In vitro tissue culture: Towards conservation of threatened desiccation sensitive *Encephalartos* cycads seeds

by

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DEDICATION

This work is dedicated to my parents;

Mr Isaac Bhekuyise Malwane

and

Mrs Nonhlanhla Elaine Malwane.

Without your support, selflessness, sacrifices, encouraging words and prayers, this wouldn't have been possible. I thank you and I love you with all my being.

DECLARATION

I, Thembeka Sebenzile Desiree Malwane, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABSTRACT

Approximately 62% of the 355 cycad species in the world are classified as threatened with extinction. The African genus, *Encephalartos*, has a total of 65 species, approximately 70% of which are threatened. This status emphasizes the need to conserve these species; however, the recalcitrant nature of cycads seeds makes it difficult to conserve using conventional seed banking methods. Recalcitrant seeds have a short lifespan and cannot be dried or stored for prolonged periods as they become non-viable when they lose moisture. While studies on cryopreservation for conserving cycad germplasm and banking these desiccation-sensitive seeds has made some advances, cycad conservation is still limited to *ex situ* living plant collections. *In vitro* tissue culture is a promising technique for conserving cycads. While attempts have been made, there have been few reported successes and, to date, there has been no successful regeneration of *Encephalartos* species. As such, this study was aimed at developing an efficient and successful *in vitro* regeneration protocol for two *Encephalartos* species. Embryo regeneration of *E. altensteinii* and *E. manikensis* was assessed, testing the effects of plant growth regulators (PGRs) - 0.5 mg/L Kinetin and 0.5 mg/L 6-BAP, alone or in combination, sucrose (0, 15 and 30 g/L) and light. Within six weeks of culture, embryos of both species were able to regenerate, however, each responded differently to the tested variables. While shoot regeneration was evident for both species during this period, this was however not explained by any of the variables assessed in this study. Rooting was highest in the treatments with 0.5 mg/L Kinetin for *E. altensteinii,* after subculture rooting was favoured by the treatments with 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP. *Encephalartos manikensis* rooting was significantly higher in PGR-free treatment in the first six weeks

of culture. After subculture, rooting was enhanced by the treatment with the highest PGR concentration of 1.0 mg/L Kinetin + 1.0 mg/L 6-BAP. Darkness enhanced rooting while 16h photoperiod enhanced shooting for both species. However, the regeneration of both roots and shoots was more prevalent in the treatments that were initiated in complete darkness as compared to the treatments initiated under 24h of light. Both species responded to sucrose; with increase in sucrose concentration, callus induction increased for *E. altensteinii* while, necrosis and contamination increased for *E. manikensis*. *In vitro*-derived *E. altensteinii* plantlets acclimatization was unsuccessful and only 3.5% of *E. manikensis* were successfully acclimatized. This study suggests that although both these species belong to the same genus, *in vitro* culture protocol should be species specific. The overall regeneration of both species was however low, thus the second study assessed the levels of phytohormones in *E. altensteinii* seed tissues (embryos and megagametophytes) of the same age as those used in the *in vitro* regeneration. Phytohormones, as well as multiple phytohormone interactions (i.e. interplay between Abscisic acid (ABA) and Gibberellins (GAs)), play a role in the germination, growth and development of a plant. The high levels of a germination inhibiting ABA compared to the low levels of cytokinins and auxins, as well as the absence of GAs obtained in the assessed seed tissue, suggest that no real germination was taking place. Thus these results suggest that *E. altensteinii* seeds have a very slow developmental process with the likely chance that at this age they may be immature.

46 **KEY WORDS**

47 *Threatened Cycads*

48 *Encephalartos altensteinii*

49 *Encephalartos manikensis*

50 Embryo

51 *In vitro*

52 Plant Growth Regulators

53 Phytohormones

54 Conservation

LIST OF ABBREVIATIONS

2,4-D - 2,4-Dichlorophenoxyacetic acid
6-BAP - 6-Benzylaminopurine
7'- OH-ABA - 7'-Hydroxy-abscisic acid
ABA - Abscisic acid
ABAGE - Abscisic acid glucose ester
AC - Activated charcoal
***c*-ABA** - *cis*-Abscisic acid
dH_2O - Distilled water
dhZ - Dihydrozeatin
dhZR - Dihydrozeatin riboside
DPA - Dihydrophaseic acid
GA - Gibberellins
IAA - Indole-3-acetic acid
IAA-Ala - N-(Indole-3-yl-acetyl)-alanine
IAA-Glu - N-(Indole-3-yl-acetyl)-glutamic acid
IAA-Leu - N-(Indole-3-yl-acetyl)-leucine
IAA-Asp - N-(Indole-3-yl-acetyl)-aspartic acid
IBA - Indole-3-butyric acid
iPR - Isopentenyladenine riboside
iP - Isopentenyladenine
Kin - Kinetin
MS - Murashige and Skoog
NAA - Naphthaleneacetic acid
NaOCl - Sodium hypochlorite
neoPA - neo-Phaseic acid
PA - Phaseic acid
PGR - Plant Growth Regulator
***t*-ABA** - *trans*-Abscisic acid
TZ - 2,3,5-tri-phenyltetrazolium chloride
Z - Zeatin
Z-O-Glu - Zeatin-O-glucoside
ZR - Zeatin riboside

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CHAPTER ONE: General introduction and literature review

Cycads are amongst the oldest known seed bearing plants (Norstog & Nicholls, 1997). They are ancient dioecious gymnosperms that emerged before the mid-Permian, in the Upper Palaeozoic era ~ 265-290 MYA (million years ago) (Jones, 2002), and reached their greatest diversity and abundance during the Jurassic–Cretaceous period of the Mesozoic era (Brenner et al., 2003). However, recent molecular work indicates that diversification of modern cycads is not more than ~12 million years old (Nagalingum et al., 2011).

