao, 2019). According to Prasad (2021), it has high porosity, provides good aeration, and high water-holding capacity. Peat moss is similar to coir in some chemical and physical properties (Wilkinson *et al*., 2014) and can be used as a soil amendment in greenhouse-grown plants (Gougoulias *et al.*, 2017). However, coir holds significantly more water and nutrients for a longer period compared to peat moss (Scagel, 2003; Holman *et al.*, 2005). For this study, coir is preferred as it holds water longer and is more generally available and sustainable, which would be important criteria for any product that is going to be used on an ongoing basis for recovery.

Vermiculite is a substrate made from hydrated magnesium iron aluminium silicate in the form of shiny flakes that range from golden brown to blackish in colour (Papadopoulos *et al.*, 2008). Due to its spongy and absorptive particles, vermiculite has been used for different purposes including soil augmentation, rooting cuttings, and seed germination and holds a significant amount of water (Handreck and Black, 2002). Vermiculite is widely used in propagation of forestry species (Bai *et al*., 2022).

Table 1.1: Physical-chemical characteristics of the material used in soilless culture. Adapted from Wilkinson *et al.*, 2014.

Substrate	Water holding capacity	Porosity
Coconut coir	High	High
Vermiculite	High	Low
Peat moss	High	High

1.4 Study Aims

The aim of this thesis is to test whether the application of horticultural techniques to improve moisture availability can reduce water stress in cycad seeds and juveniles and increase survival as one way to improve species recovery programs. The effect of substrates, watering regimes, and seed sowing depth on the seedling establishment and survival was considered (Chapter 2). Furthermore, the effectiveness of different substrates and watering regimes on the growth and survival of juvenile plants was also considered (Chapter 3).

1.5 Study objectives

The following objective forms the basis of this study:

(i) To investigate the effectiveness of different substrates on moisture retention for overcoming desiccation and increasing the survival of cycad seeds under varying conditions of water scarcity in the greenhouse (addressed in chapter 2).

(ii) To evaluate the effectiveness of the applied substrates and watering treatments on juvenile cycads establishment and provide recommendations on evidence-based species recovery guidelines of threatened cycads in the wild based on the findings of this study. This is addressed in chapter 3 where we subjected juvenile cycads growing on different substrates to varying levels of watering that would simulate periods of water stress under natural conditions.

1.6 Rationale

The recovery of wild cycad populations using seeds, seedlings or translocated plants is a challenge due to desiccation. Therefore, testing possible horticultural methods for reducing water stress in transplanted cycad juveniles and seeds has the potential to make an important contribution to cycad reintroduction and recovery strategies. Furthermore, cycad restoration efforts have had variable success and the development of guidelines, based on tested procedures and protocols for reducing desiccation, could improve the overall success of recovery efforts for cycads, particularly in arid regions.

1.7 Thesis outline

This thesis consists of four chapters, of which two of them (chapter two & three) are method chapters. The chapters are briefly described below:

- **Chapter one**: The general introduction, which comprises a statement of the research problem, background of the research problem, review of related literature, aims, and objectives of the study.

- **Chapter two**: describes experiments to test the effects of different substrates on leaf emergence and seedling establishment of *Encephalartos altensteinii* seeds under simulated drought conditions. The aim was to identify treatments that provide optimal conditions for seedling establishment.

- **Chapter three:** documents greenhouse experiments to test the effects of water-absorbing substrates on growth and survival of transplanted juvenile cycads in a simulated drought experiment. This was done by exposing cycad plants, transplanted into different growing media, to three watering regimes that would influence water availability.

- **Chapter four:** discusses the implications of this research for the recovery of cycad populations. The chapter summarises key findings of the study, presents overall conclusions, puts forward recommendations for recovery programs, and proposes further areas of research.

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CHAPTER TWO: The effects of different substrates on leaf emergence and seedling establishment of *Encephalartos altensteinii* under simulated drought conditions

Abstract

Dispersed cycad seeds face numerous challenges negatively affecting development and establishment *in situ,* including desiccation. Desiccation in cycad seeds limits seedling recruitment and can therefore negatively impact population viability. Therefore, this study evaluates the effectiveness of different horticultural techniques to overcome desiccation in seeds and thus improve seedling recruitment and development. To achieve this aim, seeds of *Encephalartos altensteinii* were sown on different substrates (Kirstenbosch Mix, Kirstenbosch Mix+Coir, Kirstenbosch Mix+Hydrogel, and Coir)), at different sowing depths (surface, 3 cm deep), and subjected to different watering treatments (once a week, once a month, once every 3 months). Different parameters such as leaf emergence, seedling survival, stomatal density and seedling growth were measured as indicators of adaptability and growth, as well as substrate moisture content to check on how well the substrates held moisture. Results showed that the Hydrogel-containing substrate had significantly higher moisture retention than substrates ($p < 0.001$), more specifically under the infrequent watering regime (once/three months). Furthermore, substrate, watering regime, and sowing depth significantly influenced seed leaf emergence and seedling survival ($p < 0.001$), with seeds sown in Coir and 3cm deep showing great resilience in establishment and survival. Therefore, for cycad recovery, using Coir or Hydrogel as substrate could be essential in improving moisture availability for seeds, which positively affects seedling establishment and growth, particularly under water scarcity conditions. Furthermore, sowing cycad seeds at least 3cm deep is important, as it provides the seeds with optimum growing conditions compared to those on the soil surface. These findings are important, as they provide valuable insights into seed-based recovery to improve cycad seedling establishment *in situ.*

2.1 Introduction

Cycads represent the most primitive lineage of seed plants dating back 330 million years ago (Ma.) at the boundary between the Early and Late Carboniferous era (Coiro *et al*., 2022). Despite this ancient lineage, cycads have been compounded with numerous challenges both *ex situ* and *in situ*, which impacts populations distribution and survival (Griffith *et al*., 2015; Donaldson, 2003). Firstly, cycad seed is reported to be recalcitrant (Deghan 1999; Nadarajan *et al*., 2018; Forsyth & Van Staden, 1983; Woodenberg *et al.*, 2007), therefore cannot be stored in seedbanks (Woodenberg *et al.*, 2014; Wyse *et al.*, 2018). Secondly, Donaldson (2008) reported that cycad seeds experience desiccation in the wild, and field recruitment studies show low seed and seedlings survival (Raimondo and Donaldson, 2003). This exposes the seeds to different environmental stresses like extreme temperatures, increasing their vulnerability to desiccation and mortality (Haj Sghaier *et al.*, 2022). Such factors negatively impact seed-based approaches for cycad recovery in the wild. The application of horticultural practices, such as the use of substrates with high moisture retention ability, could provide a solution to desiccation and improve seed survival and seedling development, in particular under water scarcity. This chapter will test whether such techniques can improve seed germination and seedling recruitment under greenhouse conditions, which simulate different levels of water stress that may be experienced under field conditions.

Cycad seeds need specific conditions for germination and establishment such as adequate light, temperature, and water (Dehgan, 1999; Woodenberg *et al.*, 2014; Pirdashti *et al.*, 2003). Providing cycad seeds with optimum growing conditions essential for seed growth and development could increase seed survival and seedling establishment. The moisture retention ability of a substrate can play a significant role in seed development and survival by providing optimum growing conditions (Abedi *et al.*, 2014; Leroy *et al.*, 2017), and according to Finch-Savage (2015) moisture availability is essential for seed germination and seedling establishment. However, cycad seed germination is reported to be slow (Nadarajan *et al.*, 2018) and may even take between 2 and 5 years in species such as *E. cycadifolius* (Raimondo and Donaldson, 2003). Therefore, providing cycad seeds with a substrate that retains its physical properties such as moisture over time is essential, particularly in arid environments (Calonje *et al*., 2010). In this context, there is limited knowledge on how different substrates influence *Encephalartos* seedling emergence and establishment under drought conditions. Therefore, the use of moisture-retaining substrates such as Coir and Hydrogel could potentially provide new direction in improving cycad recovery projects success by enhancing cycad seed development and thus seedling establishment in the wild. Furthermore, radicle emergence is one of the important stages of seed germination, as it represents the initial phase at which the developing root system begins to grow (Wolny *et al.*, 2018; Demir *et al.*, 2020; Summanen and Laurila, 2023). It is essential to present a method to increase germination percentage and seedling emergence chances (Zarchini *et al.* 2011; Masilamani *et al*., 2023).

Understanding seed response in relation to sowing depth could be essential, as it impacts leaf emergence and further seedling development (Embaye *et al.*, 2003; Masilamani *et al*., 2023). Therefore, evaluating the effectiveness of deeper sowing in cycad seeds could provide imperative information that can aid in improving cycad seed germination and thus seedling development in recovery projects. Water plays a significant role in plant survival, and water deficit results in drought stress, negatively impacting plant growth and survival (Yang et al., 2021; Seleiman *et al*., 2021). Plants have different mechanisms in response to drought stress caused by water deficiency (Peng *et al*., 2022; Gupta *et al*., 2020). According to Nunez et al. (2022), stomata and their density are important components in plant's response to mitigate drought stress. The regulation of water loss in plants is achieved through stomatal closure (Sato *et al.*, 2024; Gupta *et al*., 2020) and reduced stomatal density thereby reducing transpiration (Hasanuzzaman *et al*., 2018). Research has been done on stomata in different cycad species (Zang *et al.*, 2022; Coiro *et al*., 2021; Harwoth *et al*., 2011), however, not much work has been done in *Encephalartos* species (Woodenberg *et al*., 2019). Therefore, in this study, effects of the applied different treatments on stomatal density was tested as one important factor caused by drought stress.

This study further examined the effect of water-retaining additives and sowing depth on new leaf emergence and seedling establishment of cycad seeds under simulated conditions of varying rainfall regimes. The study seeks to determine whether the use of Coir or Hydrogel improves soil moisture content under dry conditions and whether these additions, together with sowing depth, affect seed germination and survival. It is hypothesized that i) using Coir or Hydrogel will improve moisture availability and enhance seedling growth and development under drier conditions, and ii) sowing seeds at 3cm deep will reduce desiccation and thus increase seed survival and seedling development chances.

2.2 Research methodology

Ethical considerations were taken into consideration in conducting this research. Ethical approval was obtained from the Ethics committee at Cape Peninsula University of Technology (**Ref No. 214113833/2022)** before commencing the study, ensuring that all aspects of the research adhere to ethical guidance.

2.2.1 Study species

This experiment used 240 seeds of *Encephalartos altensteinii* obtained from the Kirstenbosch Seed Room, where they were stored under controlled conditions of 15ºC temperature and 15% Relative Humidity (RH). The seeds were harvested from Kirstenbosch National Botanical Gardens.

2.2.2 Study area

All experiments were carried out at the research greenhouses of the South African National Biodiversity Institute, Kirstenbosch National Botanical Gardens, Cape Town, South Africa. The greenhouse is located 33°59'06.7"S, 18°26'09.2"E and comprises a clear polycarbonate tunnel (20m x 10m), covered with 40% shade net (grey), with slightly raised sides, an extractor fan and tornado turbine roof ventilator which allows for passive cooling.

2.2.3 Experimental design

Following guidance from Swart (2019) the viability of seeds was tested using tetrazolium (TZ) prior to seed sowing. Methods were adopted from Porter *et al*., (1947) by soaking seed into the solution of 235-Triphenyl tetrazolium chloride. The live tissue was recognized by a stain on the surface of the seed or embryo which indicates viability, and unstained indicates nonviability. Viability of 70% was observed in the tested *E. altensteinii* seeds indicating that the seed could be used for further germination and establishment experiments.

After the viability test, seeds were germinated using a method derived from Anderson (2013). Seeds were sown in clear polyethylene-sealed plastic bags (505 x 405mm) filled with Coir, and the bags were subsequently placed in a controlled growth chamber set at a temperature of 27ºC to facilitate germination. Seeds were considered ready for further experiments once the radicle had emerged from the seed. A total of 240 seeds meeting this criterion were then selected and used for testing.

Experiments to test whether Coir or Hydrogel affected soil moisture retention and leaf emergence in cycad seeds were conducted using a randomized complete block design, where a total of 240 seeds were sown into 20cm pots filled with different substrates. The experiment had 24 treatments (4 substrates x 3 watering regimes x 2 sowing depths) with 10 replicates for each treatment.

The substrate treatments comprised the standard Kirstenbosch mix (KB) as the control, Coir, KB+Coir, and KB+Hydrogel. The standard Kirstenbosch mix (KB), is typically used in the cycad nursery at Kirstenbosch and consists of equal parts fine bark, sand, compost, and loam soil, mixed with Atlantic Bio Ganic™ bounce-back (slow-release fertilizer) and bonemeal to induce root development. The Coir used here was a fine grade (6mm, obtained as a 5kg block), buffered to prevent the build-up of salts that may cause nutrient deficiency and imbalance in the substrate. The KB + Coir (KBC) consisted of equal parts (one part) of Coir and KB mix. The Hydrogel treatment (KBH) consisted of 90g Hydrogel mixed with 15 kg of KB mix, as recommended by Kumar *et al*., (2020). To make the substrate mixtures, a 120L concrete mixer was used to ensure thorough mixing of all the components. When potting, a shade net layer was placed at the base of each pot to minimize loss through the drainage holes. The three watering regimes were: once a week, once a month, and once every 3 months. For each watering event, plants were hand-watered using a watering can, each pot receiving 2L of water to ensure that the substrates were saturated, particularly under the less frequent watering regimes.

2.2.4 Effect of different treatments on substrates moisture content

The substrate's moisture content was measured weekly using a handheld moisture meter (Delta-T Devices™, ML2) and the probes were inserted into the pot near the root. According to Zheng Fu *et al.*, (2022), the critical soil moisture for plant water stress varies from $0.12 m^3.m^3$ to $0.26 m^3.m^3$. Therefore, a threshold of $0.12\ m^3.m^3$ was used to indicate low levels of water availability and this is represented as a straight line in relevant graphs (see Fig. 2.2). Although somewhat arbitrary, this threshold was included as a way to compare fluctuations in soil moisture between treatments and, particularly, the length of time that treatments experienced low levels of moisture availability.

2.2.5 Effect of different treatments on new leaf emergence at different sowing depths

In the greenhouse, new leaf emergence was assessed weekly, where the number of new leaves was counted and recorded across all the treatments. The aim was to compare leaf emergence between treatments.

2.2.6 Effect of the treatments on seedling survival at different sowing depths

Seedling survival was monitored weekly. Seeds or seedlings that showed no living tissue (e.g. dried radicle, brown leaves, dry stems, and roots) were considered dead, and all other seeds that showed some green tissues (e.g. green leaves and healthy stems) were considered alive.

2.2.7 Growth comparison between above and below-ground parts

All plants that survived were harvested after 10 months of the experiment and post-harvest measurements of leaf growth, stem diameter and fresh/dry mass were recorded. The leaf growth was measured as the full development of the leaf after harvest, where the length of the longest newly emerged leaf that had hardened-off (fully-developed with thick leaves) was measured. The measurements were obtained from the tip of the rachis to the end of the petiole. Stem diameter was measured using a Vernier digital calliper, and was measured at the widest section of the stem and 1cm below the leaf base.

Post-treatment fresh/dry mass of seedlings was measured separately for aboveground parts (leaves & stem) and belowground parts (roots). The fresh mass was obtained immediately after harvesting using an Adam Nimbus™ balance. Dry mass was obtained by placing plant parts into oven bags (Glad™, medium size) and drying in an oven at 100ºC for 48 hours (after testing different intervals at 12 and 24 hours, the 48 hours interval was the best to ensure that seedlings were thoroughly dry). Root diameter was measured as an indicator of growth, using a Vernier™ digital calliper to measure the widest section of the primary root. The root volume was obtained by placing the root into a measuring beaker with water and measuring the displacement volume. Coralloid roots, which are found in many cycad species and are formed by active growth from the apical meristems, were recorded as either present or absent.

2.2.8 Effect of different treatments on the stomatal density of juvenile cycads

Healthy leaflets of *E. altensteinii* seedlings were selected from the middle section of the leaves, given that there was no significant difference on stomatal counts of leaflets selected from the apex, middle, and base regions ($p>0.001$; $n = 10$ leaves). Since there are few or no stomata on the adaxial side of cycads leaflets (Woodenberg, 2019), leaves were thoroughly cleaned on the abaxial side using a soft cloth to remove any dust or debris. A thin layer (approx. 20mm in length) of transparent nail varnish was applied to the abaxial side of the leaflet using a fine-tipped paintbrush, and then leaflets were placed aside for at least 15 minutes to allow the nail varnish to dry completely, forming a peel (Gitz and Baker, 2009; Pathoumthong *et al.*, 2023). Using clear Sellotape, the dried nail varnish peels were gently peeled off, ensuring minimal damage to the peel. The obtained peels on the sellotape were then stuck on clean glass microscope slides and placed under the microscope at 200x magnification, Zeiss Microscope Axioskop 40. To confirm whether cells were stomata or not, the methods adopted from García-Gutiérrez *et al.*, (2019) were used, where leaflets were cut into small pieces and placed in a test tube with bleach for 12-24 hours to separate mesophyll cells. After the sample turned white and see-through on the edges, the bleach was removed by rinsing the samples with water. Using a tweezer, the abaxial side was separated from the adaxial side to isolate the side with stomata. Thereafter, the prepared samples were mounted on the microscope slides with cover slides and were then observed under a microscope. The guard cells were visible, which therefore confirmed that the circular-shaped cells were indeed stomata (Meloto *et al.,* 2008), see Fig. 2.1. The number of stomata present within the observed area were counted and recorded.

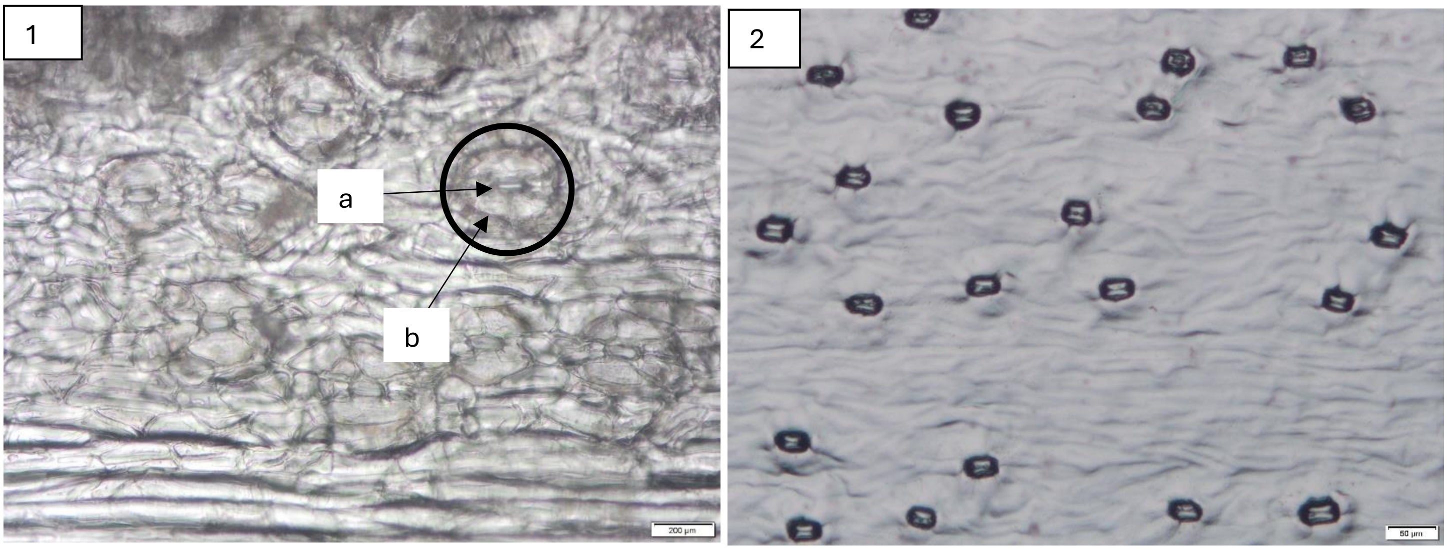

Figure 2.1: plate 1, stomata in *encephalartos altensteinii* seedlings, showing components that make up stomata, a) stomatal pore, and b) guard cell. Plate 2, shows stomatal pores, representing stomata.