Fossil records suggest that cycads were once widely distributed (Wang et al., 2005). However, extant cycads are currently restricted to the tropical and sub-tropical regions of the world: North, Central and South Americas, Africa, Asia and Australia (Donaldson, 2003; Litz et al., 2004; Anderson et al., 2007; Osborne et. al., 2012). The extant species are divided into two families, Cycadaceae and Zamiaceae with ten genera (Calonje *et al*., 2017). *Cycas* is the only genus in Cycadaceae with 117 species and Zamiaceae has a total of nine genera, *Dioon* (16), *Lepidozamia* (2), *Macrozamia* (41), *Microcycas* (1), *Zamia* (80), *Ceratozamia* (30), *Encephalartos* (65) *Bowenia* (2) and one *Stangeria* (Calonje et al., 2019; Osborne et al., 2012). This makes Zamiaceae the most diverse and widespread cycad family (Donaldson, 2003).

Threats and conservation status of cycads

Cycads are amongst the most threatened group of living organisms in the world with 62% of the 355 species classified as threatened with extinction (Donaldson, 2003;(The IUCN Red List of Threatened Species, 2010). All living cycads species are listed under

462 the Convention on International Trade in Endangered Species of Wild Fauna and Flora
463 (CITES) Appendix I and II (CITES, 2014). Furthermore, a disproportionately high
464 number of currently surviving cycads species have been assessed as either Critically
465 Endangered (CR), Endangered (EN) or Vulnerable (VU) (Fragnière et al., 2015; IUCN,
466 2016). The major causes for cycads declining in the wild include removal from the wild
467 for illegal trade and habitat destruction (Osborne, 1995; Donaldson, 2003; Donaldson
468 *et al.,* 2003). There are also naturally occurring threats that contribute towards the
469 decline of cycad populations, such as scarcity of seeds, slow rate of growth, lack of
470 pollinators and seed dispersing agents (Dehgan, 1983). Dioecious plants such as
471 cycads are dependent on pollinators for seed production since they cannot self-
472 pollinate, and hence rely on pollinator movement from male to female plants (Ohya et
473 al., 2017) and seed dispersing agents from one place to another. Pollinators and seed
474 dispersing agents play a crucial role in increasing the plant population size, while the
475 lack of pollinators and inefficiency of pollen to be transferred to female cone are one
476 of the key contributing factors that lead to the scarcity of seed (Dehgan, 1983) and
477 thus the decreased cycad population size. The scarcity of seeds, coupled with slow
478 seed germination and growth makes the growth in numbers of cycads in the wild
479 increase naturally at a relatively slow rate (Norstog & Nicholls 1997) and in small
480 populations. While the propagation of cycads has been found to be difficult (Dehgan,
481 1983), the propagation of cycads from seed can aid cycad conservation as it can
482 reduce the demand for wild collected plants (Calonje et al., 2011). For these reasons,
483 it is evident that there is an urgent need to develop an effective protocols for the mass
484 production of cycads, thus preventing the extinction of these ancient threatened plants
485 (Teixeira da Silva et al., 2014). In addition to this, the biology of cycads is not well

understood, and thus this may further contribute to the threats that lead to the decline of cycads.

Cycad conservation has mainly focused on conserving the threatened species in native habitats and as *ex situ* living plant collections (Litz et al., 2004). However current cycad conservation methods do not provide adequate protection against the threats cycads face. Hence, it has been suggested that improved cycad propagation methods could be more effective in conserving the threatened species (Dehgan & Johnson, 1983; Dehgan, 1999). The suggested propagation conservation methods include improved seed production by hand pollination, improved seed germination, root pruning in order to enhance vegetative growth rate, and enhanced procedures for vegetative propagation and the regeneration of cycads through *in vitro* tissue culture (Dehgan, 1999). The latter, has had substantial progress over the years (Litz et al., 2004). Therefore, *in vitro* tissue culture methods should be used as an important part of cycad conservation. Recent, preliminary studies show that advances in cryopreservation of cycad germplasm and seed banking of recalcitrant (desiccation-sensitive) cycads seeds, can be a potential method of conserving cycad seeds (Dandugula, 2011) as an extension the *ex situ* living plant collections that are currently the only method that is utilised.

Cycad seed biology

To date, there has not been much research conducted on cycad seeds, however, there are perceptions that cycad seeds may be recalcitrant, as some cycad seeds have been found to be ‘wet’ (Forsyth & van Staden 1983; Dehgan & Schutzman 1989).