2.2.9 Statical analysis

All statistical analyses were performed using R v 4.4.0 (R Core Team 2024) and all graphics were generated using packages 'ggplot2' (Wickham, 2016), 'ggpubr (Kassambra, 2023) and 'ggsignif' (Ahlmann-Eltze and Patil, 2021). All models were general linear models with a gaussian family distribution. The main effects for the analysis of variance for the logistic regression was calculated using package 'car' (Fox and Weisberg, 2019). The best fit model (using corrected AIC) for the model was calculated using either step command in R or package 'MuMIn' (Bartoń, 2023). The post-hoc analysis was calculated using package 'emmeans' (Lenth, 2024) or package 'multcomp' (Hothorn *et al.,* 2008).

2.4 Results

2.4.1 Effect of different treatments on substrates' moisture content

Comparisons for all substrates between regimes showed that the weekly watering regime had higher water content than both monthly and quarterly watering regimes, as well as the monthly watering regime had higher water retention than the quarterly watering regime (Table 2.1, 2.2; $p < 0.001$). For both weekly and monthly watering regimes, the control substrate had less moisture retention than the other three substrates, Coir, Kirstenbosch Mix+Coir, and Kirstenbosch Mix+Hydrogel (Table 2.1, $p < 0.001$; Fig. 2.2). For the Quarterly watering regime, however, the control had more moisture retention than the Coir and KBC substrates. The KBH substrate had significantly more moisture retention than both Coir and KBC substrates (Table 2.1, $p < 0.001$; Fig. 2.2).

Furthermore, under the weekly and monthly watering, none of the substrates were below the designated critical moisture content threshold (Fig. 2.2). Under the Quarterly watering regime, The Coir and KBC substrates had significantly less moisture over a longer period (28 weeks) than the other two substrates (control and KBH). The control was below the critical moisture content threshold for 25 weeks, while KBH was below for at least 20 weeks.

Table 2.1: Post hoc comparison for the mixed effect model for moisture content in the media of seedlings, showing the pairwise comparison between substrates for each watering regime (shown in *italics*) separately.

Treatments	Estimate	z ratio	p value
Weekly:			
Control - Coir	-0.10	-54.52	< 0.001
Control - KBC	-0.10	-54.75	< 0.001
Control - KBH	-0.12	-64.05	< 0.001
Coir - KBC	0.00	-0.24	0.995
Coir - KBH	-0.02	-9.53	< 0.001
KBC - KBH	-0.02	-9.29	< 0.001
Monthly:			
Control - Coir	-0.04	-19.99	< 0.001
Control - KBC	-0.03	-15.34	< 0.001
Control - KBH	-0.05	-28.10	< 0.001
Coir - KBC	0.01	4.65	< 0.001
Coir - KBH	-0.02	-8.11	< 0.001

KBC - KBH	-0.02	-12.76	< 0.001
Quarterly:			
Control - Coir	0.02	9.83	< 0.001
Control - KBC	0.01	6.33	< 0.001
Control - KBH	-0.03	-17.51	< 0.001
Coir - KBC	-0.01	-3.50	0.003
Coir - KBH	-0.05	-27.34	< 0.001
KBC - KBH	-0.04	-23.84	< 0.001

Table 2.2: Post hoc comparison for the mixed effect model for moisture content in the media of seedlings, showing the pairwise comparison between watering regime for each substrate (shown in *italics*) separately.

Treatments	**Estimate**	**z ratio**	**p value**
Control:			
Weekly - Monthly	0.08	45.27	< 0.001
Weekly - Quarterly	0.22	124.29	< 0.001
Monthly - Quarterly	0.14	79.03	< 0.001
Coir:			
Weekly - Monthly	0.14	81.20	< 0.001
Weekly - Quarterly	0.34	191.25	< 0.001
Monthly - Quarterly	0.20	110.05	< 0.001
KBC:			
Weekly - Monthly	0.15	86.29	< 0.001
Weekly - Quarterly	0.33	187.86	< 0.001
Monthly - Quarterly	0.18	101.58	< 0.001
KBH:			
Weekly - Monthly	0.15	82.68	< 0.001
Weekly - Quarterly	0.31	172.72	< 0.001
Monthly - Quarterly	0.16	90.04	< 0.001

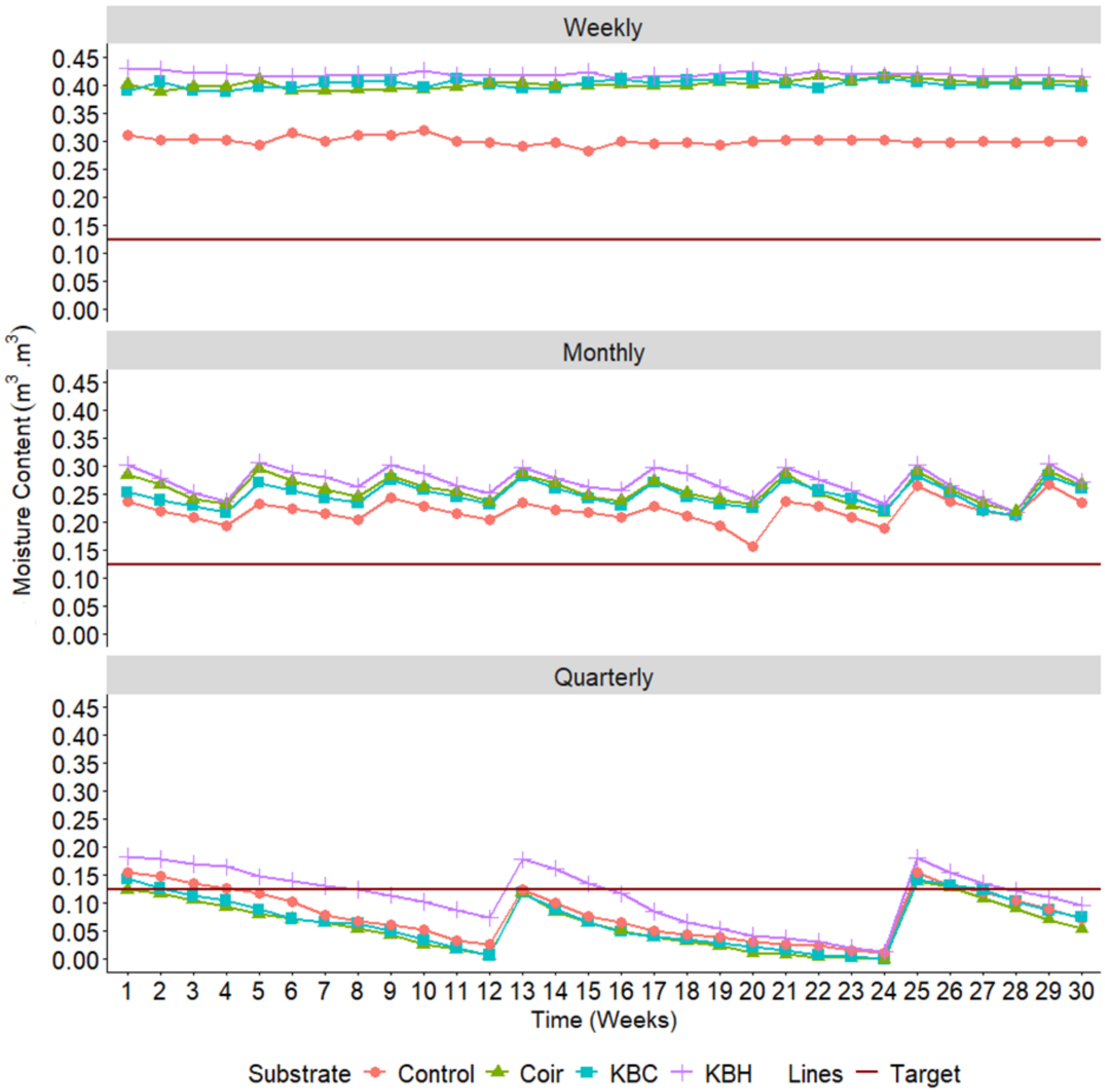

Figure 2.2: Soil moisture content in different substrates placed under different watering regimes in the greenhouse for 30 weeks. Substrates tested include: control (standard Kirstenbosch cycad mix), Coir, KBC (Kirstenbosch Mix + Coir), and KBH (Kirstenbosch Mix + Hydrogel). The target represents critical moisture threshold. Error bars represent standard deviation.

2.4.2 Effect of the treatments on seedling survival at different sowing depths

The substrate, watering regime, and depth significantly influenced seedling survival (Table 2.3, $p < 0.001$). There was a significant interaction between substrate and depth (Table 2.3, $p = 0.010$), with no significant interaction between substrate and watering regime (Table 2.3, $p = 0.130$). The seeds sown on the KBH substate had significantly lower seedling survival than those sown in the Coir and KBC under both surface and 3cm depths (Table S1, $p < 0.05$). The seeds sown in the Coir and KBC had significantly higher seedling survival seeds sown than in the control substrate (Table S2, $p < 0.05$; Fig. 2.3). Furthermore, seeds sown at 3cm deep had significantly higher seedling survival than seeds sown on the surface within all the substrates except in Coir (Table S1, $p < 0.001$; Fig. 2.4).

Table 2.3: Analysis of Variance (ANOVA) results for the Logistic regression model of seedling survival, substrate, and watering regime

	Degrees of Freedom	**Deviance**	**Residual Degrees of Freedom**	**Residual Deviance**	**p-value**
NULL			239	323.84	
Substrate	3	40.15	236	283.69	< 0.001
Regime	2	20.28	234	263.41	< 0.001
Depth	1	65.14	233	198.27	< 0.001
Substrate:Regime	6	9.87	227	188.40	0.130
Substrate:Depth	3	11.36	224	177.04	0.010

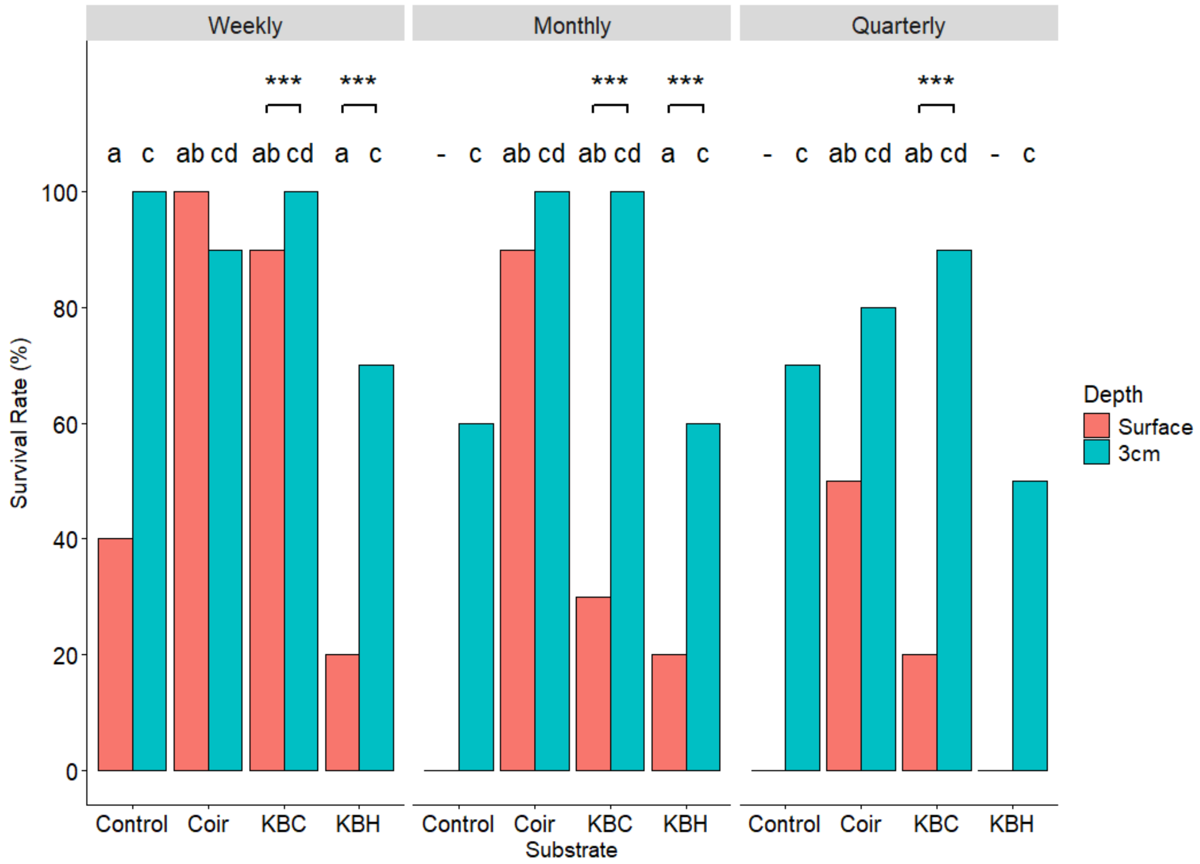

Figure 2.3: Seed and seedling survival rate over 10 months across all 12 treatments in the greenhouse. Bars represent the percentage (%) of survival, n=10. The bars and stars represent significant differences between the depth for each substrate and the letters represent whether the substrate is significantly different (bars that have the same letter are not significantly different) for each depth.

2.4.3 Effect of different substrates, watering regimes, and sowing depths on new leaf emergence

Substate, watering regime, and sowing depth all significantly influence the new leaf emergence with a significant three-way interaction between substrate, watering regime and depth (Table 2.4, $p = 0.004$). There were two-way interactions between substrate & regime and substrate & depth (Table 2.4, $p < 0.05$) but no significant interaction between regime and depth (Table 2.4, $p = 0.243$).

Leaf emergence was significantly higher on seeds sown at 3cm depths than at the surface (Table S3, $p < 0.05$). The leaf emergence on seeds sown on Coir and KBC was significantly higher than the control at 3cm under the weekly and monthly watering regime, and on surface under the monthly watering regime (Table S3, $p < 0.05$). Seeds sown on the surface in Coir and KBC had significantly higher leaf emergence than the KBH substrate for weekly and monthly watering regimes (Table S3, $p < 0.05$). Overall seeds sown on the substrates containing Coir under the 3cm sowing depth proved to support new leaf emergence (Table S3; Fig 2.5).

Table 2.4: ANOVA (Type III) results for the non-parametric ranked ANOVA for leaf emergence at different substrates, watering regimes and depths (package car).

Response: rank(Leaf)	Sum of Squares	Degrees of freedom	F value	p value
(Intercept)	3484860	1	1576.44	<0.001
Substrate	126742	3	19.11	<0.001
Regime	173245	2	39.19	<0.001
Depth	152258	1	68.88	<0.001
Substrate:Regime	39915	6	3.01	0.008
Substrate:Depth	25305	3	3.82	0.011
Regime:Depth	6294	2	1.42	0.243
Substrate:Regime: Depth	43860	6	3.31	0.004
Residuals	477488	216		

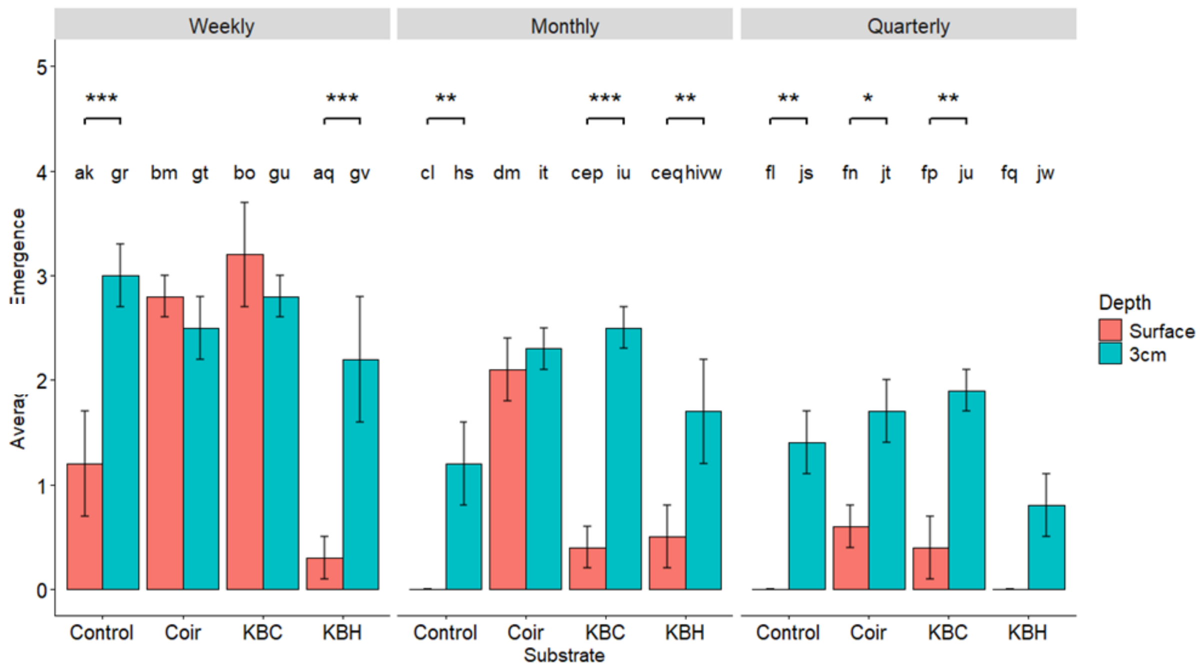

Figure 2.4: New leaf emergence on cycad seeds sown on different substrates and depths, under different watering regimes. Error bars represent the standard error. Letters a to j represent significant differences between substrates by watering regime and depth, letters k to w represent significant differences between watering regimes by substrates and depth (bars with the same letters are not significantly different). The horizontal bars show

significant differences between depths for each substrate and watering regime, level of significance is indicated by *.

2.4.4 Growth comparison between above and below-ground parts in cycad seedlings

a) Leaves

There was a significant interaction between the watering regime and sowing depth, and they significantly influence the length of leaves ($F = 6.87$, $df = 3$, $df_{residual} = 129$, $p = 0.001$). The interaction between substrate, watering regime, and sowing depth did not significantly influence the length of leaves (Table S4; $p > 0.05$), the same was found for the interaction between substrate and sowing depth (Table S4; $p > 0.05$). However, the quarterly watering regime would have significantly lower leaf lengths than in the weekly and monthly watering regimes for a surface sowing depth (Table S4, $p \leq 0.001$). The results further showed that the 3cm sowing depth had significantly higher leaf lengths than at the surface ($t = -4.31$, $p < 0.001$).

b) Stems

There was a significant interaction between watering regime and sowing depth, and they significantly influence the square root length of stems ($F = 6.87$, $df = 3$, $df_{residual} = 129$, $p = 0.001$). Substrate, watering regime and sowing depth did not significantly influence the square root length of stems ($p > 0.05$), the same was found for the interaction between substrate and sowing depth ($p > 0.05$). The control plants at 3cm sowing depth had significantly thicker stem diameters than those in Coir and KBC substrates (Table S5, $p < 0.05$). Plants in the KBH substrate had significantly thicker stems at 3 cm sowing depth than those in the Coir substrate [$t(131) = -2.64$, $p = 0.046$]. The weekly watering regime yielded significantly thicker stem diameters than both monthly and quarterly watering regimes (Table S5, $p < 0.05$).

c) Roots

There was a significant three-way interaction between substrate, watering regime and sowing depth that significantly influenced the root diameter ($F = 4.81$, $df = 3$, $df_{residual} = 120$, $p = 0.03$). Watering regime [$F(3) = 6.44$, $p < 0.001$] and substrate [$F(2) = 25.90$, $p < 0.001$] significantly influenced the root diameter independently. The significant interaction between substrate and sowing depth also significantly influenced root diameter [$F(3) = 5.18$, $p < 0.002$] .