509 Further studies conducted by Woodenberg et al. (2007) have highlighted that
510 *Encephalartos natalensis* and *E. gratus* seeds are indeed recalcitrant (desiccation-
511 sensitive), losing viability when megagametophytes and embryos are subjected to
512 dehydration. While not all cycad seeds have been assessed for their recalcitrance, the
513 occurrence of recalcitrant seeds is a common feature in extant gymnosperms from
514 tropical and subtropical areas (Berjak et al., 1989; Dehgan & Schutzman, 1989; Attree
515 & Fowke, 1993 Forsyth and van Staden 1983; Woodenberg et al., 2007) and as such,
516 it is likely that most cycads, if not all have recalcitrant seeds. These types of seeds
517 undergo no maturation drying during the final stages of development, tolerating little
518 post-shedding desiccation and are also sensitive to chilling (Roberts, 1973). Hence
519 cycad seeds have a high moisture content and do not survive when subjected to drying
520 or long term storage at low temperatures (Attree & Fowke, 1993; Forsyth & van
521 Staden, 1983; Dehgan & Schutzman, 1989). Recalcitrant seeds typically have a short
522 lifespan even when stored under hydrated conditions (Berjak et al., 1989; Woodenberg
523 et al., 2014; Berjak & Pammenter, 2013). Unlike orthodox seeds, which can survive
524 drying and prolonged storage at sub-zero temperature, recalcitrant seeds do not
525 survive desiccation and therefore cannot be stored at sub-zero temperatures because
526 water in the cells forms ice crystals which results to death (Walters et al., 2013). Cycad
527 seeds, if indeed are all recalcitrant, are therefore, not suitable for long term *ex situ*
528 conservation, and hence the search for alternative methods for successfully storing
529 cycad seeds and conserving cycad seed germplasm is crucial.

530 **The African genus, *Encephalartos***

531 The genus *Encephalartos* has a total of 65 species which are documented as 27%
532 Critically Endangered (CR), 24% Vulnerable (VU), 15% Endangered (EN), 6% Extinct

533 in the Wild (EW), 20% Near Threatened (NT) and only 9% are of Least Concern (LC)
534 according to the IUCN Red List of Threatened Species, 2014. Thirty-eight of the 65
535 *Encephalartos* cycads are of South African origin, as a result South Africa has the
536 highest cycad diversity in the whole of Africa (Donaldson, 2003; Osborne et al., 2012).
537 South African *Encephalartos* species are also highly threatened and have been
538 classified as CR (12), EN (4), VU (8), EW (3), NT (8) with only three species of the 38
539 species are categorised as LC (Calonje et al., 2019; Osborne et al., 2012).

540 **Integrated cycad conservation**

541 During the 9th International Conference of Cycad Biology in 2011 held at Shenzhen,
542 China, an integrated conservation strategy was suggested as one that would be more
543 effective and successful in the conservation of cycads (Pritchard *et al.,* 2011). The
544 integration of *in situ* and *ex situ* conservation methods is necessary for much needed
545 improved conservation of threatened cycads. However, according to Nadarajan *et al.,*
546 (2018), there is a shortfall in the understating of cycad biology, hence there is a need
547 to generate knowledge for better understanding of cycad biology i.e. cycad pollen,
548 seed and tissues in relation to their conservation. Hence, this study will help improve
549 and build-up knowledge on *Encephalartos* cycad seeds, and the ways in which they
550 regenerate in *in vitro* tissue culture.

551 Apart from *ex situ* living plant collections, there is very little ongoing research
552 based on cycad seed conservation. The lack of ongoing research is primarily due to
553 the lack of research material (seeds) for experimental purposes (Dehgan &
554 Schutzman, 1989;) as well as the protective laws that govern the acquisition of cycad
555 seeds (Schlegel, 1991).

Aims and Objectives

This thesis primarily investigates an alternative effective conservation method for cycads, using embryos obtained from seeds that were stored *ex situ* for a short term period between 8-12 months post-harvest. The focus of this research is to develop an effective protocol for regeneration of *Encephalartos altensteinii* and *Encephalartos manikensis* embryos in *in vitro* culture as a precursor to further research on long term embryo storage and cryopreservation. A final component of this thesis covers the analysis of the phytohormones in megagametophytes and embryological tissues of *E. altensteinii*, to determine which phytohormones are prevalent in 8-12 month old seeds. Knowing the presence and concentrations of the prevalent phytohormones could potentially help determine which plant growth regulators could be used in *in vitro* for the regeneration of the embryos. Therefore, the overall objective of this study is to broaden the already existing knowledge on *Encephalartos* cycad species. The main research aims were as follows:

Chapter Two

(i) Investigate the effect of various sucrose concentrations on the regeneration, growth and development of cycad embryos in *in vitro* culture,

(ii) Investigate the effects of treating embryos with plant growth regulators (PGRs) (Kinetin and 6-BAP) alone and in combination, for improved embryo regeneration,

(iii) Investigate the effects of light on embryo regeneration

(iv) Acclimatise *in vitro* regenerated plantlets, from cycad seed embryos and,

Chapter Three

(v) Investigate what hormones are prevalent in mature (8 -12 month old) seeds (embryos and megagametophytes) through phytohormone profiling.

This study consist of four chapters: Chapter One is the general introduction and literature review on cycads, Chapter Two and Three are experimental chapters (outlined above), and Chapter Four is a synthesis and discussion of the findings and includes recommendations for future tissue culture of these species.

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676

CHAPTER TWO: *In vitro* regeneration of *Encephalartos altensteinii* and *Encephalartos manikensis*

Abstract

Cycads are one of the most threatened groups of plants in the world, with current *ex situ* conservation strategies limited to living plant collections. While seed banking techniques have been used for other plant species, this technique is limited by the desiccation-sensitive nature of most cycad species. Over the years it has become apparent that there is a need to find alternative *ex situ* conservation methods for cycads. Thus this study was aimed at investigating *in vitro* tissue culture as a method that would be integrated with living collections for improved conservation of threatened *Encephalartos* cycad species. *In vitro* tissue culture experiments were carried out for *Encephalartos altensteinii* and *Encephalartos manikensis*, assessing the effects of plant growth regulators (PGRs), sucrose and light. Both species were able to regenerate roots and shoots, and were positively affected by the presence of PGRs; however, they each responded differently to the assessed PGRs. *Encephalartos altensteinii* rooting was significantly higher in the treatments with 0.5 mg/L Kinetin. In the first six weeks of the experiment *E. manikensis* rooting was higher in the PGR-free treatment, but was enhanced by the combination between Kinetin and 6-BAP when the concentrations were increased to 1.0 mg/L. High sucrose concentration (30 g/L) also had different effects on each species - which resulted in increased callus induction for *E. altensteinii* and increased necrosis and contamination for *E. manikensis*. In both species, rooting was higher in darkness compared to the light. However, the alternation of darkness and light was most beneficial for embryo rooting and shooting after subculture, however, this was most effective in treatments that were initiated in

701 darkness. Acclimatization of *in vitro*-derived *E. altensteinii* plantlets was unsuccessful.
702 In contrast, 3.5% of *E. manikensis in vitro*-derived plantlets underwent hardening and
703 acclimatization and survived 300 days form initial culture. This study suggests that
704 although both these species are from the same genus, *in vitro* culture methods may
705 need to be species specific.

2.1. Introduction

All living cells have the potential to regenerate into an entire organism (Haberlandt, 1902). Over the years, this has enabled researchers to regenerate whole plants through *in vitro* techniques using different explants i.e. cells, tissues or organs (Delporte et al., 2012). However, explant's response largely depends on environmental and cellular signals such as: light, temperature, pH, O_2 and CO_2 concentrations, genetic make-up of a plant, plant growth media, nutrients: water, macro and micro elements and sugar, vitamins and PGRs (Murashige, 1977; Pan & van Staden, 1998). These elements control cell division, elongation, differentiation and polarity (Dodeman et al., 1997). Almost every part of a plant can be used in tissue culture to regenerate whole plants but, according to Elhiti and Stasolla (2011), embryos are the most stable and effective explants for difficult to regenerate species such as cycads.

Explant response in culture medium is largely dependent on the presence of plant growth regulators (PGRs) (Murashige, 1977; Pan & van Staden, 1998). Plant growth regulators promote the growth and differentiation of plant cells, tissues and organs and are grouped into six naturally occurring categories: auxins, cytokinins, gibberellins, brassinosteroids, abscisic acid, and ethylene, with auxins and cytokinins as the most widely used for explant regeneration *in vitro* tissue culture (Gaspar et al., 1996). Pioneering *in vitro* studies on tobacco have illustrated that a high ratio of auxin to cytokinin led to root regeneration and a high ratio of cytokinin to auxin led to the promotion of shoot regeneration (Skoog & Miller, 1957). Since then, researchers have been manipulating cytokinin and auxin concentrations and ratios in order to achieve desired outcomes with plant cells, tissues and organs (Smigocki & Owens 1989; Lee et al., 2011). Research shows that ultimately the presence, combination and

731 concentration of PGR determines whether explants generate through organogenesis
732 or somatic embryogenesis (directly or indirectly through callus formation) (Gray &
733 Trigiano, 2004). These techniques have proved to be beneficial in the conservation of
734 threatened species (Fay, 1992; Mikulík, 1999; Sarasan et al., 2006), and could
735 therefore also be useful in conserving threatened cycads species.

736 Cycads are amongst the most threatened group of living organisms in the world
737 and of the 355 known cycad species, 65 are of African origin belonging to the genus
738 *Encephalartos* (IUCN Red List of Threatened Species, 2014). The genus
739 *Encephalartos* currently has 46 species (70%) that are classified as threatened
740 (Calonje et al., 2019) and are therefore a priority for conservation. While current cycad
741 *ex situ* conservation is limited to living plant collections (Litz et al., 2004), another
742 limiting factor for other forms of *ex situ* conservation is the recalcitrant nature of most
743 cycad species seeds (Attree & Fowke, 1993; Forsyth & van Staden 1983; Woodenberg
744 et al*.,* 2007). Recalcitrant seeds are difficult to conserve using conventional seed
745 banking methods (Attree & Fowke, 1993; Forsyth & van Staden, 1983; Dehgan &
746 Schutzman, 1989). Several studies have worked on *in vitro* tissue culture in some
747 cycad species (Table 2.1) with promising success; however, for improved
748 conservation of cycad species, integration of tissue culture and *ex situ* conservation
749 has been proposed (Pritchard et al., 2011). In spite of the high threatened status of
750 *Encephalartos*, limited propagation research has been undertaken.