For the weekly watering regime and the 3cm sowing depth the control and KBH substrates would have significantly thicker root diameters than in the Coir and KBC substrates (Table S6, $p < 0.05$). Surface planted seeds would have significantly thinner root diameters in the KBH substrate than in the KBC substrate, while they would have significantly thicker roots in the KBH substrate than in the Coir substrate during the monthly watering regime (Table S6, $p < 0.05$).

2.4.5 Fresh and dry mass in cycad seedlings

a) Leaves

Watering regime ($F = 31.48$, $df = 2$, $df_{residual} = 138$, $p < 0.001$) and sowing depth of seeds ($F = 5.82$, $df = 1$, $df_{residual} = 137$, $p = 0.017$) influenced the water content in leaves. Seeds planted at 3cm depth have significantly higher moisture content in their leaves than those planted at the surface ($t = 2.41$, $p < 0.05$).

b) Stems

Substrate ($F = 2.79$, $df = 3$, $df_{residual} = 137$, $p < 0.05$) and watering regime ($F = 25.9$, $df = 2$, $df_{residual} = 135$, $p < 0.001$) significantly influenced the moisture content in the stems. Plants in the weekly watering regime had significantly higher moisture content than those in both the monthly and quarterly watering regimes ($t=132$, $p < 0.001$). Even though the ANOVA showed that substrates significantly influenced the moisture content the influence was not big enough to show differences between the substrates in the post hoc analysis (Table S7, $p > 0.05$).

c) Roots

Watering regime significantly influenced the water content in roots ($F = 73.80$, $df = 2$, $df_{residual} = 138$, $p < 0.001$), but sowing depth did not have a significant influence ($F = 0.14$, $df = 1$, $df_{residual} = 137$, $p = 0.707$). The post hoc results showed that the weekly watering regime would yield significantly higher moisture content than both the monthly and quarterly watering regime and the monthly watering regime would yield higher moisture content in the roots than the quarterly watering regime $t=137$, $p < 0.001$; Fig. 2.6.

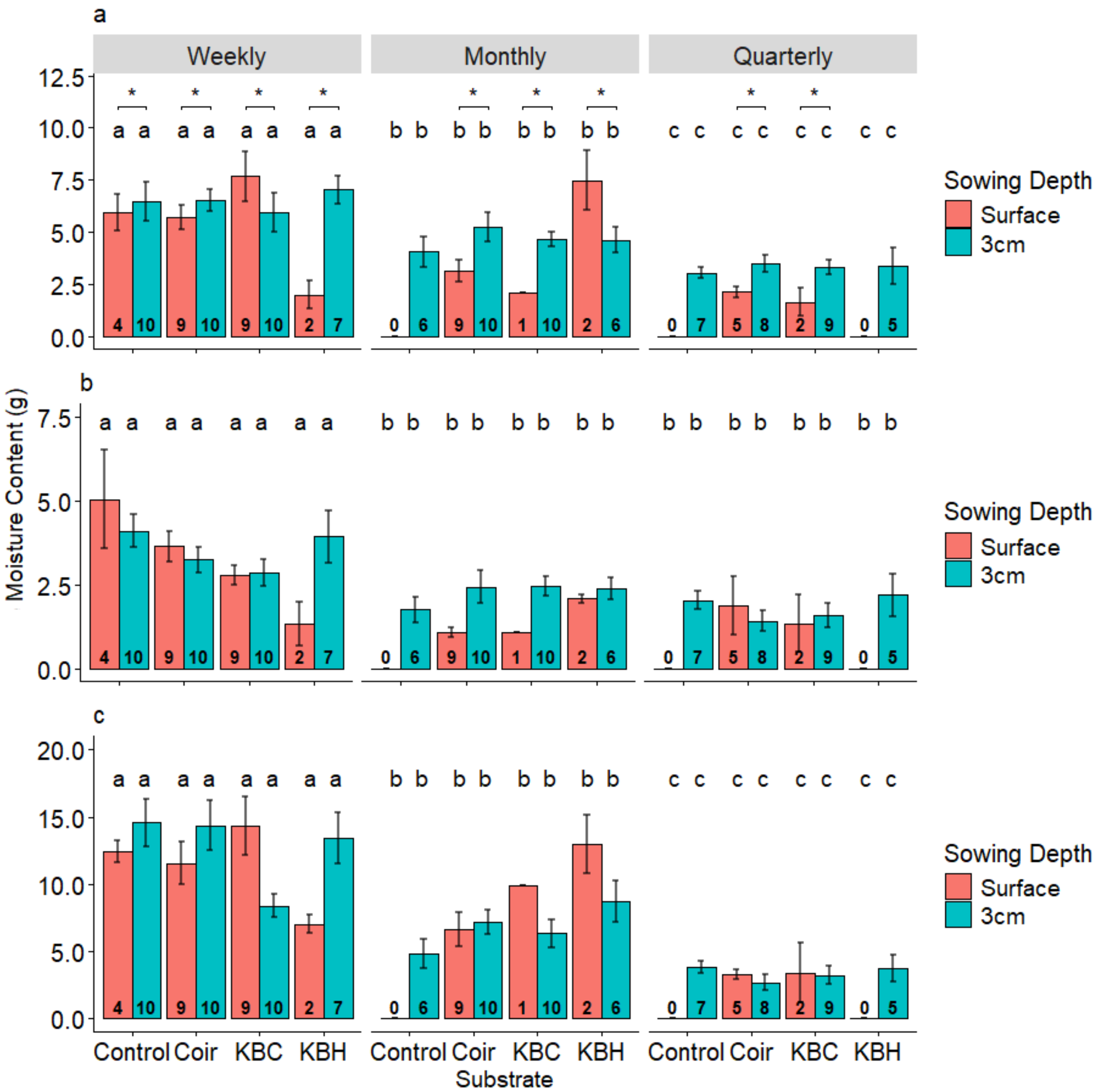

Figure 2.5: a) Average leaf moisture content, b) Average stem moisture content and c) Average root moisture content by substrate, watering regime and sowing depth in *Encephalartos* seeds. The error bars represent the standard error. Different letters represent whether the watering regime is significantly different (bars that have the same letter is not significantly different). Horizontal error bars represent significant differences between sowing depths.

2.4.6 Coralloid roots development in cycad seedlings

The ANOVA showed both substrate ($F = 5.29$, $df = 3$, $df_{residual} = 134$, $p = 0.002$) and watering regimes ($F = 14.22$, $df = 2$, $df_{residual} = 134$, $p < 0.001$) significantly influenced the formation of coralloid roots in seedlings.

The development of coralloid roots was significantly greater in plants watered weekly across all the substrates and sowing depths (Table 2.5, P< 0.05; Fig. 2.7) with only a small percentage of plants developing coralloid roots when watered monthly (only in Coir substrates, Fig. 2.8) or quarterly (Coir and KBC substrates). When watered weekly, seeds planted on the surface generally had a higher percentage with coralloid roots than those planted at 3cm, but this was not significantly different

Table 2.5: Post hoc analysis for the logistic regression of coralloid root formation in relation to substrate and watering regime.

Substrate	Coefficient Estimate	Standard Error	z value	p value
Coir - Control == 0	2.64	0.87	3.03	0.012
KBC - Control == 0	0.95	0.89	1.06	0.707
KBH - Control == 0	0.99	1.01	0.98	0.757
KBC - Coir == 0	-1.69	0.63	-2.68	0.036
KBH - Coir == 0	-1.65	0.79	-2.10	0.150
KBH - KBC == 0	0.04	0.82	0.05	1.000

Watering Regime	Coefficient Estimate	Standard Error	z value	p value
Monthly - Weekly == 0	-2.70	0.72	-3.75	< 0.001
Quarterly - Weekly == 0	-2.78	0.83	-3.35	0.002
Quarterly - Monthly == 0	-0.08	0.96	-0.08	0.996

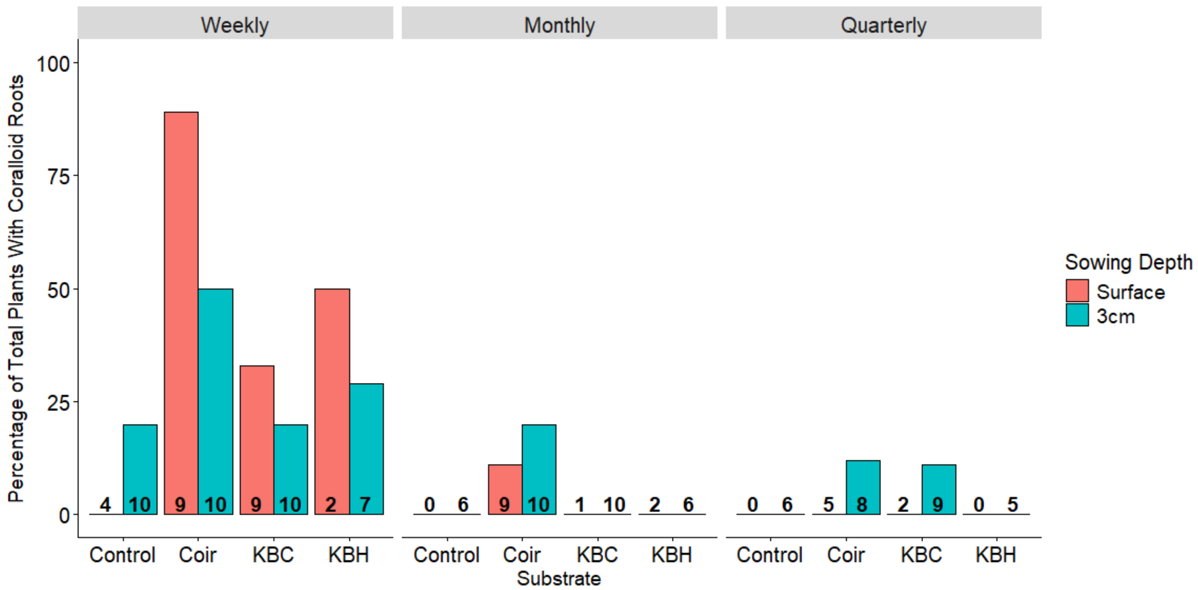

Figure 2.6: Percentage of total plants that developed coralloid roots, for substrate by watering regime and sowing depth. The numbers on the bars indicate the sample size of plants that survived the experiments.

Figure 2.7: Coralloid development on *E. alteinsteinii* seedling resulted from the seeds sown on Coir, at 3cm deep, and watered weekly.

2.4.7 Root volume in cycad seedlings

The results for root volume (Table S8, Fig. 2.9) showed a significant three-way interaction between substrate, watering regime, and sowing depth ($F = 5.70$, $df = 3$, $df_{residual} = 120$, $p = 0.001$) as well as a significant two-way interaction between substrate and sowing depth ($F = 3.00$, $df = 3$, $df_{residual} = 125$, $p = 0.033$). As a general trend, root volume declined under drier conditions, most notably in the control, Coir and KBH substrates (Fig. 2.9). Sowing depth had variable effects, with significantly greater root volume in one treatment sown on the surface (KBC-weekly), and in five treatments where seeds were sown at 3cm (Coir-weekly; KBH-weekly; and control, KBC, KBH watered quarterly). Seeds planted in KBC at 3cm showed the highest root volume for all treatments under the quarterly watering regime (Fig. 2.9).

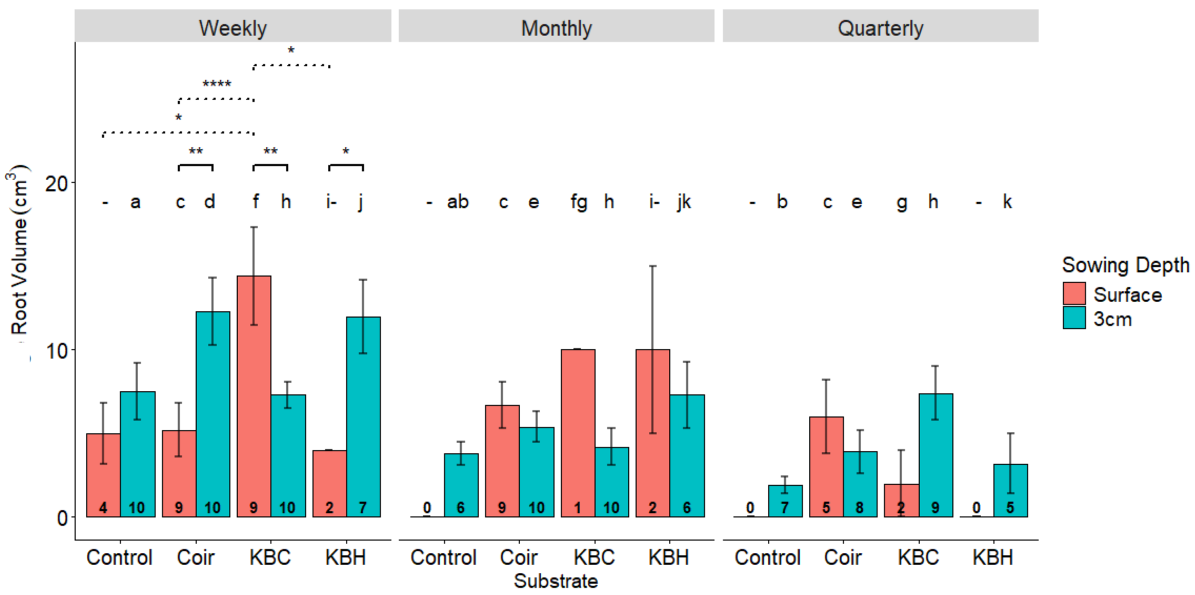

Figure 2.8: Average root volume (cm) in *Encephalartos* from seeds planted on different substrates, sowing depths, and watering regimes. The error bars represent the standard error. Different letters represent whether the watering regime is significantly different for specific substrate and sowing depths (bars that have the same letter is not significantly different, bars with "–" indicate no comparison was possible). Horizontal error bars represent significant differences between sowing depths (solid lines) and substrates (dotted lines), with level of significance indicated by *.

2.4.8 Effect of different treatments on stomatal density in cycad seedlings

The results showed that both substrate ($F = 2.81$, $df = 3$, $df_{residual} = 116$, $p = 0.043$) and watering regime ($F = 29.31$, $df = 2$, $df_{residual} = 114$, $p < 0.001$) significantly influenced the stomatal density in cycad seedlings. There was a significant interaction between substrate and watering regime ($F = 29.31$, $df = 2$, $df_{residual} = 114$, $p < 0.001$). The control had significantly higher stomatal density than Coir, KBC, and KBH on the Weekly watering regime (Table 2.6, $p < 0.05$). For the Monthly watering regime KBC had a significantly higher stomatal density than in the Coir and KBH substrate (Table 2.6, $p < 0.05$). For the Quarterly watering regime, Coir had significantly more stomata than in the control, KBC or KBH substrate (Table 2.6, $p < 0.05$). The results further showed that, in all tested substrates, stomatal density was significantly higher for the weekly watering regime than in both the monthly and quarterly watering regime and significantly higher in the monthly watering regime than in the quarterly regime (Table S9, $p < 0.001$; Fig. 2.10).

Table 2.6: Post hoc Tukey comparison for the GLM of stomatal density for the interaction between substrate, and watering regime showing the pairwise comparisons between substrates for each watering regime. Level of significance is indicated with the p-value.

Contrast	Estimate	Standard Error	degrees of freedom	t.ratio	p.value
Weekly					
Control - Coir	14.1	5.09	108	2.77	0.033
Control - KBC	25.7	5.09	108	5.05	< 0.001
Control - KBH	19.4	5.09	108	3.81	0.001
Coir - KBC	11.6	5.09	108	2.28	0.109
Coir - KBH	5.3	5.09	108	1.04	0.725
KBC - KBH	-6.3	5.09	108	-1.24	0.604
Monthly					
Control - Coir	8.0	5.09	108	1.57	0.399
Control - KBC	-8.8	5.09	108	-1.73	0.314
Control - KBH	4.6	5.09	108	0.90	0.803
Coir - KBC	-16.8	5.09	108	-3.30	0.007
Coir - KBH	-3.4	5.09	108	-0.67	0.909
KBC - KBH	13.4	5.09	108	2.63	0.047
Quarterly					
Control - Coir	-16.8	5.09	108	-3.30	0.007
Control - KBC	0.9	5.09	108	0.18	0.998
Control - KBH	-1.7	5.09	108	-0.33	0.987
Coir - KBC	17.7	5.09	108	3.48	0.004
Coir - KBH	15.1	5.09	108	2.97	0.019
KBC - KBH	-2.6	5.09	108	-0.51	0.956

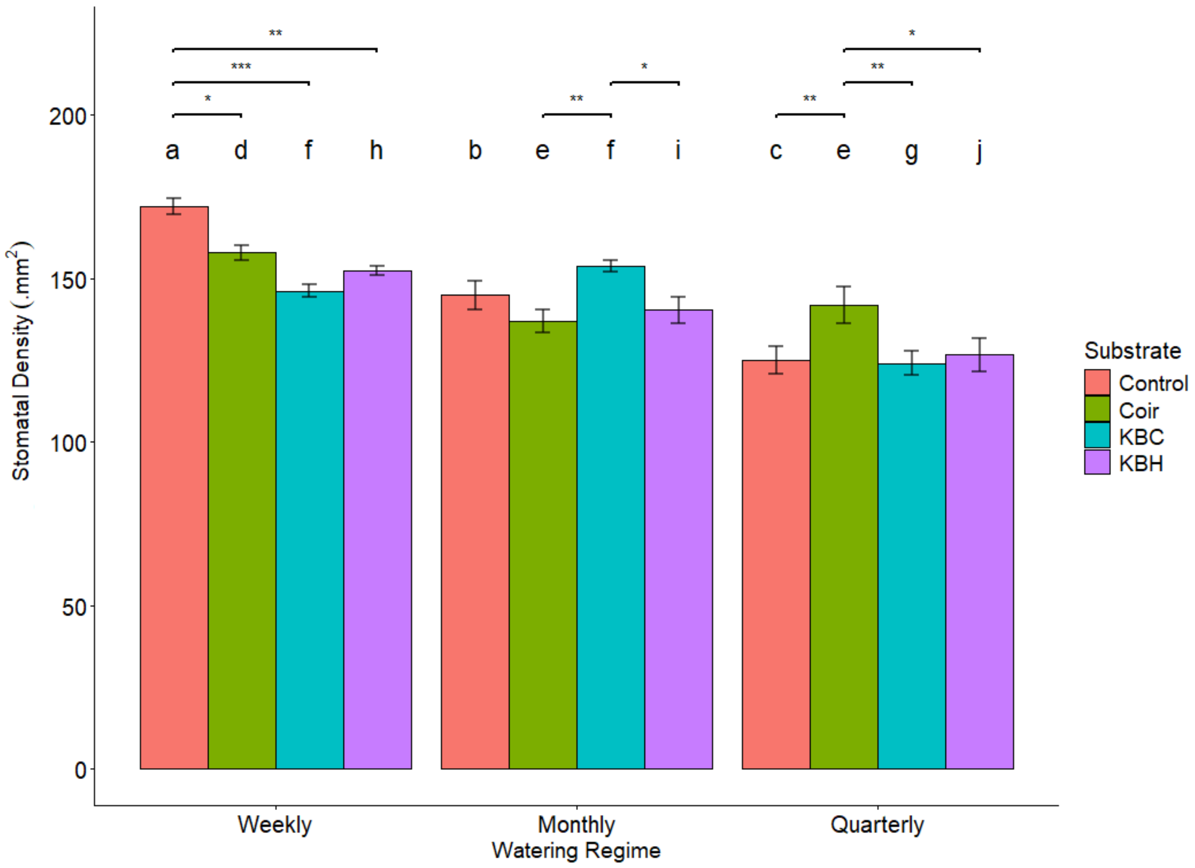

Figure 2.9: Mean stomatal density in cycad seedlings under different treatments in the greenhouse, n=10 per treatment. The bars represents the mean number of stomata, the error bars represent the standard error, and the bars and stars represent significant differences between the substrates for each watering regime and the letters represent whether the watering regime is significantly different (bars that have the same letter in each substrate is not significantly different) for each substrate.