751 Table 2.1: Summary of *in vitro* studies conducted on different cycad species, across the two cycad families, as well as the different
752 choice of explants.

Family	Genus	Species	Explant	Authors
Zamiaceae	*Stangeria*	*S. eriopus*	Roots of seedlings	Osborne & van Staden 1987
	Dioon	*D. edule*	Zygotic embryos	Chavez & Litz, 1999
	Ceratozamia	*C. hildae, C. Mexicana*	Zygotic embryos	Chávez et al*.,* 1992a
		C. hildae,	Zygotic embryos	Litz et al*.,* 1995a
		C. Mexicana	Zygotic embryos	De Luca et al*.,*1979
			Megagametophytes	Chávez et al., 1992c
			Megagametophytes	Chávez et al., 1995
	Zamia	*Z. floridana*	Megagametophytes	La Rue, 1948; La Rue, 1953
		Z. integrifolia (=pumila)	Megagametophytes	Norstog, 1965
		Z. integrifolia	Seed, cotyledon, squama (plant scales) and shoot tips of *in vitro* seedling	Lin et al., 2007
			Zygotic embryos	Dhiman et al., 2000
		Z. latifoliolata	Zygotic embryos	Webb & Rivera, 1981
		Z. fischeri, Z. pumila, Z. furfuracea	Zygotic embryos	Chávez et al., 1992b
		Z. furfuracea	Megagametophytes	Webb et al., 1984
		E. umbeluziensis	Megagametophytes	De Luca et al.,1979
		E. altensteinii	Megagametophytes	Webb et al., 1984

	Encephalartos	*E. cycadifolius*	Zygotic embryos	Jäger & van Staden, 1996a
		E. dyerianus *E. natalensis*	Zygotic embryos	Jäger & van Staden, 1996b
Cycadaceae	*Cycas*	*C. euryphylidia*	Young leaf flushes	Chávez et al., 1998
		C. circinalis	Megagametophytes	Dhiman *et al*., 2000
		C. guizhouensis	Roots, stem, squama (plant scales)	Zai-qi, 2011
		C. revoluta	Megagametophytes	De Luca et al.,1979
			Megagametophytes	Webb et al., 1984
			Seed, cotyledon, squama (plant scales) and shoot tips of *in vitro* seedling	Lin et al., 2007
			Megagametophytes	De Luca & Sebato, 1980
			Zygotic embryos	Rinaldi & Leva, 1995
			Seedling epicotyls and hypocotyls	Tadera et al., 1995
			Sections of epicotyl and cotyledonary leaves	Rinaldi & Rivera, 1995 Rinaldi, 1999
			Bulbil inner bulb-scales	Chaplot & Jasrai, 2000
			Zygotic embryos	Kiong et al., 2008
			Zygotic embryos	Motahashi et al., 2008
			Leaf midrib	Pan et al., 2013
			Zygotic embryos	Naderi et al., 2015

753

754 Most of the *in vitro* work on cycads has largely focused on *Zamia*, *Ceratozamia*
755 and *Cycas* species, with *Cycas revoluta* as the most studied cycad species (Table 2.1;
756 Teixeira da Silva et al., 2014). To date there have been only a few *in vitro* studies
757 documented on *Encephalartos* species (Table 2.1). One of the earlier studies was
758 conducted by De Luca et al. (1979), investigating regeneration of cycad
759 megagametophytes in *C. mexicana, C. revoluta* and *E. umbeluziensis* where
760 megagametophytes were induced to proliferate *in vitro* in different plant growth media
761 i.e. White's medium (1943) and media 8, 13 of Norstog (1965); 21, 56, 63, 78 of
762 Norstog and Rhamstine (1967); and 56 bis (analogous to 56 but lacking in casein
763 hydrolisate). De Luca et al. (1979) observed callusing in all three species, with callus
764 in *C. mexicana* and *C. revoluta* producing adventive embryos which became
765 monocotyledonous plantlets bearing the first leaf and haploid roots in *C. mexicana* and
766 *C. revoluta* respectively. While*, E. umbeluziensis* callus did not further develop, the
767 calli formed were different depending on the medium and temperature in which the
768 megagametophytes were incubated. Under light conditions at 25 ºC on medium 8, *E.*
769 *umbeluziensis* megagametophytes became light green, producing spherical masses
770 of callus which in some cases grew, becoming crisp and snow-white coloured. This
771 suggests the importance of light for photosynthesis, thus changing the colour to green.
772 On White's medium *E. umbeluziensis* megagametophytes became green, slightly
773 swelled and large brown callus was observed on medium 78. Lastly *E. umbeluziensis*
774 megagametophytes, incubated on medium 8 and White's medium at 35 ºC under light
775 conditions, produced small irregular overgrowths of callus. This raises questions
776 whether other *Encephalartos* species would respond similarly to *E. umbeluziensis* in
777 *in vitro* culture.