2.6 Discussion

This study found that moisture content significantly increases seedling survival rate, thus supporting the hypothesis that adding water-retaining compounds into the substrate could increase moisture availability and seedling survival in cycad seeds. Furthermore, cycad seeds may be placed on the surface or halfway buried on a moisture retaining medium (Tang *et al*., 2019; Calonje *et al*., 2011). So, this study found that sowing seed at a depth of 3cm significantly increased seed survival and leaf emergence under less frequent watering regimes, indicating that this practice may provide better conditions for the establishment of *Encephalartos altensteinii* seedlings. This supports the hypothesis that sowing seeds at 3cm deep would reduce desiccation and thus improve leaf emergence chance and overall seedling establishment than sowing on the surface, as Schutzman (2015) stated that there are very few cycad seeds that develop into mature seedlings in the wild.

Substrates comprising water-absorbing compounds resulted in consistently higher moisture content than the control under frequent watering conditions. However, under less water availability (3 months) the difference between the control and the other substrates diminished. At quarterly watering intervals (3 months), only KBH resulted in consistently higher moisture content than the control. This shows that the incorporation of water-absorbing compounds such as Hydrogel into the medium plays a pivotal role in moisture retention under less water availability. These findings underline the importance of substrate utilization in the recovery of cycad seedlings. Additionally, the enhanced moisture retention in KBH may relate to the effectiveness of the polymers within the hydrogel, a factor also identified in past research as crucial for water retention (Jnanesha *et al.*,2021; Yangirova *et al*., 2021). These findings indicate that selecting appropriate substrates in cycad recovery is important to maximize moisture retention during seed sowing and thus seedling establishment. Frequent watering seems beneficial, but where this is not possible, substrates such as Coir and KBH can be utilized to reduce the need for frequent watering while at the same time providing more moisture for the seedlings. Furthermore, studies have shown that substrates like Coir are known for high water-holding capacity ability due to their fibrous structure (Jahromi *et al*., 2020), which is consistent with the results observed in this study. Ampitiyawatta and Weerasuriya (2021) also found that the utilization of moisture-absorbing substrates allows for significant improvement in soil moisture conservation. The findings of this study are important as they give insight into substrate selection for improved moisture retention, which could possibly reduce the need for frequent watering of cycad seedlings in the wild. This may further have a key role in informing cycad recovery project in the wild, particularly in areas where water is a limiting factor.

According to Singh *et al.*, (2017) seed sowing depth is important for seedling establishment. The results of the study suggest that sowing seed at 3cm deep is more useful than on the surface. This was supported by the higher new leaf emergence and seedling survival obtained on seeds sown 3cm deeper. Snow and Walter (2007) reported that cycad seeds in the wild often remain on the ground for long periods, and any wound may increase the rate of embryo desiccation or entry of pathogens, thus resulting in mortality. Therefore, the 3cm sowing depth possibly provided the seeds with optimum growing conditions essential for further seedling development. Similar results were reported by other studies (Becerra *et al*., 2022; Guo *et al*., 2010; Snow and Walter 2007; Odeleye *et al.,* 2007). Furthermore sowing seeds deeper could provide protection and optimum moisture, and also seeds are less exposed to harsh environmental conditions than seeds on the surface (Guo *et al*., 2010). Therefore, the hypothesis that sowing cycad seeds 3cm deep improve seedling survival is therefore supported by the results of this study.

The higher survival obtained under the Coir and KBC is indicative that Coir-containing substrates are more critical in ensuring seedling survival. This suggests that Coir may provide better conditions for radicle further development, leading to improved seedling survival, as similarly reported by Mariotti *et al.* (2023). The better leaf emergence in the moisture-retentive substrates, such as Coir, indicates that these conditions would provide optimum conditions for *Encephalartos altensteinii* seed germination. Thus, for cycad recovery, the careful selection of substrates such as Coir with better moisture retention would be some of the effective strategies to improve seedlings' establishment under unfavourable environmental conditions of less water availability.

Furthermore, adequate water availability improved leaf development, which can also assist seedling survival in the early stages of seedling growth. This shows the importance of water in leaf growth, as it was identified that leaf growth was particularly sensitive to water deficits (Bañón *et al*., 2022). A study by Masetto *et al.*, (2011) also reported that as the substrate water availability decreased, there was a consistent reduction in the formation of seedlings. Similar results were obtained from the stem growth. The lack of significant influence of substrate on leaf and stem growth could be the result of the fact that leaf and stem growth is more influenced by adequate water availability or by substrate properties. Unlike in my study, the substrates such as Coir significantly influenced leaf growth (Mariotti *et al*., 2023). Additionally, Tuckeldoe *et al.*, (2023) found that stem growth and development is associated with the nutrient composition of the soil. Therefore, future studies could assess the effect of substrate's physical and chemical properties on leaf growth and stem growth in cycad seedlings.

Water availability and sowing depth significantly influenced water content in cycad seedlings leaves, stems, and roots. My results are consistent with Li *et al.* (2009) who also reported that leaf water content of other plant species such as, *Sophora davidii* significantly dropped with water availability. These results suggest that adequate watering is critical to optimize the water content of seedlings for better survival success in recovery efforts of cycads, particularly in arid regions. The study findings further showed that substrate and water availability are important factors that affect the water content of the stems of cycads seedlings. These results point out the need for adequate water availability to keep the substrate at optimal moisture levels for the growth and establishment of the cycads, particularly in recovery project.

Root size has a direct relationship with a plant's ability to cope with water stress, particularly in arid environments (Kulkarni and Phalke, 2009). Thicker roots were observed under certain conditions, more specifically with the KBH substrate and frequent watering. This was similarly reported in a study by Park *et al. (*2020), where substrate and watering significantly affected the root growth of *Crepidiastrum denticulatum* seedlings. However, a study by Shahbani *et al*., (2021) had findings in contrast with my study findings as there was no significant in stem diameter of *Passiflora quadrangularis* seedlings grown on different substrates. Meanwhile, seeds in KBH produced thinner but significantly thicker stems than seeds sown in Coir roots and watered monthly. This is supported by Munroe *et al*., (2018) findings that higher proportions of Coir adversely affect root growth in gymnosperm species.

Grossnickle (2005) states that a well-developed root system is important for seedlings to aid with the retention of available water in the soil, which is critical for survival and growth. My results imply that substrate selection together with adequate water supply, are critical in optimizing root development in cycad recovery using seeds. The findings show that KBC substrate offers greater root development in cycad seedlings, particularly where seeds are sown on the surface and with frequent watering, which might be critical for seedling establishment. On the other hand, Coir and KBH were better when the seed is sown at 3 cm depth though the produced root volume is still less than that of KBC. Therefore, adding Coir to the cycad mix should be the preferred substrate for the recovery of cycad seeds in the wild to increase root volume. This is supported by the findings of Mariotti *et al.* (2020), where Coir significantly improved root development in *Quercus* species.

The highest water content in roots was obtained from the weekly watering, followed by monthly and quarterly watering. This finding was similarly reported by Li *et al.,* (2009) where root mass was highest in seedlings subjected to more water availability. However, contrary to my study, Park *et al*., (2020) found that fresh and dry weights of the leaf and root increased significantly under less frequent watering but decreased under more frequent watering. This could have been affected by different factors such as substrates, watering regimes, and species used in the studies.

According to Restrepo *et al*., (2020) soil significantly impacts the development of coralloid roots in cycads, and in my study similar results were obtained where coralloid roots formation was influenced by substrates. Coralloid roots were most common in plants grown in Coir under the highest soil moisture conditions. Marler (2023) stated that coralloid roots mostly develop in soils with nutrient limitation and Coir may possibly supported the development of coralloid roots due to its value as a nutrient source. It was evident that the weekly-watered seedlings had significantly higher development of coralloid root compared to monthly and quarterly watered, suggesting that continuous soil moisture is an essential requirement for the development of coralloid roots. Nonetheless, this result may have been heavily influenced by the small sample size of seeds surviving long enough to develop coralloid roots in other substrates and watering regimes.

The findings of my study suggest that stomatal development is influenced by substrates and the frequency of watering. This may be decisive in improving drought resistance and seedlings' survival in *Encephalartos* restoration in the wild. The Coir had more stomatal density under infrequent watering and might support cycads under drought conditions. This study finding is consistent with numerous studies (Xu and Zhou, 2008; Driesen *et al.*, 2023). The higher stomatal density with more frequent watering may indicate that cycads develop their stomata according to water use efficiency under drought conditions, this could be further studied with emphasis on water use efficiency.

2.7 Conclusions

The results of this simulation study could potentially provide a practical approach to maximize seed survival and thus seedling establishment in the wild. The findings of this study revealed that sowing seeds 3cm deep is more effective than sowing on the surface. This is due to the fact that seeds on the surface are more exposed to harsh environmental conditions and other factors such as desiccation that could possibly limit seedling establishment. Numerous studies have identified that cycad seeds face the challenge of damage by rodents and animals in the wild (Donaldson, 2008; Yáñez-Espinosa *et al.*, 2014; Nadarajan *et al.*, 2018; Swart, 2019; Bezuidenhout, 2020). Therefore, sowing seeds at least 3cm deep would further protect the seeds from predation and damage by rodents and baboons. Furthermore, it would be essential to utilize Hydrogel or Coir as a substrate to improve cycad seed survival chances and thus seedling establishment, as both offers cycad seeds with optimum moisture conditions essential for seedling development. According to Londra *et al*. (2018) method, timing, and amount of irrigation water play an important role in defining a substrate as "ideal" or not. Therefore, the findings revealed the importance of understanding the interplay of the water availability and substrate for improving the establishment and resilience of cycad seedlings in the wild. This is crucial, as the information could aid in improving the success rate of cycad seeds in recovery projects by ensuring optimization of the first-stage growth.

2.8 Limitations and future studies

For this study, I used seeds from one species (*E. altensteinii)* and tested one sowing depth (3cm). That could potentially affect the results, as some species may respond differently to the similar conditions. Future studies could assess different sowing depths in order to make an informed decision as to which sowing depth is most recommended for optimum growing conditions for seeds. For this study, there was one water amount applied under different watering regimes. Testing different water amounts could provide more insight in understanding adequate water supply for cycad seeds and thus seedling establishment. Furthermore, Falquetto-Gomes *et al.* (2023) found that environmental factors such as light and CO_2 concentration influence plant’s stomatal density. Therefore, future studies could look at how these factors influence cycad seedling's stomatal density and thus water use efficiency in the wild.

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CHAPTER THREE: Effects of water-absorbing additives on growth and survival of transplanted juvenile cycads in a simulated drought experiment

Abstract

In situ restoration or recovery of cycad populations is difficult and has often been unsuccessful due to high seedling mortality caused by desiccation, particularly under drought conditions. Therefore, this study investigated the use of horticultural techniques to improve the growth and establishment of *E. altensteinii* juveniles grown under simulated drought conditions. To achieve this, methods to increase moisture retention and thus seedling survival were employed by adding water-absorbing compounds, specifically, Hydrogel and Coir into the growing media of juvenile transplanted cycads. In the greenhouse, a 4x3 factorial experiment was employed, where *E. altensteinii* seedlings were grown in four different substrates and were subjected to three watering regimes. The results showed that there was a significant interaction between substrates and watering regimes ($p < 0.05$), indicating that the effectiveness of a substrate is tied to water availability. Nonetheless, neither substrate nor watering regime alone significantly affected seedlings' survival ($p > 0.05$), which is in contrary with the hypothesis that the addition of water-retaining compounds will improve seedling survival. Among the substrates tested. Both Hydrogel and Coir significantly influenced seedlings' stomatal density, as stomatal density dropped with watering frequency on plants cultivated on both Hydrogel and Coir. The decrease in stomatal density under water scarcity conditions aid in supporting the plant to minimize drought stress. The findings of this study provide practical implications for developing recovery protocols to enhance seedling survival and growth *in situ*, especially in drought-prone areas. In conclusion, the study suggests that Hydrogel is most optimal for drought-prone areas where water is limited, while Coir works best in managed irrigation environments with water sources, providing practical guidelines for the recovery of cycad species in the wild.

3.1. Introduction

Cycads have adapted to various habitats (Webb and Osborne, 1989; Donaldson, 2003). However, cycad growth and survival is affected by numerous environmental factors, one of which is drought induced desiccation (Raimondo & Donaldson, 2003), particularly in the juvenile stage. According to Miller *et al.*, (2020), desiccation in young seedlings is a period where seedlings experience dehydration stress. This often occurs when seedlings lose more water through transpiration and evaporation than they absorb from their environment (Santos *et al.,* 2022). Furthermore, cycads are prone to desiccation, particularly at the juvenile stage of growth and development (Stevenson, 1980), which makes restoration a challenge. In *Encephalartos* species (*E. cycadifolius),* it was reported that seed and seedling survival is low due to fire, however, desiccation was also one of the factors impacting seedling survival (Raimondo & Donaldson, 2003). Moreover, seedling mortality due to desiccation was problematic during the reintroduction of the cycad *Dioon edule* (Vovides *et al.*, 2010). This indicates that desiccation is one of the limiting factors in cycad seedlings development in the wild. Despite the decline of cycads in the wild (Okubamichael *et al.*, 2016; Mankga and Yessoufou, 2017), there are limited published protocols currently in place to guide the recovery of cycad species in the wild. Employing horticultural techniques offers a possible way to overcome desiccation and improve the survival of nursery grown *Encephalartos* juveniles when they are transplanted into the wild. Such treatments could include the addition of water-absorbing compounds into the growing media to improve moisture availability and soil water holding capacity (WHC). Improving soil WHC could provide a solution in overcoming plant water stress under water scarcity conditions (Abdallah, 2019), thereby improving seedling development and survival (Farrell *et al.*, 2013) in the wild.

Soil water holding capacity is defined as the amount of water that a given substrate can hold for plant use (Zhang *et al.*, 2021). Substrates with insufficient water-holding capacity result in plant desiccation or require frequent watering, while substrates with high water-holding capacity require less watering (Mahangade & Butala, 2023; Zahao *et al.,* 2022). In addition to water-holding capacity, it is known that cycads require well-drained soils with adequate moisture (Whitelock, 2002). According to Driesen *et al.,* (2023), water movement is regulated by stomata present in leaves. The total number of stomata present in a leaf area is referred to as stomatal density (Lawson and Blatt, 2014), and according to Hasanuzzaman *et al.* (2023), stomata protect plants against desiccation by minimizing water loss, particularly during drought period. The reduction in stomatal density is one of the mechanisms for plants in response to drought tolerance to minimize water loss (Bertolino *et al*., 2019; Caine *et al.*, 2019). Therefore, the inclusion of water-retaining compounds for cycad recovery could potentially aid in improving cycad juveniles adaptability to drought conditions by influencing stomatal density under water scarcity.

Numerous types of water-retaining compounds and substrates, such as polymers, peat, coir, perlite, and vermiculite can potentially improve substrates water availability (Xu *et al.*, 2023; Malik *et al.*, 2022;Wang *et al.*, 2021; Farrell *et al.*, 2013). For this study, Hydrogel and Coir have been selected for the following reasons. Firstly, Hydrogel is often used to improve substrate WHC and holds a significant amount of water during a dry period (Montesano *et al.*, 2015; Ullah *et al.*, 2015; Madduma-Bandarage, 2020). The use of hydrogel in plantings has been proven to lower irrigation frequency (Montesano *et al.*, 2015; Kumar *et al.*, 2020) and also improve seedling growth and survival chances in cultivation (Narjary, 2014; Ullah *et al.*, 2015) and in restoration (Crous, 2016; de Almeida *et al.,* 2022). Secondly, the use of soilless substrates is also effective in improving WHC (Barrett *et al.*, 2016; Kazemi and Mohorko, 2017). Coir is a soilless substrate made from coconut fibre (Abad *et al.*, 2005; Gougoulias *et al.*, 2017) and according to Kukal *et al.* (2012), coir is excellent in retaining moisture, as it has high water retention ability (Rajan and Abraham, 2008). Plants grown in containers need good water retention and drainage for optimal growth (Ingram *et al.*, 1993; Hentges *et al.*, 2019). Therefore, using substrates with improved water retention could be beneficial for the cycads juveniles in the greenhouse experiment.

This chapter focuses on evaluating the effects of various water-absorbing additives on the growth and survival of transplanted cycad juveniles under simulated drought conditions. Here I aim to evaluate whether adding Coir and Hydrogel to the growing medium surrounding nursery-grown cycads improves the moisture retention period and secondly, whether the properties of these additives promote plant growth and survival under conditions of reduced water availability.

The hypothesis is that the addition of Coir and Hydrogel to the growing media will significantly improve (i) substrate moisture retention and, (ii) juvenile cycads growth, establishment, stomatal density and survival. This study is intended to contribute to the development of protocols to guide future reintroduction and recovery programs where seedlings are planted out to address declines in wild populations.

3.2. Methods and Materials

Ethical considerations were taken into consideration in conducting this research. Ethical approval was obtained from the Ethics committee at Cape Peninsula University of Technology (**Ref No. 214113833/2022)** before commencing the study, ensuring that all aspects of the research adhere to ethical guidance.

3.2.1 Study species

Given that it is difficult to obtain propagules from highly threatened cycad species, the more common *Encephalartos altensteinii* was used. The rationale for using this species is that *E. altensteinii* shares biological and ecological similarities with some of the threatened cycad species, such as growth form and environmental preference. For example, it has a similar growth form to *E. inopinus* (CR), *E. natalensis* (VU) and *E. woodii* (EW), and occurs in the same environment as *E. latifrons* (CR). *Encephalartos altensteinii* is listed as Vulnerable (VU) in the IUCN RedList of Threatened Species (IUCN, 2022) and occurs from the Bushman's River in the Eastern Cape to near the border with Kwa-Zulu Natal (Bösenberg and Donaldson, 2020).

Boyd (1995) recommended that plants to be used for cycad reintroduction should be 5 years or older. For this study, potted juvenile cycads of *E. altensteinii* (8 years old) were obtained from the cycad nursery at Kirstenbosch National Botanical Gardens and were then repotted in pots with different substrates, to simulate planting out in the field. A permit for obtaining the plants was received from Kirstenbosch National Botanical Garden cycad specialist (permit number: S65803).

3.2.2 Study area

All experiments were carried out at the research greenhouses of the South African National Biodiversity Institute, Kirstenbosch National Botanical Gardens, Cape Town, South Africa. The greenhouse is located 33°59'06.7"S, 18°26'09.2"E and comprises a clear polycarbonate tunnel (20m x 10m), covered with 40% shade net (grey), with slightly raised sides, an extractor fan and tornado turbine roof ventilator which allows for passive cooling. The temperature within the greenhouse was not controlled, but rather remained at ambient levels.

3.2.3 Greenhouse: seedling growth and survival under simulated drought conditions

The greenhouse experiment was designed to examine seedling growth and survival under different simulated rainfall regimes and using different substrates. For this experiment, plants grown in 5L-sized bags were bare-rooted, and the length of the longest leaf, stem diameter, and fresh mass were measured. The plants were then transferred into 120mm x 300mm Ellepot biodegradable bags filled with standard cycad potting substrate. A period of 5 weeks was allocated for acclimatization within the nursery. Following this acclimation period, each plant, while still in the biodegradable bag, was planted into a 35L BATO pot. The substrate in the BATO pot comprised the substrate treatment. The 35L pots were chosen because they allow the addition of approximately 20L of substrate around the plant, thus allowing free seedling root growth. The experiments comprised a complete randomized 4x3 design, with four substrates and three watering regimes. The substrate treatments comprised the standard Kirstenbosch mix (KB) as the Control, Coir, KB + Coir, and KB + Hydrogel. The rationale for using Coir separately, not Hydrogel, was that Coir is normally used alone as it possesses physical properties contributing to water retention. Hydrogel, on the other hand, is normally used as an additive with other substrates to aid in enhancing moisture retention. The four substrate treatments comprised: (i), the standard KB, which is typically used in the cycad nursery and represents the control. The KB mix consists of equal parts fine bark, sand, compost, and loam soil, mixed with Atlantic Bio Ganic bounce-back (slow-release fertilizer) and bonemeal to induce root development; (ii) Coir: a fine grade 6mm x 5kg block, buffered to prevent the build-up of salts that may cause nutrient deficiency and imbalance in the substrate; (iii) Coir + KB mix (KBC): which consists of equal parts (one part) of Coir and KB mix; and (iv) Hydrogel treatment (KBH): which consists of 90g Hydrogel mixed with 15 kg of KB mix, as recommended by Kumar *et al*., (2020). To make the substrate mixtures, a 120L concrete mixer was used to ensure thorough mixing of all the components for each substrate mixed. Furthermore, to minimize the loss of the substrate through drainage holes, a shade cloth layer was laid at the base of each pot prior to pouring in the substrate.

The three watering regimes were: once a week, once a month, and once every 3 months, to test the effects of the substrates under different levels of water availability. For each watering event, plants were hand-watered using a 10L watering can, each plant receiving 20L of water to ensure that the substrates were saturated, particularly under the less frequent watering regimes. Watering once a week is considered optimal for cycad seedlings' growth and survival (pers comm, Xaba), therefore it was used as the standard regime compared to the other treatments. *E. altensteinii* occurs in the Eastern Cape, extending to the border with KwaZulu-Natal, South Africa (Bösenberg and Donaldson, 2020). The species occurs from near sea level up to 600 masl (metres above sea level) and according to Zwane (2023), the region receives an annual rainfall between 550–700mm. Therefore, the monthly and 3-months watering regimes were designed as treatments, represents infrequent rain, especially in summer, with the 3-months being more extreme level of infrequent rainfall.

In the greenhouse, substrate moisture content, plant growth and physiological responses to the addition of moisture-retaining compounds were measured. Substrate moisture content was measured close to the root ball in the original potting bag using a handheld moisture meter (Delta-t Devices, PR2x). Plant growth was determined from measurements of leaf emergence, maximum leaf length, stem diameter, root diameter, root volume, and differences in fresh and dry mass. The stomatal density was measured as a potential indicator of plant responses to avoid water loss. A total of 168 juveniles of *E. altensteinii* were used for this experiment, comprising 14 plants for each of the 12 experimental treatments.

3.2.4 Substrates moisture content retention ability under different watering regimes

Substrates' moisture content readings were measured weekly close to the root ball in the original potting bag using a handheld moisture meter (Delta-T Devices™ , PR2). The moisture meter probe comprises four sensors positioned at distinct substrate depths, and for this study, moisture content readings were taken at 400mm below the soil surface, as this region was the deepest and closest to the root ball. The results were similar to those obtained from chapter 2 above.

3.2.5 Impact of different treatments on survival of juvenile cycads

The plant survival in the juvenile cycads was monitored weekly, and dead plants were recorded when observed across all treatments. Plants which showed some green tissues (e.g. green leaves and healthy stems) were considered to have survived, and all other plants which showed no living tissue (e.g. brown leaves, dry stems and roots), were considered as dead plants.

3.2.6 Effect of different treatments on growth and development of juvenile cycads

The plants that survived were harvested and different post-harvest measurements were obtained and recorded. The length of the longest newly emerged leaf that had hardened-off was measured. The measurements were obtained from the tip of the rachis to the end of the petiole. Using a Vernier digital caliper, the stem diameter was obtained at the widest section and 1cm below the leaf base as a standard measure.

Post-harvest fresh mass, root, and stem diameter were compared with pre-planting measurements to determine plant growth in response to the treatments. Furthermore, the newly emerged leaves across different treatments were observed and recorded weekly. New leaves were identified when a petiole and rachis with opposite to alternate pairs of leaflets were observed.

To obtain post-treatment fresh mass, the seedlings were separated into aboveground parts (leaves & stem) and belowground parts (roots). The mass was obtained using an Adam Nimbus balance. The parts were then put into Glad medium oven bags and dried in an oven at 100ºC for 72 hours (it was predetermined that they were thoroughly dry after checking at 24 and 48 hours respectively). The dried plants were then separately weighed to obtain dry mass.

Root diameter was measured as an indicator of growth. Using a Vernier digital caliper, root diameter was measured at the widest section of the primary root. The root volume was obtained by placing the root into a measuring glass beaker partially filled with water and measuring the displacement volume. Coralloid roots are found in many cycad species and are formed by active growth from the apical meristems. For this study, plants with coralloid roots were identified and recorded to compare the effect of different treatments on the growth and development of coralloid roots.

3.2.7 Statical analysis

A mixed effect linear model was fitted to the soil moisture content for substrate and watering regime data using package ‘lme4’ (Bates *et al.,* 2015) for both seeds and seedlings. The R^2 value for the model was calculated using the package ‘MuMIn’ (Bartoń, 2023). The main effects for the analysis of variance were calculated using package ‘car’ (Fox and Weisberg, 2019). The post-hoc analysis for the two-way interaction between substrate and watering regime was calculated using package ‘emmeans’ (Lenth, 2024). A logistic regression model was fitted to the survival data to investigate how substrate and watering regime influenced cycad seedling survival. A generalized linear model (GLM) was fitted to the leaf emergence, and stomatal density for substrate and watering regime. The best fit model for the GLM included an interaction between the substrate and watering regime for leaf emergence and stomatal density. Thereafter a post hoc Tukey test, using the package ‘multcomp’ (Hothorn *et al*., 2008) was run to see where the significant differences were between groups. All statistical analyses were performed using R statistics software v 4.4.0 (R Core Team 2024) and all graphics were generated using packages ‘ggplot2’ (Wickham 2016), ‘ggpubr (Kassambra 2023) and ‘ggsignif’ (Ahlmann-Eltze and Patil 2021).

3.3 Results

3.3.1 Impact of different treatments on survival of juvenile cycads

The control substrate had high survival rates across all watering regimes, with the highest survival (100%) under the weekly and monthly watering regimes and a slight decline under the three-month (78.57%). But neither substrate ($\chi^2 = 5.66$, df = 3, p = 0.129) nor watering regime ($\chi^2 = 3.03$, df = 2, p = 0.220) significantly influenced seedling survival. The ANOVA showed that there was a significant interaction between substrate and watering regime ($\chi^2 = 13.31$, df = 6, p = 0.038). However, the interaction was not big enough to be reflected in the logistic regression output (Table S10, p > 0.05) or a pairwise Tukey comparison.

3.3.2 Effect of different treatments on juvenile cycad's growth and development

a) Leaf emergence

The watering regime significantly influenced the number of leaves ($\chi^2 = 10.35$, df = 2, p = 0.006), whereas the substrate had no significant influence on the number of leaves ($\chi^2 = 7.46$, df = 3, p = 0.059). The quarterly watering regime resulted in significantly fewer leaves compared to the weekly and monthly regimes for all treatments (Table 3.1, p = 0.016, p = 0.011; Fig 3.1). There was no significant difference in leaf emergence between weekly and monthly watering regimes (Table 3.1, p = 0.994; Fig 3.1).

Table 3.1: Post hoc Tukey comparison on the effect of the watering regime on leaf emergence (using package "multcomp") in juvenile cycads.

Comparison	Estimate	Standard Error	z value	p-value
Monthly – Weekly == 0	0.01	0.11	0.11	0.994
Quarterly – Weekly == 0	-0.35	0.13	-2.75	0.016
Quarterly – Monthly == 0	-0.37	0.13	-2.88	0.011

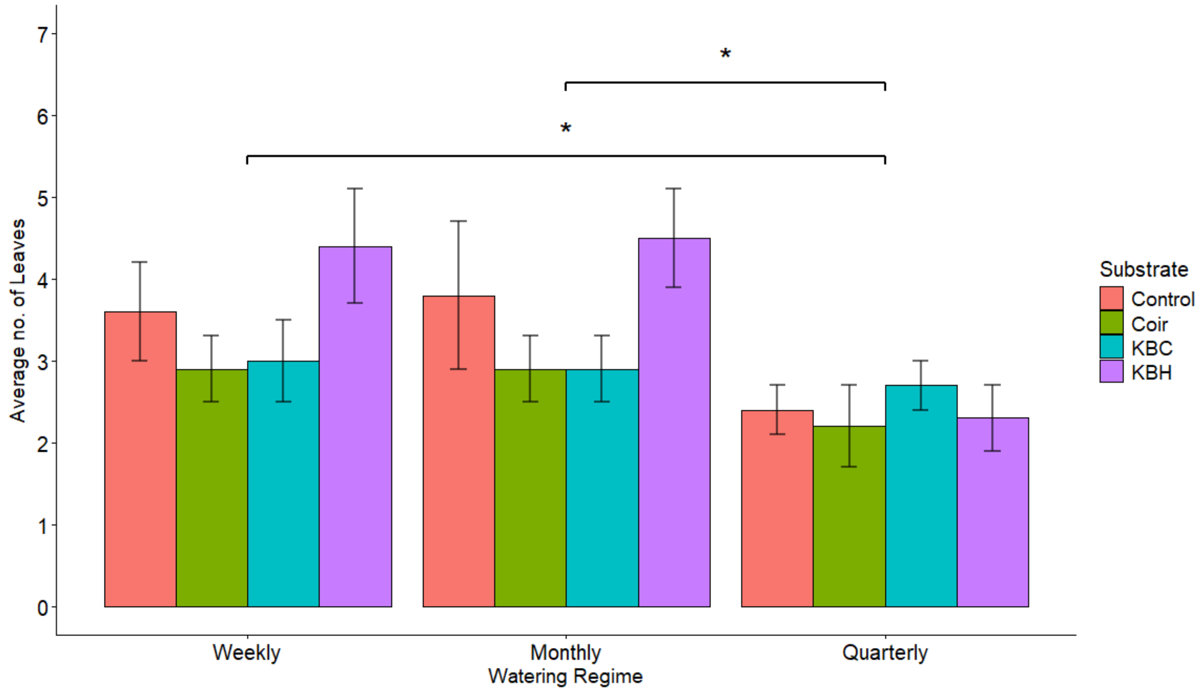

Figure 3.10: Leaf emergence in *E. altensteinii* seedlings under different substrates and watering regimes in the greenhouse, n=14 per treatment. Error bars represent the standard error of the mean. The level of significance difference in watering regime is indicated by *

b) Changes in fresh mass

The best-fit model included substrate and watering regime but no interaction between the two factors. There was no significant difference in fresh mass between the substrates ($F = 1.18$, $df = 3$, $df_{residual} = 125$, $p = 0.322$). The watering regime did show a significant difference with the mass of plants watered quarterly being significantly lower than both the weekly and monthly watering regimes (Table 3.2, $p < 0.05$; Fig 3.2).

For stem diameter the best fit model included substrate and watering regime. There was no interaction between the two factors and neither substrate ($F = 1.18$, $df = 3$, $df_{residual} = 125$, $p = 0.322$) nor watering regime ($F = 3.06$, $df = 2$, $df_{residual} = 123$, $p = 0.051$) was significant. There was also no significant difference in root diameter between the substrates ($F = 1.53$, $df = 3$, $df_{residual} = 125$, $p = 0.210$; Fig 3.3).

Table 3.2: Post hoc analysis for the GLM of juvenile cycads fresh mass comparisons between pre-planting and post-harvest under different watering regimes.

Treatments	**estimate**	**Standard Error**	**df**	**t.ratio**	**p.value**

Weekly - Monthly	2.12	0.88	123	2.40	0.046
Weekly - Quarterly	5.07	0.95	123	5.35	< 0.001
Monthly - Quarterly	2.95	0.96	123	3.09	0.007

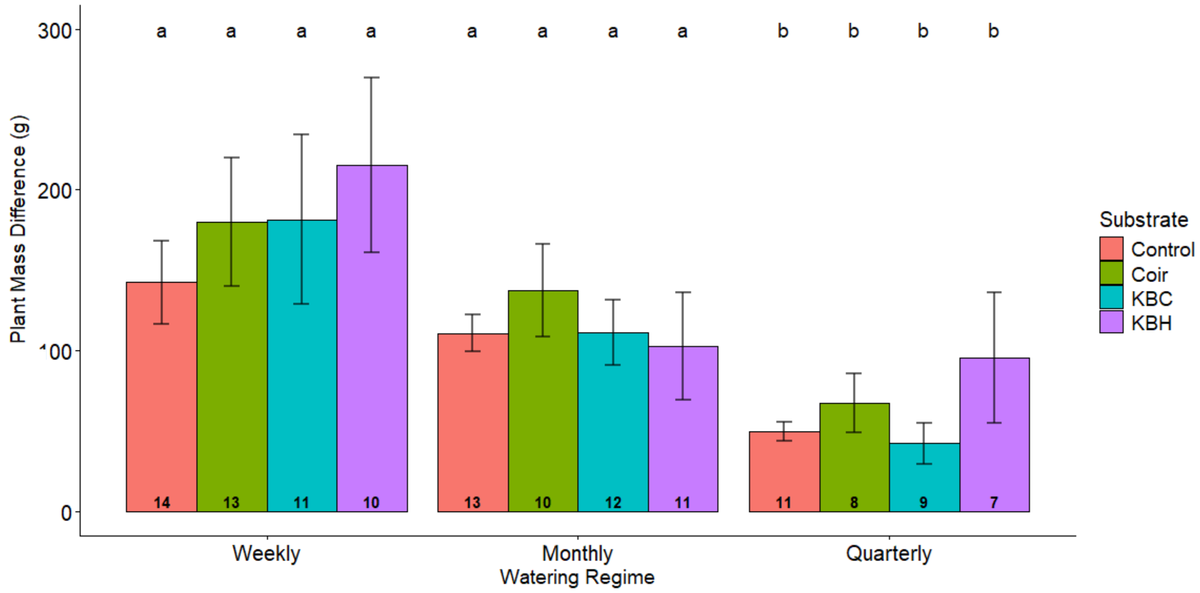

Figure 3.11: Net gain in fresh mass (g) of *Encephalartos* seedlings under different substrate and watering regimes. The plant mass difference is from before and after the experiment mass. The bars represent the mean and standard error for each treatment and sample size is represented by the number inside the bars. Letters above the bars designate whether there is a significant difference between bars (bars that have the same letter are not significantly different).

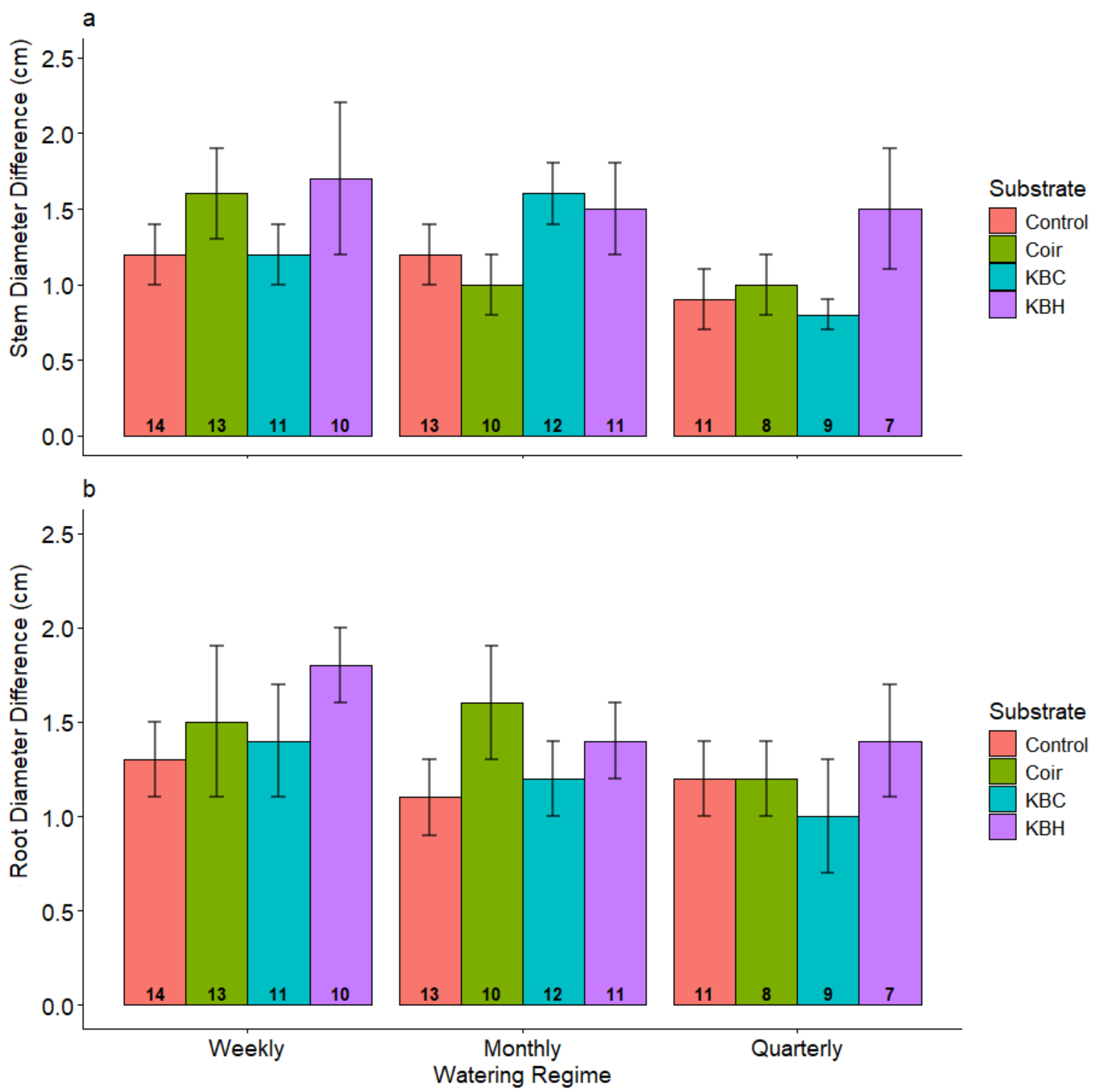

Figure 3.12: Net change in stem (a) and root (b) diameter for *E. altensteinii* seedlings subjected to different substrates and watering regimes. The data represent the mean and standard error for each of the 12 treatments and sample size is provided inside each bar.

c) Difference in fresh and dry mass

The best-fit model included substrate and watering regime as important variables that influenced the water content but showed no interaction between the two factors. Substrate significantly influenced moisture content in the leaves ($F = 2.99$, $df = 3$, $df_{residual} = 133$, $p = 0.033$) and the stems ($F = 4.32$, $df = 3$, $df_{residual} = 133$, $p = 0.006$), with the KBH yielding higher moisture content in plant leaves and stems than the control substrate across all watering regimes (Table 3.3, $p = 0.041$; Fig 3.4). The ANOVA showed that both substrates ($F = 1.27$, $df = 3$,

$df_{residual}$ = 133, p = 0.287) and the watering regime (F = 2.26, df = 2, $df_{residual}$ = 131, p = 0.108) did not significantly influence the water content in roots.