Subsequent studies of somatic embryogenesis and organogenesis were conducted by Jäger and van Staden (1996 a & b) on *E. cycadifolious*, *E. dyerianus* and *E. natalensis,* where zygotic embryos were used as explants. In these three species Jäger and van Staden (1996 a & b) initiated callus on medium composed of Gamborg B5 major salts (Gamborg et al., 1968) and MS basal media (Murashige & Skoog, 1962) with minor salts plus vitamins. They further supplemented medium with various amino acids, sucrose and PGRs, and maintained in 26 ºC in darkness. After 5 months of culture, they used the initiated callus to induce somatic embryogenesis on a medium supplemented with various combinations and concentrations of 2,4-D and Kinetin, thereafter cultures containing suspensor stage embryos were transferred to 16h photoperiod. Furthermore, they carried out embryo maturation in medium supplemented with abscisic acid (ABA) and lastly, rooting in PGR free medium. Jäger and van Staden (1996 a & b) acquired rapid callus growth in induction medium for *E. cycadifolious,* with the embryos turning green with exposure to light. The suspensors however desiccated when the embryos were transferred to a maturation medium with ABA, resulting in 10% of *E. cycadifolious* embryos successfully rooting within 4-5 weeks of incubation in PGR-free medium. *Encephalartos dyerianus* formed somatic embryos on media with low concentrations of PGRs or no PGR and in *E. natalensis* somatic embryos were formed in media with the same concentration of 2,4-D and Kinetin as the induction medium (Jäger & van Staden, 1996a). Somatic embryos of both *E. dyerianus* and *E. natalensis* began as proembryos, developing suspensors and finally a dicotyledonary embryo developed at the distal end of the suspensor. Maturation of the embryos with radicle development was not acquired (Jäger & van Staden, 1996b). Although there were no successful plantlet regenerations, followed by acclimatization achieved by Jäger and van Staden (1996 a & b) on *Encephalartos*

803 species, the induction of embryogenic cultures and recovery of somatic embryos was
804 possible. This indicates a potential for the successful regeneration of *Encephalartos*
805 species in future studies, as such, *in vitro* culture can be used to improve the
806 propagation of highly threatened cycads.
807
808 Numerous plant parts have been used as explants for *in vitro* research on
809 cycads, with a majority of studies using megagametophytes and zygotic embryos as
810 explants (Table 2.1). The responses in each of these studies differs on account of
811 differences in the explants being used, the focal species, media type, supplementation
812 with PGRs at varied concentrations, and different light conditions. Most commonly
813 used PGRs in cycad *in vitro* studies are cytokinins (Kinetin and 6-Benzylaminopurine-
814 6-BAP), and auxins (2,4-Dichlorophenoxyacetic acid (2,4-D), 1-Naphthaleneacetic
815 acid (NAA), Indole-3-butyric acid (IBA) and Indole-3-acetic acid (IAA)). Cytokinins are
816 predominantly for cell division and shoot formation while auxins are associated with
817 bud formation and root initiation (Müller & Leyser, 2011). With the cycad species
818 tabulated in Table 2.1, it is evident that each species responds differently to PGR and
819 various PGR concentrations used. As such, it would be beneficial to determine the
820 optimum PGRs, light and nutrient sources (sucrose) for specific cycad species.
821
822 The induction of callus reported in several studies that used
823 megagametophytes and zygotic embryos as explants, was caused by the presence of
824 PGRs such as Kinetin, 6-BAP and NAA in plant growth medium (La Rue, 1950; La
825 Rue, 1954; Webb et al., 1983 and Pan et al., 2003). Other PGRs such as 2,4-D have
826 demonstrated a negative effect when used in high concentrations, such as the
827 inhibition of root development in *E. umbeluziensis* (De Luca et al., 1979). However, in

828 *D. edule* megagametophytes and zygotic embryos, the presence of 2,4-D in
829 combination with Kinetin was shown to result in callus induction in both explants, which
830 later formed shoots via indirect organogenesis. In *C. revoluta, a*dventitious shoot
831 regeneration through direct and indirect organogenesis was reported in the presence
832 of 0.5 mg/L 6-BAP after 35 days in culture (Naderi et al., 2015), whereas in preliminary
833 studies (Malwane, unpublished data) on *Encephalartos* species, the same
834 concentration of 6-BAP was shown to induce callus with no further regeneration.
835 These results are clearly indicating the role PGRs in *in vitro* culture and how the same
836 PGR can have different growth response on different species.

837 The effect of light has a crucial role in *in vitro* culture. The effects of light and
838 darkness were demonstrated on seedlings of *C. revoluta*, *E. altensteinii* and *Z.*
839 *furfuracea* by Webb et al., (1984), where they showed that secondary root formation
840 and root elongation was inhibited for seedlings that were maintained under fluorescent
841 light. Similar results on light inhibiting root formation have also been demonstrated in
842 *Z. pumila* and *D. edule* seedlings (Webb 1982a; Webb 1982b). Further *in vitro* studies
843 conducted by Rinaldi (1999), tested different factors that affect shoot regeneration of
844 *C. revoluta*, from zygotic embryo and seedling explants – in which darkness was
845 reported to promote callus induction, while light promoted shoot induction. All these
846 previous studies demonstrated that light and darkness have significant roles in the
847 regeneration of explants, therefore, these factors will be tested in this study on the
848 regeneration of *Encephalartos* embryos.