Table 3.3: Post hoc results for moisture content in leaves, df represents the degrees of freedom for each test.

contrast	Coefficient Estimate	Standard Error	df	t.ratio	p.value
Control - Coir	-0.01	0.15	131	-0.04	1.000
Control - KBC	-0.20	0.14	131	-1.38	0.517
Control - KBH	-0.39	0.14	131	-2.68	0.041
Coir - KBC	-0.19	0.15	131	-1.27	0.581
Coir - KBH	-0.38	0.15	131	-2.51	0.063
KBC - KBH	-0.19	0.15	131	-1.25	0.595

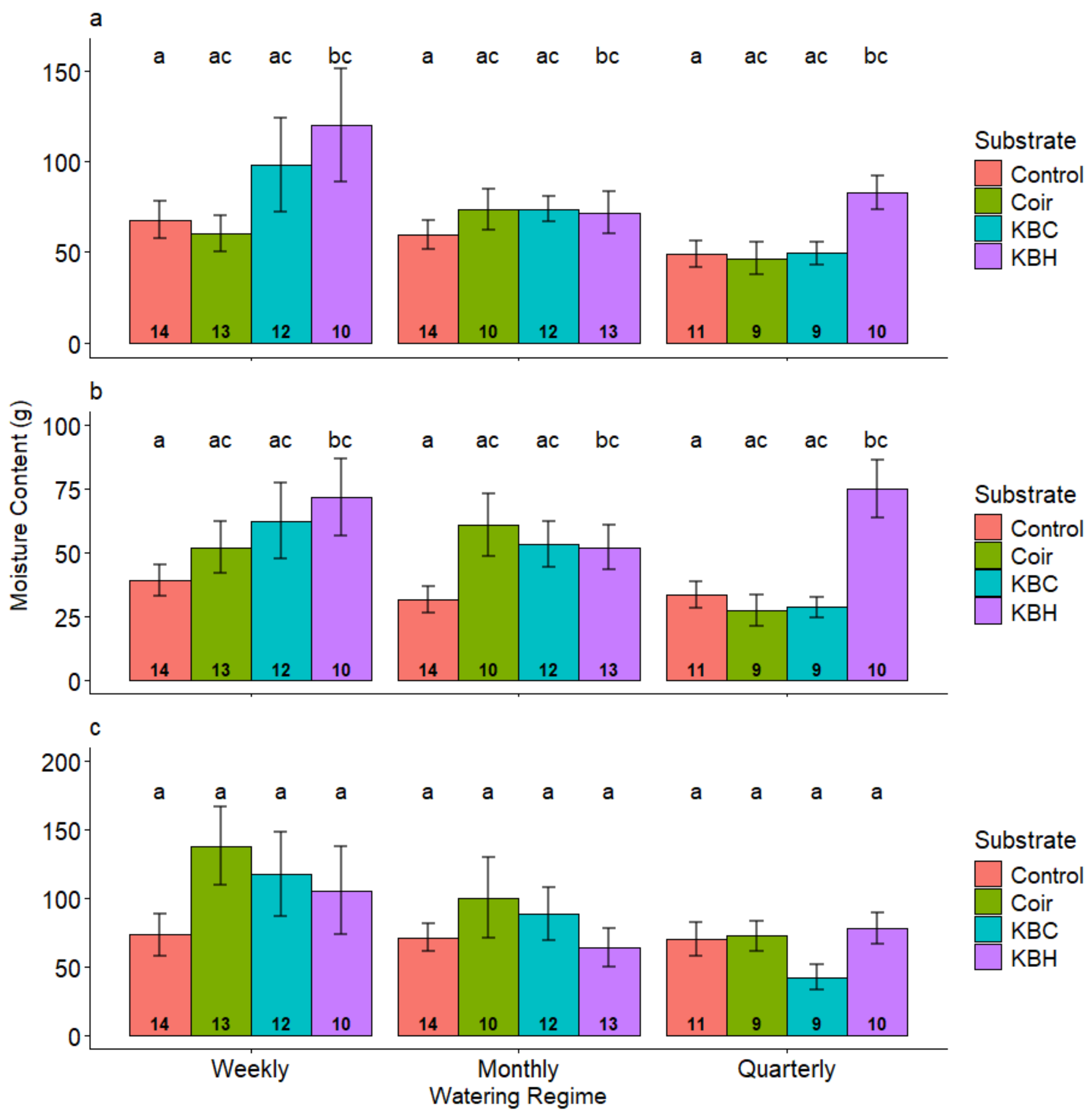

Figure 3.13: Difference in fresh and dry mass of different plant parts in E. altensteinii seedlings. a) represent mean leaf moisture content, b) represent mean stem moisture content, and c) represent root moisture content under different substrates and watering regimes in the greenhouse. The error bars represent the standard error. Different letters represent whether the substrate is significantly different (bars that have the same letter are not significantly different).

d) Root volume

The best fit model included watering regime but removed substrate as an important variable that influenced the root volume. With no interaction between the two. There was no significant difference in the root volume between the watering regimes ($F = 0.07$, $df = 2$, $df_{residual} = 134$, $p = 0.935$; Fig 3.5).

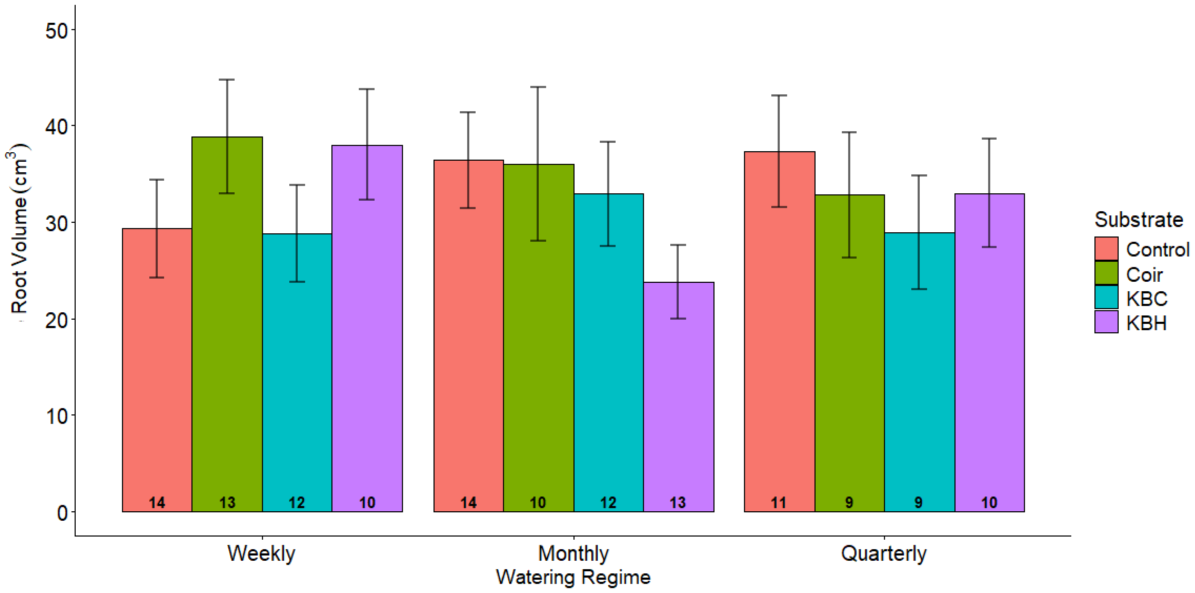

Figure 3.14: Mean of root volume (cm³) in *Encephalartos altensteinii* seedlings under different substrates and watering regimes. The sample size is represented inside the bars and the error bars represent the standard error of the mean.

e) Coralloid roots

The results showed that the watering regime significantly influenced the formation of coralloid roots ($F = 7.49$, $df = 2$, $df_{residual} = 125$, $p = 0.001$), but the substrate did not ($F = 1.45$, $df = 3$, $df_{residual} = 125$, $p = 0.725$). However, there was a significant interaction between the watering regime and substrate ($F = 2.26$, $df = 6$, $df_{residual} = 125$, $p = 0.042$). Under the weekly watering the plants developed significantly more coralloid roots than in the monthly for the control (Table S11, $p = 0.039$; Fig. 3.6) and quarterly for both Coir and KBH substrates (Table S11, $p < 0.05$; Fig. 3.6). This shows that Coir and KBH substrates would be more likely to develop coralloid roots than control under moderate water availability, but not under longer intervals without water (Fig. 3.6).

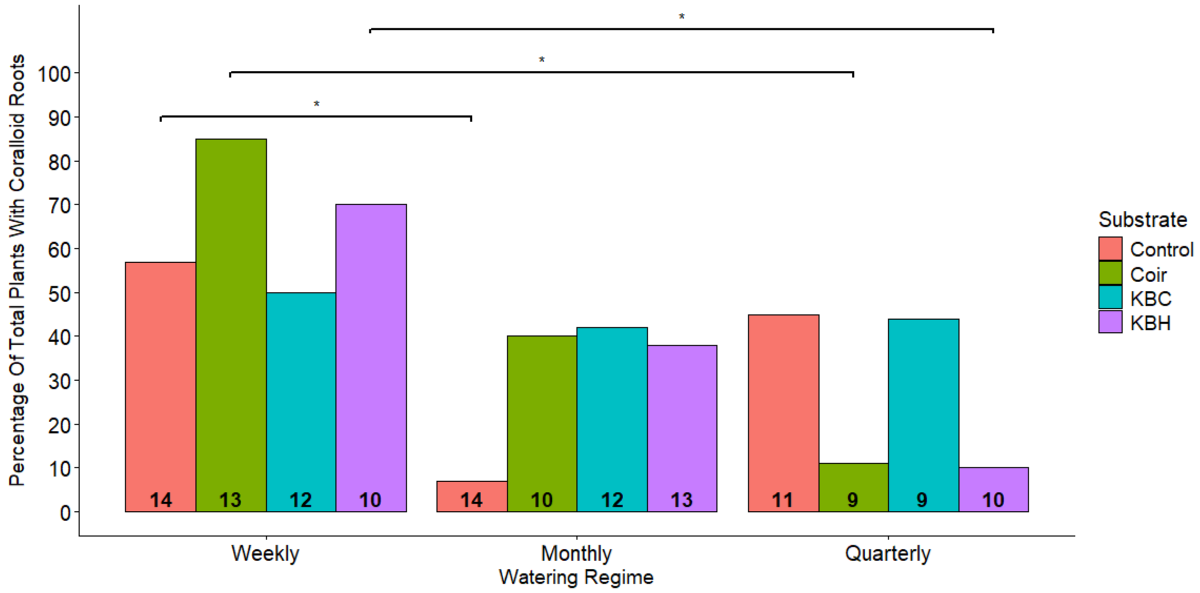

Figure 3.15: The total number of plants (%) with coralloid roots in *Encephalartos altensteinii* seedlings under different substrates and watering regimes. Sample size represented inside the bars. The level of significant difference in watering regime by substrate is indicated by *

3.3.3 Effect of different treatments on the stomatal density of cycad juveniles

The model showed that both substrate and watering regime significantly influenced stomatal density (Table 3.4, $p < 0.001$). It also showed a significant interaction between substrate and watering regime (Table 3.4, $p < 0.001$).

<u>Watering regime</u>

Under the weekly watering regime, all three substrate treatments had a significantly higher stomatal density than the control (Table 3.5, $p \leq 0.001$). For the quarterly watering regime, KBH had significantly more stomatal density than the control substrate (Table 3.5, $p = 0.033$). This therefore shows that plants grown in KBH maintain higher levels of stomatal density even under reduced watering frequency (Fig. 3.7).

<u>Substrates</u>

Plants grown in the control substrate had significantly higher stomatal density under the monthly watering regime as compared to the weekly and quarterly watering regimes (Table S12, $p < 0.001$). On the other side, Coir, KBC, and KBH substrates had significantly higher stomatal density under the weekly watering regime compared to the quarterly watering regime (Table S12, $p < 0.05$; Fig 3.7).

Table 3.4: Analysis of Variance (ANOVA) results for the Generalized Linear Model of stomatal density in juvenile cycads under different substrate and watering regime.

	Degrees of freedom	Deviance	Residual Degrees of freedom	Residual Deviance	F	p-value
NULL			119	10887.70		
Substrate	3	1004.57	116	9883.13	7.09	$2.16e^{-04}$
Regime	2	2313.05	114	7570.08	24.47	$1.72e^{-09}$
Substrate:Regime	6	2466.28	108	5103.80	8.70	$1.00e^{-07}$

Table 3.5: Post hoc Tukey pairwise comparison for the GLM of stomatal density in juvenile cycads under different substrate and watering regimes, showing the comparisons between substrates for each watering regime.

Contrast	Estimate	Standard Error	degrees of freedom	t.ratio	p.value
Weekly					
Control - Coir	-15.90	3.07	108	-5.17	< 0.001
Control - KBC	-23.50	3.07	108	-7.64	< 0.001
Control - KBH	-16.10	3.07	108	-5.24	< 0.001
Coir - KBC	-7.60	3.07	108	-2.47	0.070
Coir - KBH	-0.20	3.07	108	-0.07	1.000
KBC - KBH	7.40	3.07	108	2.41	0.082
Monthly					
Control - Coir	4.50	3.07	108	1.46	0.463
Control - KBC	4.30	3.07	108	1.40	0.503
Control - KBH	4.70	3.07	108	1.53	0.424
Coir - KBC	-0.20	3.07	108	-0.07	1.000
Coir - KBH	0.20	3.07	108	0.07	1.000
KBC - KBH	0.40	3.07	108	0.13	0.999

Quarterly					
Control - Coir	-2.90	3.07	108	-0.94	0.782
Control - KBC	-3.20	3.07	108	-1.04	0.726
Control - KBH	-8.50	3.07	108	-2.77	0.033
Coir - KBC	-0.30	3.07	108	-0.10	1.000
Coir - KBH	-5.60	3.07	108	-1.82	0.269
KBC - KBH	-5.30	3.07	108	-1.72	0.316

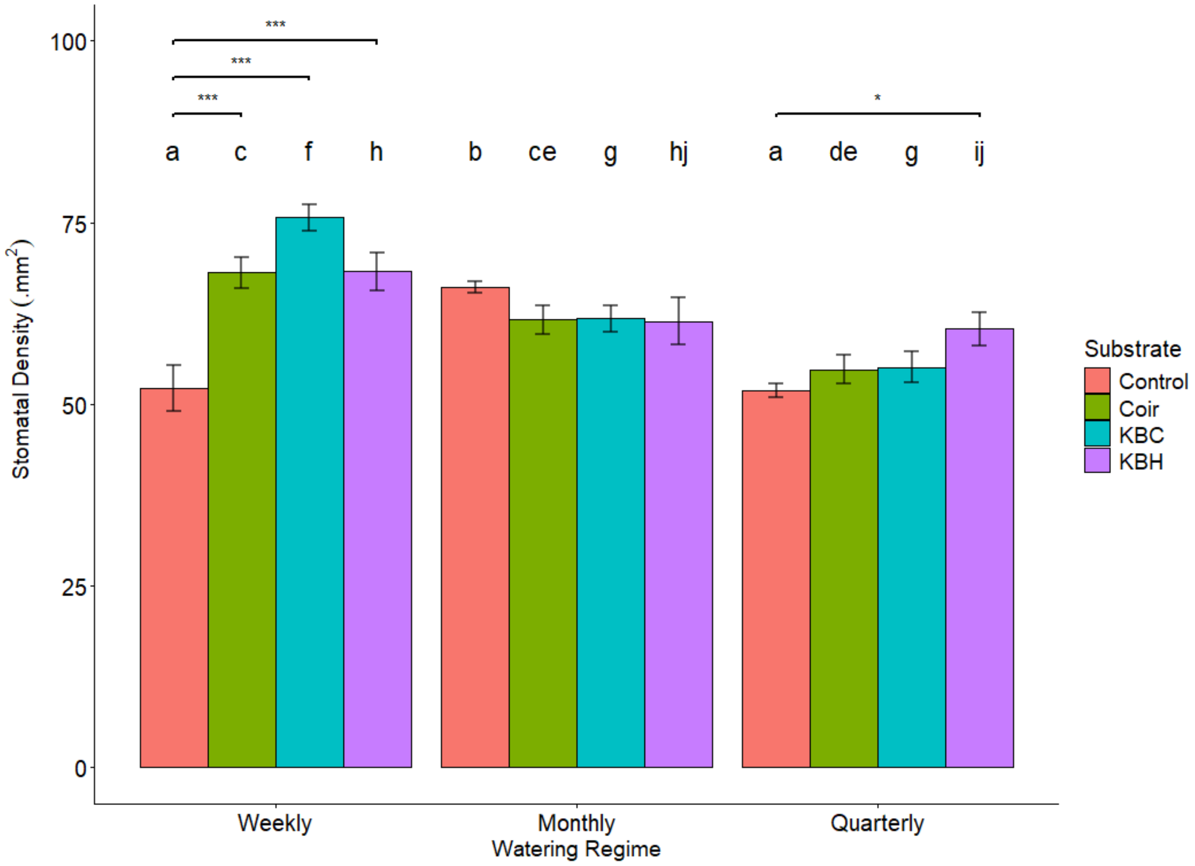

Figure 3.16: Stomatal density in *E. altensteinii* seedlings grown under different substrate and watering regimes in the greenhouse. The data represents the mean for 10 replicates per treatment and the error bars represent the standard error of the mean. The lines and stars above the bars represent significant differences between the substrates for each watering regime. The letters represent whether plants grown under different watering regimes are significantly different for each substrate (bars that have the same letter in each substrate are not significantly different).

3.5 Discussion

3.5.1 Impact of different treatments on survival of juvenile cycads

Results from this study revealed that neither substrate nor watering frequency in the greenhouse affected survival in transplanted cycads. This result was not expected, given that substrate properties such as WHC often play a critical role in seedling survival (Gavrilescu, 2021). Similar results were reported in a study by (Silva *et al.*, 2015), where none of the tested substrates significantly influenced plant survival, including Hydrogel. In my study, the significant interaction between substrate and water availability shows that seedling survival does not rely on the substrate or how often plants get watered alone, but rather on how the two factors interplay. In other words, certain substrates may be effective under certain water availability conditions. For instance, the control substrate had high survival under the more frequent watering, indicating the effectiveness of substrate and watering interaction. This was similarly reported by Dutra *et al.* 2018, wherein the interaction between both substrates and frequency was significant for seedling survival in *Luehea divaricata*.

Water scarcity for plants results in extended periods of drought stress which is likely to cause desiccation and negatively impact seedling survival (Engelbrecht *et al.*, 2005; Martinez-Vilalta *et al.*, 2019). Therefore, to optimize cycad seedling survival, the findings implies that the effectiveness of substrate choice could be dependent on the water availability of the restoration site. For instance, in environments with low water availability, adding water-retaining additives that improve moisture retention ability (e.g. Hydrogel and Coir) into the substrates could be utilized to aid in seedling survival by optimizing moisture retention.

3.5.2 Effect of different treatments on juvenile cycad's growth and development

The study's findings revealed that only watering frequency affected new leaf emergence in cycad seedlings, while substrate did not. This is consistent with previous studies where adequate water supply attained through proper irrigation intervals proved to be instrumental in the emergence of new leaves of species including *Cycas revoluta* (Sorour 2021), *Amaranthus sp.*, and *Bidens pilosa* (Sinasson and Shackleton, 2023). In particular, the three-month watering regime had significantly lower leaf emergence than the weekly and monthly watering regimes, which agrees with Mukhtar *et al.*, (2016), who reported that infrequent watering can lead to water stress, thus negatively affecting seedlings' vegetative growth. Although the alternative substrates improved moisture retention, the improvement was not sufficient to result in a meaningful difference in growth parameters.

This study further found that seedling fresh mass (pre and post planting) was only influenced by watering regimes, not by substrates, which raises a question whether the lack of significant differences between substrates indicate that the seedlings are relatively tolerant of the different types of substrates; this needs to be further studied. McElrone *et al.,* (2013) reported that water is one of the most important factors influencing plant growth, perhaps, the findings could suggest that water availability is more of a critical factor in seedlings' fresh mass increase than substrates. The findings of this study are consistent with Gutezeit (2006), where watering significantly influenced increase in seedling fresh mass. However, this study's findings are in contrast with Dede *et al.,* (2006), where only substrates significantly influenced seedling mass, not watering. The substrates and watering did not influence stem and root growth, this could suggest that the response may vary depending on the species and treatments utilized, as other factors not assessed in this study, such as light or even temperature, could potentially influence the stem and root growth of *E. altensteinii* juveniles. Furthermore, results for differences in fresh and dry mass on above and below-ground plant parts showed that substrate influenced the water content of above parts (leaves and stems), not below-ground parts (i.e. roots). The higher water content obtained from seedlings planted in KBH compared to those planted in control across all watering regimes, suggests that Hydrogel should be most preferred for cycad species recovery.

The results suggest that the development of the coralloid roots in *Encephalartos altensteinii* seedlings is dependent on water availability and not on the type of substrate alone. However, the significant interaction between substrates and watering regimes indicated that seedlings grown under frequent watering in substrates such as Coir and Hydrogel promote the formation of coralloid roots. This could suggest that the symbiotic relationship with cyanobacteria is more effective under good moisture availability, which facilitates either the development of roots or the colonization of roots by cyanobacteria. According to Chang *et al*., (2019), in a symbiotic relationship, cyanobacteria fix nitrogen for their hosts, and this is no different in cycads (Nicholls and Norstog, 1997). The nitrogen fixed becomes important to support cycad survival under harsh environmental conditions (Wang *et al*., 2023; Nicholls and Norstog, 1997).

The findings of this study suggest that the addition of Hydrogels to soils provides the best outcomes under the conditions tested. These findings could assist in improving seedling growth and development on cycad recovery.

3.5.3 Effect of different treatments on the stomatal density of cycad juveniles

This study showed that the watering frequency and substrates are critical factors influencing the physiological response of cycad juveniles, in terms of stomatal density. Compared with the control substrate, the increased stomatal density exhibited by plants growing in Coir, KBC, and KBH substrates suggests that these substrates offer better conditions for physiological functioning in cycad seedling. The results of this study are consistent with previous studies by Hasanuzzaman *et al.*, (2023) and Lavergne *et al.*, (2020), which demonstrated that substrate and water significantly increased stomatal density and overall seedling survival of the tested species. Previous studies have found that high stomatal density is often influenced by water availability (Doheny-Adams *et al.*, 2012; Roberston *et al.*, 2023), and this agrees with the findings of my study where higher stomatal density obtained on seedlings cultivated on Coir, KBC, and KBH, substrates with high water retention ability. This response could be an indication of the fact that cycad seedlings utilize the water absorbed from substrates with high moisture retention for physiological processes.

The findings of this study, therefore, demonstrate how substrate choice can influence physiological response in cycad seedlings. Utilizing substrates such as Coir or Hydrogel that enhance stomatal density could potentially result in improved growth and survival rates in cycad seedlings

3.6 Conclusions

The findings of this study on growth and development in juvenile cycad reveals a number of important findings that have implications for recovery efforts. To maximize moisture availability, the results suggest that appropriate selection of substrate selection based on water availability of the restoration site is critical for restoration, as this is essential for seedling establishment. The incorporation of the water-retaining additives into the substrate can further enhance moisture retention and thus influence seedling establishment, as previously observed in a study by Jialin et al., (2017). Therefore, given that Hydrogel had significantly high moisture content across all watering regimes indicates that it could be beneficial in improving moisture availability, particularly in habitats with less frequent rainfall. The same applies to seedling survival, the study findings suggest that while the choice of substrate and watering regime may not independently affect the survival of seedlings, their interaction could play a role. To improve survival, this means that recovery efforts should focus more on the combined effect of substrate and water availability than on them independently.

The different watering regimes significantly affected leaf emergence, with fewer leaves emerging under the infrequent watering (three months) compared with weekly and monthly frequencies. It is indicative that more water availability promotes leaf growth in juvenile cycads. Furthermore, the fresh mass of the quarterly watered seedlings was lower compared to all other watering regimes, indicating that infrequent watering may adversely affect biomass accumulation, thereby reducing the chances of juvenile cycads survival in arid environments. The study's findings further suggest that adequate and frequent watering might be important for the promotion of active growth in juvenile cycad, especially in recovery projects where the aim is not just survival, but also to ensure vigorous juvenile plant growth toward the establishment of a viable population. The incorporation of the water-retaining additives (Coir and Hydrogel) into the growing medium proved to play an important role a as KBC and KBH substrates showed some promise in extending water availability for juvenile cycad under drier conditions. In addition, under conditions of water stress, Coir and KBH should be utilized as both proven to be crucial in inducing coralloid roots for the long-term resilience of the juvenile cycads under water scarcity.

In summary, moderate water availability ensures seedling establishment and growth. However, under less water availability conditions, substrates such as KBH or Coir are conducive to water retention, stomatal density, and development of coralloid roots together, essential for establishment under unfavourable environmental conditions.

3.7 Limitations

For this study, I used three watering regimes with 20L of water only. I also used the KB mix for transplanting the cycad juveniles, as it is a standard mix that is optimal for cycad growth. Furthermore, this mix also contains nutrients and other components that may also influence WHC, survival and growth of cycads. Moreover, this study did not consider other influential parameters relating to humidity and nutrient availability among other factors that might impact the development and moisture retention of cycads.

3.8 Future studies

Long-term and field trials involving multiple cycad species would further validate the applicability of findings in the restoration of cycads over different climatic zones in the wild. This could provide further insight into the dynamics of seedling survival, leading to a better choice of substrate selection for restoring cycads in different climatic regions. Furthermore, in the wild, seedlings are often planted on different areas on the site with different light exposure, which could affect moisture availability. Therefore, future studies should assess the effect of light exposure on moisture availability. Finally, this could be more deeply explored by integrating physiological measurements, such as chlorophyll content, stomatal conductance or photosynthesis rates, to explain mechanisms underlying how substrate and water availability may influence cycad seedlings growth.

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CHAPTER FOUR: Conclusions and Recommendations

Optimizing species recovery techniques for cycad seeds and juveniles establishment

Ultimately, the aim was to identify methods that can be employed under field conditions to improve moisture availability and thus, the growth and survival of cycad seeds and juveniles through recovery conservation efforts. It was hypothesized that the addition of water-retaining compounds such as Coir and Hydrogel into the growing medium would improve moisture availability and increase seed and juvenile establishment and survival under drought conditions. Through different method chapters (2 & 3), the study examined the effectiveness of the applied treatments on seeds and juvenile cycads (*Encephalartos altensteinii*). The results proved the hypothesis correct under conditions in which both substrates and watering frequency showed a significant effect on the moisture content for both, seeds and juveniles. In contrast, for conditions related to the survival of cycad seedlings, it was not supported, and for new leaf emergence, it was partly supported, where only watering frequency proved to be a significant factor. However, for seeds, the survival proved the hypothesis correct, as all the factors substrates, watering regimes and sowing depth significantly influenced survival. These findings may aid the understanding of the relationship between substrate selection and water availability for cycad recovery *in situ*.

4.1 Implications for seed-based species recovery

In chapter 2, the study examined the effectiveness of the applied substrates and watering treatments on cycad seeds sown at two different sowing depths. I found that sowing seeds at least 3cm deep improved seed survival and seedling development. Moreover, survival and growth were generally better in Coir substrates. As a result, using Coir for seed-based recovery and planting seeds at 3cm depth could improve seed survival chances in the early stages of development under water scarce conditions. The early stages of seed development, begin with water uptake and end with embryo elongation (Rajjou *et al.*, 2012; Xaba, 2014), and are an important phase of the plant life cycle (Biswas *et al.*, 2018) which can determine the success or failure of the seed (Finch-Savage and Bassel, 2016). Therefore, utilizing substrates such as Coir with high moisture retention could influence longer term growth and survival.

4.2 Implications for juvenile-based species establishment

In chapter 3, the study examined the effectiveness of the substrates and watering regime on juvenile cycads (8 years old), and I found that, to maximize substrate moisture retention, my findings suggests that using Hydrogel for recovery of juvenile cycads could be essential for survival in areas with limited water resources or where it is not possible to hand water. However, in areas where there is a water source or labour, Coir can be utilized as a substrate for cycad seedling recovery, as this substrate holds significant amounts of water under frequent watering (weekly and monthly). The areas with no water source could be areas such as near the mountains and forests, for an example, then where there is labour or irrigation in place could be protected areas such as nature and game reserves. Given that there are limited published guidelines for cycad recovery, the findings of my study provide some options for the application of moisture retaining compounds and substrates to improve moisture availability. This could further improve seedlings and juvenile cycads survival in the wild, thereby impactfully contributing to cycad conservation. Furthermore, substrate selection proved to be essential in maximizing plant growth and development, including physiological factors such as stomatal density, as Coir or Hydrogel as substrate proved to be essential in minimizing water loss through reduced stomatal density under water scarcity.

4.3 Site suitability and natural habitat considerations

My study findings further showed the importance of substrate choice tailored to a given environmental water availability condition to improve cycad seedling survival chances during species recovery. Boyd (1993) suggested that cycad juveniles grown *ex situ* should be reintroduced when exceeding the age of 5 years in order to ensure that the juveniles are well-established and important for reintroduction *in situ.* Therefore, choosing a suitable site that could support seedling establishment (e.g. rocky outcrop) is important when planning a restoration project for cycads (Marler, 2021; Swart *et al*., 2018), to ensure survival given that several cycad species grow naturally in slopes amongst rocks in scrub vegetations that are fire-prone (Whitelock, 2002; Jones, 2002). According to Fujita and Mizuno (2015), rocks appear to have positive nurse effects on the establishment of woody plants in other southern African biomes, and this could be impactful on seedling establishment and development for the recovery of cycads *in situ*.

4.4 Adaptive monitoring and management plan for effective cycad conservation restoration

Lastly, proper management of the project is required after all the work is done to limit the chance of losing the restored species (IUCN, 2013; Godefroid *et al*., 2011; Vaughn *et al*., 2010). So, there should be continuous monitoring of seedling growth in response to the substrates applied, so the practices may be altered as appropriate. This kind of adaptive conservation monitoring may be important in the establishment of cycad seeds and juveniles under varied environmental conditions, particularly under water scarcity.

In conclusion, this study provides a practical approach for cycad species recovery, with emphasis on substrates selection and water management for seeds and juveniles establishment. The recommendations provided based on the findings of this study could therefore serve as guidance in the development of restoration protocols threatened cycads in the wild. The protocols could play an important role in increasing seed and juvenile survival chances in the wild, as the results provide a more practical approach for cycad species recovery *in situ*.

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Supplementary material

Table S1: Post hoc Tukey comparison for the GLM of the seedling survival for the interaction between substrate, and depth.

Depth	**Estimate**	**SE**	**z.ratio**	**p.value**
Surface:				
Control - Coir	-14.24	791.02	-0.02	1.000
Control - KBC	-12.44	791.02	-0.02	1.000
Control - KBH	-10.51	791.02	-0.01	1.000
Coir - KBC	1.80	0.77	2.33	0.091
Coir - KBH	3.73	0.81	4.63	< 0.001
KBC - KBH	1.93	0.75	2.58	0.049
3cm:				
Control - Coir	3.93	395.51	0.01	1.000
Control - KBC	2.49	395.51	0.01	1.000
Control - KBH	6.23	395.51	0.02	1.000
Coir - KBC	-1.44	1.34	-1.07	0.707
Coir - KBH	2.31	0.84	2.73	0.032
KBC - KBH	3.74	1.18	3.18	0.008
Substrate	**Estimate**	**SE**	**z.ratio**	**p.value**
Control				
Surface - 3cm	-19.13	1186.54	-0.02	0.987
Coir:				
Surface - 3cm	-0.96	0.83	-1.16	0.246
KBC:				
Surface - 3cm	-4.20	1.19	-3.54	< 0.001
KBH:				
Surface - 3cm	-2.39	0.68	-3.52	< 0.001

Table S2: Logistic regression results for seedling survival on different substrate and watering regimes.

Coefficients:	**Estimate**	**Std. Error**	**z value**	**p-value**	**Significance**
(Intercept)	-0.41	0.65	-0.63	0.530	
SubstrateCoir	2.97	1.24	2.39	0.017	*
SubstrateKBC	2.62	1.23	2.12	0.034	*
SubstrateKBH	-1.07	0.92	-1.16	0.247	
RegimeMonthly	-18.32	1186.54	-0.02	0.988	
RegimeQuarterly	-17.88	1186.54	-0.02	0.988	
Depth3cm	19.13	1186.54	0.02	0.987	
SubstrateCoir:RegimeMonthly	18.32	1186.54	0.02	0.988	
SubstrateKBC:RegimeMonthly	15.40	1186.54	0.01	0.990	
SubstrateKBH:RegimeMonthly	18.04	1186.54	0.02	0.988	
SubstrateCoir:RegimeQuarterly	15.49	1186.54	0.01	0.990	
SubstrateKBC:RegimeQuarterly	14.07	1186.54	0.01	0.991	
SubstrateKBH:RegimeQuarterly	16.70	1186.54	0.01	0.989	
SubstrateCoir:Depth3cm	-18.17	1186.54	-0.02	0.988	
SubstrateKBC:Depth3cm	-14.93	1186.54	-0.01	0.990	
SubstrateKBH:Depth3cm	-16.75	1186.54	-0.01	0.989	

Table S3: Post hoc test for interaction between Substrate:Watering Regime:Depth and their effects on shoot emergence. Results represent pairwise comparisons for non-parametric ranked ANOVA.

Substrate	Regime	contrast	estimate	SE	df	t.ratio	p.value
Control	Weekly	Surface - 3cm	-80.70	21	216	-3.84	< 0.001
Control	Monthly	Surface - 3cm	-58.50	21	216	-2.79	0.006
Control	Quarterly	Surface - 3cm	-68.00	21	216	-3.23	0.001
Coir	Weekly	Surface - 3cm	14.40	21	216	0.69	0.493
Coir	Monthly	Surface - 3cm	-11.30	21	216	-0.54	0.592
Coir	Quarterly	Surface - 3cm	-51.00	21	216	-2.43	0.016
KBC	Weekly	Surface - 3cm	8.90	21	216	0.42	0.673
KBC	Monthly	Surface - 3cm	-102.00	21	216	-4.85	< 0.001
KBC	Quarterly	Surface - 3cm	-70.00	21	216	-3.33	0.001
KBH	Weekly	Surface - 3cm	-86.30	21	216	-4.10	< 0.001
KBH	Monthly	Surface - 3cm	-60.40	21	216	-2.87	0.005
KBH	Quarterly	Surface - 3cm	-39.60	21	216	-1.89	0.061
Substrate	**Depth**	**contrast**	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
Control	Surface	Weekly - Monthly	57.90	21	216	2.75	0.018
Control	Surface	Weekly - Quarterly	57.90	21	216	2.75	0.018
Control	Surface	Monthly - Quarterly	0.00	21	216	0.00	1.000
Coir	Surface	Weekly - Monthly	37.05	21	216	1.76	0.185
Coir	Surface	Weekly - Quarterly	107.00	21	216	5.09	< 0.001
Coir	Surface	Monthly - Quarterly	69.95	21	216	3.33	0.003
KBC	Surface	Weekly - Monthly	122.85	21	216	5.84	< 0.001
KBC	Surface	Weekly - Quarterly	122.85	21	216	5.84	< 0.001
KBC	Surface	Monthly - Quarterly	0.00	21	216	0.00	1.000
KBH	Surface	Weekly - Monthly	-9.45	21	216	-0.45	0.895
KBH	Surface	Weekly - Quarterly	15.10	21	216	0.72	0.753
KBH	Surface	Monthly - Quarterly	24.55	21	216	1.17	0.474
Control	3cm	Weekly - Monthly	80.00	21	216	3.81	0.001
Control	3cm	Weekly - Quarterly	70.55	21	216	3.36	0.003
Control	3cm	Monthly - Quarterly	-9.45	21	216	-0.45	0.895
Coir	3cm	Weekly - Monthly	11.30	21	216	0.54	0.853
Coir	3cm	Weekly - Quarterly	41.50	21	216	1.97	0.121
Coir	3cm	Monthly - Quarterly	30.20	21	216	1.44	0.324

KBC	3cm	Weekly - Monthly	11.95	21	216	0.57	0.837
KBC	3cm	Weekly - Quarterly	44.00	21	216	2.09	0.094
KBC	3cm	Monthly - Quarterly	32.05	21	216	1.52	0.282
KBH	3cm	Weekly - Monthly	16.45	21	216	0.78	0.714
KBH	3cm	Weekly - Quarterly	61.75	21	216	2.94	0.010
KBH	3cm	Monthly - Quarterly	45.30	21	216	2.15	0.082

Table S3(cont). Post hoc test for interaction between Substrate:Watering Regime:Depth and their effects on shoot emergence. Results represent pairwise comparisons for non-parametric ranked ANOVA.

Substrate	Regime	contrast	estimate	SE	df	t.ratio	p.value
Control	Weekly	Surface - 3cm	-80.70	21	216	-3.84	< 0.001
Control	Monthly	Surface - 3cm	-58.50	21	216	-2.79	0.006
Control	Quarterly	Surface - 3cm	-68.00	21	216	-3.23	0.001
Coir	Weekly	Surface - 3cm	14.40	21	216	0.69	0.493
Coir	Monthly	Surface - 3cm	-11.30	21	216	-0.54	0.592
Coir	Quarterly	Surface - 3cm	-51.00	21	216	-2.43	0.016
KBC	Weekly	Surface - 3cm	8.90	21	216	0.42	0.673
KBC	Monthly	Surface - 3cm	-102.00	21	216	-4.85	< 0.001
KBC	Quarterly	Surface - 3cm	-70.00	21	216	-3.33	0.001
KBH	Weekly	Surface - 3cm	-86.30	21	216	-4.10	< 0.001
KBH	Monthly	Surface - 3cm	-60.40	21	216	-2.87	0.005
KBH	Quarterly	Surface - 3cm	-39.60	21	216	-1.89	0.061
Substrate	**Depth**	**contrast**	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
Control	Surface	Weekly - Monthly	57.90	21	216	2.75	0.018
Control	Surface	Weekly - Quarterly	57.90	21	216	2.75	0.018
Control	Surface	Monthly - Quarterly	0.00	21	216	0.00	1.000
Coir	Surface	Weekly - Monthly	37.05	21	216	1.76	0.185
Coir	Surface	Weekly - Quarterly	107.00	21	216	5.09	< 0.001
Coir	Surface	Monthly - Quarterly	69.95	21	216	3.33	0.003
KBC	Surface	Weekly - Monthly	122.85	21	216	5.84	< 0.001
KBC	Surface	Weekly - Quarterly	122.85	21	216	5.84	< 0.001
KBC	Surface	Monthly - Quarterly	0.00	21	216	0.00	1.000
KBH	Surface	Weekly - Monthly	-9.45	21	216	-0.45	0.895
KBH	Surface	Weekly - Quarterly	15.10	21	216	0.72	0.753
KBH	Surface	Monthly - Quarterly	24.55	21	216	1.17	0.474
Control	3cm	Weekly - Monthly	80.00	21	216	3.81	0.001
Control	3cm	Weekly - Quarterly	70.55	21	216	3.36	0.003
Control	3cm	Monthly - Quarterly	-9.45	21	216	-0.45	0.895
Coir	3cm	Weekly - Monthly	11.30	21	216	0.54	0.853
Coir	3cm	Weekly - Quarterly	41.50	21	216	1.97	0.121
Coir	3cm	Monthly - Quarterly	30.20	21	216	1.44	0.324

KBC	3cm	Weekly - Monthly	11.95	21	216	0.57	0.837
KBC	3cm	Weekly - Quarterly	44.00	21	216	2.09	0.094
KBC	3cm	Monthly - Quarterly	32.05	21	216	1.52	0.282
KBH	3cm	Weekly - Monthly	16.45	21	216	0.78	0.714
KBH	3cm	Weekly - Quarterly	61.75	21	216	2.94	0.010
KBH	3cm	Monthly - Quarterly	45.30	21	216	2.15	0.082

Table S4: Post hoc analysis for the two- way interaction between watering regime and sowing depth on leaf length (square root of leaf length).