849 To date, only two studies have successfully acclimatised cycad plantlets grown
850 *in vitro,* with both studies on *C. revoluta.* In the first study, mature zygotic embryos
851 were induced and grown in Schenk and Hildebrandt (SH) medium supplemented with
852 3.0% sucrose, 20% coconut milk, 0.6% agar and PGRs 6-BAP (0.2 - 2.0 mg/L) and

2,4-D (0 - 0.2 mg/L) in combination. It was reported that regenerated plantlets were successfully acclimatized 196 days after culture initiation (Motohashi et al., 2008). In the second study, Naderi et al. (2015) published a simplified regeneration protocol for *C. revoluta* Thunb. using mature zygotic embryos. Explants were incubated on half-strength Murashige and Skoog (½ MS) basal medium supplemented with 0.5 mg/L (6-BAP, Kinetin and 2,4-D), alone or in combination. They observed adventitious shoot regeneration after 35 days of incubation in the presence of 0.5 mg/L 6-BAP. Thereafter, they achieved root induction in ½ MS at 0.1 mg/L NAA. Regenerated plantlets were successfully acclimatised after 185 days of culture, resulting in 10% success rate (Naderi et al., 2015). The successful regeneration of *C. revoluta* suggests that *in vitro* regeneration for threatened *Encephalartos* species is possible. However, the protocol used for *C. revoluta* may need to be adjusted to suit regeneration of *Encephalartos* species.

The level of research conducted on *in vitro* tissue culture of cycads indicates that *in vitro* techniques is promising method for the mass production and conservation of threatened cycad germplasm. The success reported on the regeneration of *C. revoluta* zygotic embryos by Naderi et al. (2015), is an important reference point for this current research. Although, currently no known cycad grower is reported to be using the *in vitro* technique in the commercial industry. Furthermore, the protocol was found to be ineffective in preliminary testing for *Encephalartos* species as it resulted in less than 10% embryo regeneration (Malwane, unpublished data). Several problems were identified; such as high percentage of contamination, death of embryos due to browning (necrosis) as well as the induction of callus that did not regenerate any structures. However, by adjusting the protocol used for *C. revoluta,* these problems can potentially be resolved.

As a result of the above noted observations, for this current research, adjustments will include; a) increased time to sterilize explants to minimize contamination, b) light to promote embryo callusing, root and shoot induction, c) the addition of various sucrose concentrations and d) addition of activated charcoal (AC) in culture medium to alleviate necrosis and media browning. The ultimate goal of this study is to achieve an *ex situ* conservation method for *Encephalartos* species through cryopreservation of embryos or germplasm. However, this is the longer term objective which will be informed by the results of the current work. This study aims to develop a protocol for the regeneration of *E. altensteinii* and *E. manikensis* embryos through *in vitro* tissue culture. Because embryo growth is controlled by a number of factors, such as, but not limited to light, nutrients, and phytohormones, it is also important to know and assess the levels endogenous phytohormone of the embryos, as these levels would allow or hinder embryos from regenerating in *in vitro* tissue culture, therefore, chapter 3 will cover the phytohormone profiling of *Encephalartos altensteinii* seeds.

The seeds of the species selected were between 8-12 months old, postharvest. According to Giddy (1974) and Woodenberg et al. (2007), *Encephalartos* seeds, depending on species, mature between four to seven months after disintegrating from the cones and thereafter are ready for germination. Since the selected seeds for these experiments are older than the predicted ages where they are ready for germination, they are assumed to be mature and ready for germination and therefore expected to regenerate in *in vitro* tissue culture. *Encephalartos altenstinii* and *E. manikensis* were selected based on seed availability and adequacy at Kirstenbosch National Botanical Gardens (KNBG). Furthermore, these two species are from different *Encephalartos* clades, originating from different climates, and are therefore potentially good representatives for the *Encephalartos* genus.

To achieve the objective of this study, the following questions were asked; 1) Can *E. altensteinii* and *E. manikensis* embryos regenerate using known media for *in vitro* tissue culture?; 2) Do different sucrose concentrations, PGRs and light influence the regeneration, growth and development of embryos in culture? and; 3) Can *in vitro* regenerated *E. altensteinii and E. manikensis* plantlets be successfully acclimatized and survive *ex vitro*?

2.2. Materials and Methods

Seed collection and viability

This study was conducted at Kirstenbosch Research Centre (KRC), South African National Biodiversity Institute (SANBI), Cape Town between 2017 and 2018. Seeds used for this study were sourced from *E. altensteinii* and *E. manikensis* cultivated at Kirstenbosch National Botanical Gardens (KNBG). *Encephalartos* seeds were artificially pollinated using the wet pollination method (Calonje et al., 2011). *Encephalartos* cones were collected upon natural cone disintegration, the sarcotesta was cleaned off and the seeds were subjected to a water floating test, by placing all *Encephalartos* seeds in a large container filled with water, such that water covered all seeds. Seeds were allowed to stand for a few minutes. Floating seeds were regarded as non-viable, as the floating may have been due to seeds having unfilled spaces, no embryos or damaged due to pest or pathogens. The floaters/non-viable seeds were discarded, while the sinking seeds were regarded as potentially viable. Potentially viable seeds were then smeared with Virikop Efekto Fungi-Nill 500 WP ® Captab (Dicarboximide) and placed in a breathable plastic mesh bag. Finally the seeds were stored for 8-12 months under dry conditions, in the seed room maintained at 15°C and 15% relative humidity (RH). The water float viability test was repeated again after seed storage, prior to use. However, many of these seeds did not contain embryos (see below 2.2.1.1).