Sowing Depth	**contrast**	**Coefficient estimate**	**Standard Error**	**degrees of freedom**	**t.ratio**	**p.value**
Surface	Weekly - Monthly	-0.13	0.20	129	-0.64	0.799
Surface	Weekly - Quarterly	0.83	0.23	129	3.69	0.001
Surface	Monthly - Quarterly	0.96	0.25	129	3.91	< 0.001
3cm	Weekly - Monthly	-0.12	0.12	129	-0.98	0.589
3cm	Weekly - Quarterly	-0.06	0.13	129	-0.46	0.891
3cm	Monthly - Quarterly	0.06	0.13	129	0.49	0.878

Watering regime	**contrast**	**Coefficient estimate**	**Standard Error**	**degrees of freedom**	**t.ratio**	**p.value**
Weekly	Surface - 3cm	-0.13	0.14	129	-0.93	0.353
Monthly	Surface - 3cm	-0.13	0.20	129	-0.65	0.517
Quarterly	Surface - 3cm	-1.02	0.24	129	-4.31	< 0.001

Table S5: Post hoc analysis for the two-way interaction between substrate and sowing depth for the stem growth averaged over watering regime, as well as the post hoc analysis for watering regime averaged over substrate and sowing depth.

Sowing Depth	contrast	Coefficient estimate	Standard Error	degrees of freedom	t.ratio	p.value
Surface	Control - Coir	-0.27	0.22	131	-1.23	0.607
Surface	Control - KBC	-0.59	0.23	131	-2.58	0.053
Surface	Control - KBH	-0.09	0.28	131	-0.31	0.990
Surface	Coir - KBC	-0.32	0.14	131	-2.24	0.117
Surface	Coir - KBH	0.18	0.21	131	0.85	0.830
Surface	KBC - KBH	0.50	0.23	131	2.19	0.132
3cm	Control - Coir	0.35	0.11	131	3.17	0.010
3cm	Control - KBC	0.29	0.11	131	2.68	0.041
3cm	Control - KBH	0.04	0.12	131	0.31	0.990
3cm	Coir - KBC	-0.06	0.10	131	-0.54	0.948
3cm	Coir - KBH	-0.31	0.12	131	-2.64	0.046
3cm	KBC - KBH	-0.26	0.12	131	-2.18	0.136

Substrate	contrast	Coefficient estimate	Standard Error	degrees of freedom	t.ratio	p.value
Control	Surface - 3cm	-0.60	0.22	131	-2.77	0.006
Coir	Surface - 3cm	0.02	0.11	131	0.17	0.869
KBC	Surface - 3cm	0.28	0.14	131	2.04	0.043
KBH	Surface - 3cm	-0.47	0.22	131	-2.18	0.031

contrast	Coefficient estimate	Standard Error	degrees of freedom	t.ratio	p.value
Weekly - Monthly	0.24	0.08	131	2.93	0.011
Weekly - Quarterly	0.52	0.08	131	6.19	<0.001
Monthly - Quarterly	0.29	0.09	131	3.25	0.004

Table S6: Post hoc results for the three-way interaction between watering regime, substrate, and sowing depths on root diameter. Df represents is degrees of freedom. Results where no comparisons were possible has been omitted.

Watering Regime	Sowing Depth	contrast	Coefficient Estimate	Standard Error	df	t.ratio	p.value
Weekly	Surface	Control - Coir	-0.12	0.77	120	-0.16	0.999
Weekly	Surface	Control - KBC	-1.39	0.77	120	-1.82	0.271
Weekly	Surface	Control - KBH	1.92	1.11	120	1.74	0.310
Weekly	Surface	Coir - KBC	-1.27	0.60	120	-2.11	0.157
Weekly	Surface	Coir - KBH	2.04	1.00	120	2.05	0.176
Weekly	Surface	KBC - KBH	3.31	1.00	120	3.32	0.007
Monthly	Surface	Coir - KBC	-2.13	1.35	120	-1.58	0.257
Monthly	Surface	Coir - KBH	-2.52	1.00	120	-2.53	0.034
Monthly	Surface	KBC - KBH	-0.40	1.56	120	-0.25	0.965
Quarterly	Surface	Coir - KBC	-1.03	1.07	120	-0.97	0.335
Weekly	3cm	Control - Coir	2.09	0.57	120	3.67	0.002
Weekly	3cm	Control - KBC	1.91	0.57	120	3.35	0.006
Weekly	3cm	Control - KBH	-0.15	0.63	120	-0.24	0.995
Weekly	3cm	Coir - KBC	-0.18	0.57	120	-0.32	0.989
Weekly	3cm	Coir - KBH	-2.24	0.63	120	-3.57	0.003
Weekly	3cm	KBC - KBH	-2.06	0.63	120	-3.28	0.007
Monthly	3cm	Control - Coir	-0.27	0.66	120	-0.42	0.976
Monthly	3cm	Control - KBC	-0.54	0.66	120	-0.82	0.847
Monthly	3cm	Control - KBH	-0.97	0.74	120	-1.32	0.554

Monthly	3cm	Coir - KBC	-0.26	0.57	120	-0.46	0.967
Monthly	3cm	Coir - KBH	-0.70	0.66	120	-1.06	0.717
Monthly	3cm	KBC - KBH	-0.43	0.66	120	-0.66	0.913
Quarterly	3cm	Control - Coir	1.24	0.66	120	1.87	0.246
Quarterly	3cm	Control - KBC	0.47	0.64	120	0.72	0.888
Quarterly	3cm	Control - KBH	0.34	0.75	120	0.46	0.968
Quarterly	3cm	Coir - KBC	-0.77	0.62	120	-1.24	0.602
Quarterly	3cm	Coir - KBH	-0.90	0.73	120	-1.23	0.609
Quarterly	3cm	KBC - KBH	-0.13	0.71	120	-0.18	0.998

Watering Regime	**Substrate**	**contrast**	**Coefficient Estimate**	**Standard Error**	**df**	**t.ratio**	**p.value**
Weekly	Control	Surface - 3cm	-1.30	0.76	120	-1.72	0.088
Weekly	Coir	Surface - 3cm	0.92	0.59	120	1.57	0.119
Weekly	KBC	Surface - 3cm	2.01	0.59	120	3.42	0.001
Weekly	KBH	Surface - 3cm	-3.37	1.02	120	-3.29	0.001
Monthly	Coir	Surface - 3cm	-0.52	0.59	120	-0.88	0.380
Monthly	KBC	Surface - 3cm	1.35	1.34	120	1.01	0.316
Monthly	KBH	Surface - 3cm	1.31	1.04	120	1.26	0.211
Quarterly	Coir	Surface - 3cm	0.27	0.73	120	0.37	0.713
Quarterly	KBC	Surface - 3cm	0.53	1.00	120	0.53	0.596

Sowing Depth	**Substrate**	**contrast**	**Coefficient Estimate**	**Standard Error**	**df**	**t.ratio**	**p.value**
3cm	Control	Weekly - Monthly	2.49	0.66	120	3.78	0.001
3cm	Control	Weekly - Quarterly	2.37	0.63	120	3.77	0.001
3cm	Control	Monthly - Quarterly	-0.12	0.71	120	-0.17	0.985
Surface	Coir	Weekly - Monthly	1.56	0.60	120	2.59	0.029
Surface	Coir	Weekly - Quarterly	2.16	0.71	120	3.04	0.008
Surface	Coir	Monthly - Quarterly	0.61	0.71	120	0.85	0.672
3cm	Coir	Weekly - Monthly	0.12	0.57	120	0.21	0.975

3cm	Coir	Weekly - Quarterly	1.51	0.61	120	2.50	0.036
3cm	Coir	Monthly - Quarterly	1.39	0.61	120	2.30	0.060
Surface	KBC	Weekly - Monthly	0.70	1.35	120	0.52	0.862
Surface	KBC	Weekly - Quarterly	2.40	1.00	120	2.41	0.046
Surface	KBC	Monthly - Quarterly	1.70	1.56	120	1.09	0.522
3cm	KBC	Weekly - Monthly	0.04	0.57	120	0.07	0.997
3cm	KBC	Weekly - Quarterly	0.92	0.59	120	1.58	0.260
3cm	KBC	Monthly - Quarterly	0.88	0.59	120	1.51	0.290
Surface	KBH	Weekly - Monthly	-3.01	1.28	120	-2.36	0.020
3cm	KBH	Weekly - Monthly	1.67	0.71	120	2.35	0.053
3cm	KBH	Weekly - Quarterly	2.86	0.75	120	3.83	0.001

Table S7: Post hoc results for moisture content in stems, df represents the degrees of freedom for each test.

Watering Regime comparison	Coefficient Estimate	Standard Error	df	t.ratio	p.value
Weekly - Monthly	0.51	0.09	132	5.95	< 0.001
Weekly - Quarterly	0.55	0.10	132	5.54	< 0.001
Monthly - Quarterly	0.03	0.11	132	0.31	0.947

Substrate Comparison	Coefficient Estimate	Standard Error	df	t.ratio	p.value
Control - Coir	0.14	0.10	132	1.40	0.500
Control - KBC	0.19	0.10	132	1.91	0.227
Control - KBH	0.06	0.11	132	0.52	0.955
Coir - KBC	0.05	0.09	132	0.59	0.934
Coir - KBH	-0.08	0.10	132	-0.77	0.868
KBC - KBH	-0.13	0.11	132	-1.23	0.607

Table S8: Post hoc results for the three-way interaction between watering regime, substrate, and sowing depths on root volume. Df represents is degrees of freedom. Results where no comparisons were possible has been omitted.

Watering Regime	**Sowing Depth**	**contrast**	**Coefficient Estimate**	**Standard Error**	**df**	**t.ratio**	**p.value**
Weekly	Surface	Control - Coir	-0.07	0.94	120	-0.08	1.000
Weekly	Surface	Control - KBC	-3.15	0.94	120	-3.33	0.006
Weekly	Surface	Control - KBH	0.33	1.36	120	0.25	0.995
Weekly	Surface	Coir - KBC	-3.07	0.74	120	-4.15	< 0.001
Weekly	Surface	Coir - KBH	0.41	1.23	120	0.33	0.987
Weekly	Surface	KBC - KBH	3.48	1.23	120	2.83	0.027
Monthly	Surface	Coir - KBC	-1.11	1.66	120	-0.67	0.781
Monthly	Surface	Coir - KBH	-1.11	1.23	120	-0.90	0.639
Monthly	Surface	KBC - KBH	0.00	1.93	120	0.00	1.000
Quarterly	Surface	Coir - KBC	1.33	1.32	120	1.01	0.313
Weekly	3cm	Control - Coir	-1.60	0.70	120	-2.28	0.109
Weekly	3cm	Control - KBC	0.07	0.70	120	0.10	1.000
Weekly	3cm	Control - KBH	-1.50	0.77	120	-1.94	0.218
Weekly	3cm	Coir - KBC	1.67	0.70	120	2.37	0.088
Weekly	3cm	Coir - KBH	0.10	0.77	120	0.13	0.999
Weekly	3cm	KBC - KBH	-1.57	0.77	120	-2.02	0.185
Monthly	3cm	Control - Coir	-0.52	0.81	120	-0.64	0.918
Monthly	3cm	Control - KBC	-0.12	0.81	120	-0.15	0.999
Monthly	3cm	Control - KBH	-1.17	0.91	120	-1.29	0.574
Monthly	3cm	Coir - KBC	0.40	0.70	120	0.57	0.941
Monthly	3cm	Coir - KBH	-0.64	0.81	120	-0.79	0.857
Monthly	3cm	KBC - KBH	-1.04	0.81	120	-1.29	0.573
Quarterly	3cm	Control - Coir	-0.67	0.81	120	-0.83	0.842
Quarterly	3cm	Control - KBC	-1.86	0.79	120	-2.35	0.092
Quarterly	3cm	Control - KBH	-0.45	0.92	120	-0.49	0.962
Quarterly	3cm	Coir - KBC	-1.19	0.76	120	-1.56	0.407
Quarterly	3cm	Coir - KBH	0.23	0.90	120	0.25	0.994
Quarterly	3cm	KBC - KBH	1.41	0.88	120	1.61	0.375

Substrate	**Watering regime**	**contrast**	**Coefficient Estimate**	**Standard Error**	**df**	**t.ratio**	**p.value**
Control	Weekly	Surface - 3cm	-0.83	0.93	120	-0.90	0.372
Coir	Weekly	Surface - 3cm	-2.36	0.72	120	-3.27	0.001
KBC	Weekly	Surface - 3cm	2.38	0.72	120	3.30	0.001
KBH	Weekly	Surface - 3cm	-2.67	1.26	120	-2.12	0.036
Coir	Monthly	Surface - 3cm	0.42	0.72	120	0.59	0.560
KBC	Monthly	Surface - 3cm	1.93	1.65	120	1.17	0.243
KBH	Monthly	Surface - 3cm	0.89	1.28	120	0.69	0.490
Coir	Quarterly	Surface - 3cm	0.71	0.90	120	0.79	0.431
KBC	Quarterly	Surface - 3cm	-1.82	1.23	120	-1.48	0.142

Sowing Depth	**Substrate**	**contrast**	**Coefficient Estimate**	**Standard Error**	**df**	**t.ratio**	**p.value**
3cm	Control	Weekly - Monthly	1.22	0.81	120	1.51	0.292

3cm	Control	Weekly - Quarterly	1.88	0.77	120	2.43	0.044
3cm	Control	Monthly - Quarterly	0.66	0.87	120	0.75	0.732
Surface	Coir	Weekly - Monthly	-0.48	0.74	120	-0.65	0.793
Surface	Coir	Weekly - Quarterly	-0.26	0.88	120	-0.30	0.953
Surface	Coir	Monthly - Quarterly	0.22	0.88	120	0.25	0.965
3cm	Coir	Weekly - Monthly	2.30	0.70	120	3.27	0.004
3cm	Coir	Weekly - Quarterly	2.81	0.75	120	3.77	0.001
3cm	Coir	Monthly - Quarterly	0.51	0.75	120	0.68	0.775
Surface	KBC	Weekly - Monthly	1.48	1.66	120	0.89	0.645
Surface	KBC	Weekly - Quarterly	4.15	1.23	120	3.38	0.003
Surface	KBC	Monthly - Quarterly	2.67	1.93	120	1.39	0.352
3cm	KBC	Weekly - Monthly	1.03	0.70	120	1.47	0.309
3cm	KBC	Weekly - Quarterly	-0.05	0.72	120	-0.07	0.998
3cm	KBC	Monthly - Quarterly	-1.08	0.72	120	-1.50	0.296
Surface	KBH	Weekly - Monthly	-2.00	1.57	120	-1.27	0.206
3cm	KBH	Weekly - Monthly	1.56	0.87	120	1.78	0.181
3cm	KBH	Weekly - Quarterly	2.93	0.92	120	3.19	0.005
3cm	KBH	Monthly - Quarterly	1.38	0.95	120	1.45	0.320

Table S9: Post hoc Tukey comparison for the GLM of stomatal density for the interaction between substrate, and watering regime showing the pairwise comparisons between watering regimes for each substrate.

Contrast	**Estimate**	**Standard Error**	**Degrees of freedom**	**t.ratio**	**p.value**
Control:					

Weekly - Monthly	27.0	5.09	108	5.31	< 0.001
Weekly - Quarterly	46.9	5.09	108	9.22	< 0.001
Monthly - Quarterly	19.9	5.09	108	3.91	< 0.001
Coir:					
Weekly - Monthly	20.9	5.09	108	4.11	< 0.001
Weekly - Quarterly	16.0	5.09	108	3.14	0.006
Monthly - Quarterly	-4.9	5.09	108	-0.96	0.602
KBC:					
Weekly - Monthly	-7.5	5.09	108	-1.47	0.307
Weekly - Quarterly	22.1	5.09	108	4.34	< 0.001
Monthly - Quarterly	29.6	5.09	108	5.82	< 0.001
KBH:					
Weekly - Monthly	12.2	5.09	108	2.40	0.048
Weekly - Quarterly	25.8	5.09	108	5.07	< 0.001
Monthly - Quarterly	13.6	5.09	108	2.67	0.023

Table S10: Logistic regression results for seedling survival under different substrate and watering regime

Coefficients:	**Estimate**	**Standard error**	**z value**	**p-value**
(Intercept)	18.57	1743.00	0.01	0.992
SubstrateCoir	-16.00	1743.00	-0.01	0.993
SubstrateKBC	-16.77	1743.00	-0.01	0.992
SubstrateKBH	-17.65	1743.00	-0.01	0.992
RegimeMonthly	0.00	2465.00	0.00	1.000
RegimeQuarterly	-17.27	1743.00	-0.01	0.992
SubstrateCoir:RegimeMonthly	-1.65	2465.00	0.00	0.999
SubstrateKBC:RegimeMonthly	-1.20	2465.00	0.00	1.000
SubstrateKBH:RegimeMonthly	1.65	2465.00	0.00	0.999

SubstrateCoir:RegimeQuarterly	15.62	1743.00	0.01	0.993
SubstrateKBC:RegimeQuarterly	17.27	1743.00	0.01	0.992
SubstrateKBH:RegimeQuarterly	16.94	1743.00	0.01	0.992

Table S11: Logistic regression model for the presence of Coralloid roots. LCI and UCI represent the lower confidence interval and upper confidence interval, respectively.

Substrate	contrast	Odds ratio	asymp. LCL	asymp. UCL	SE	null	z ratio	p value
Control	Weekly / Monthly	17.33	1.12	268.93	20.28	1	2.44	0.039
Control	Weekly / Quarterly	1.60	0.24	10.71	1.30	1	0.58	0.831
Control	Monthly / Quarterly	0.09	0.01	1.54	0.11	1	-1.98	0.116
Coir	Weekly / Monthly	8.25	0.78	86.73	8.28	1	2.10	0.089
Coir	Weekly / Quarterly	44.00	2.04	947.92	57.64	1	2.89	0.011
Coir	Monthly / Quarterly	5.33	0.29	97.91	6.62	1	1.35	0.369
KBC	Weekly / Monthly	1.40	0.20	9.62	1.15	1	0.41	0.912
KBC	Weekly / Quarterly	1.25	0.16	9.95	1.11	1	0.25	0.966
KBC	Monthly / Quarterly	0.89	0.11	7.20	0.80	1	-0.13	0.991
KBH	Weekly / Monthly	3.73	0.46	30.42	3.34	1	1.47	0.305
KBH	Weekly / Quarterly	21.00	1.10	402.34	26.46	1	2.42	0.042
KBH	Monthly / Quarterly	5.63	0.34	93.30	6.74	1	1.44	0.320

Table S12: Post hoc Tukey pairwise comparison for the GLM of stomatal density between watering regimes for each substrate.

Contrast	Estimate	Standard Error	Degrees of freedom	t.ratio	p.value
Control:					
Weekly - Monthly	-13.90	3.07	108	-4.52	< 0.001
Weekly - Quarterly	0.30	3.07	108	0.10	0.995
Monthly - Quarterly	14.20	3.07	108	4.62	< 0.001
Coir:					
Weekly - Monthly	6.50	3.07	108	2.11	0.092
Weekly - Quarterly	13.30	3.07	108	4.33	< 0.001
Monthly - Quarterly	6.80	3.07	108	2.21	0.074
KBC:					
Weekly - Monthly	13.90	3.07	108	4.52	< 0.001
Weekly - Quarterly	20.60	3.07	108	6.70	< 0.001
Monthly - Quarterly	6.70	3.07	108	2.18	0.079
KBH:					
Weekly - Monthly	6.90	3.07	108	2.24	0.068
Weekly - Quarterly	7.90	3.07	108	2.57	0.031
Monthly - Quarterly	1.00	3.07	108	0.33	0.943