Embryo viability testing

Tetrazolium (TZ), 2,3,5-tri-phenyltetrazolium chloride is commonly used as a redox indicator of cellular respiration (Berjak & Pammenter, 2014); Roistacher et al., 2014).

In this study, TZ was used to differentiate between metabolically active and inactive tissues to determine viability. A solution of 1% 2,3,5-triphenyltetrazolium chloride was prepared using distilled water. Seeds (5 per species) of both *E. altensteinii* and *E. manikensis* were randomly selected and cracked open using a nut cracker in order to separate the sclerotesta (seed coat) from the whole seed. The megagametophytes were sectioned longitudinally and the embryos were rescued. Five embryos per species were placed in a vial containing 20 ml of the TZ solution. The vials were sealed and embryos allowed to soak for 30 min prior to examination. Sample size (n = 5) for each species.

Preparation of Plant Growth Regulator stock solutions

Stock solutions for Indole-3-butyric acid (IBA), Kinetin and 6-Benzylaminopurine (6-BAP) were prepared in a similar manner. Each stock solution with a concentration of 100 mg/ml was prepared by dissolving a 100 mg of the PGR powder in 2 - 5 ml 1N NaOH, this being brought to a volume of 100 ml with dH_2O. The stock solutions were stored at 4 ºC and utilized within a week of preparation.

Media preparation

Murashige and Skoog (MS) basal culture medium at half the concentration (2.2 g/L) with vitamins and macro- and microelements (Murashige & Skoog, 1962) was prepared, with minor amendments according to that outlined in Naderi et al., (2015). Amendments included use of three different sucrose concentrations (0, 15 and 30 g/L) in order to establish the optimum sucrose concentration suitable for *Encephalartos* embryo growth and development and the use of 5.8 g/L activated charcoal to minimize embryo browning. Two PGRs (Kinetin and 6-BAP, individually and in combination,

each at 0.5 mg/L) were supplemented to the media. All media were used within a week of preparation. A summary of protocols used is shown in Table 2.2 and 2.3.

Table 2.2: *Encephalartos altensteinii* and *E. manikensis* embryos cultured and maintained in growth chambers with 24h of darkness and light for six weeks. Plant growth media was supplemented with sucrose concentrations at (0, 15 and 30 g/L) and plant growth regulators (PGRs) Kinetin and 6-BAP either individually or in combination at 0.5 mg/L. The sample size of each treatment was (n = 7) and each treatment was repeated three times. Treatments carried out are represented by x on the table.

PGR (0.5 mg/L)	**Treatments**	**24h Light Growth Chamber**			**24h Dark Growth Chamber**		
		Sucrose (g/L)					
		0	**15**	**30**	**0**	**15**	**30**
E. altensteinii							
Control	1	x			x		
	2		x			x	
No PGR	3			x			x
Kinetin + 6-BAP	4	x			x		
	5		x			x	
	6			x			x
	7	x			x		
Kinetin	8		x			x	
	9		x			x	
6-BAP	10			x			x
E. manikensis							
Control	1	x			x		
	2		x			x	
No PGR	3			x			x
Kinetin + 6-BAP	5	x			x		
	6		x			x	
	7			x			x
6-BAP	8	x			x		

Table 2.3: *Encephalartos altensteinii* and *E. manikensis embryos* cultured and maintained in growth chambers with 16h photoperiod for six weeks. Plant growth media was supplemented with 15 g/L sucrose and plant growth regulators (PGRs) Kinetin and 6-BAP either individually or in combination at 0.5 mg/L. The sample size of each treatment was (*n* = 10) and each treatment was repeated three times. Treatments carried out are represented by x on the table.

16h Photoperiod Growth Chamber		
2.2 g/L MS Basal Media **10 g/L Agar** **15 g/L Sucrose**		
Treatments	*E. altensteinii*	*E. manikensis*
Control (No PGR)	x	x
0.5 mg/L Kinetin	x	
0.5 mg/L 6-BAP	x	
0.5 mg/L Kinetin + 0.5 mg/L 6-BAP	x	x
1.0 mg/L Kinetin + 1.0 mg/L 6-BAP		x

Explant Sterilization

Seeds were rinsed under running tap water for a minute before being placed in 30% sodium hypochlorite (NaOCl) and allowed to surface sterilise for 30 min, shaking at 140 revolutions per minute (rpm). The sclerotesta were removed under a lamina flow and megagametophytes were further sterilized in 70% ethanol for two minutes, followed by another rinse in 50% dilution of NaOCl containing 2-3 drops of Tween-20 for 30 minutes. The megagametophytes were further rinsed three times with sterile dH_20 for 10 minute each. This protocol was adopted from (Naderi *et al.,* 2015), however, surface sterilization of the seeds in 30% NaOCl was increased from 20 min to 30 min.

Initiation and maintenance of *Encephalartos* embryos in culture medium

2.2.1.1. Initiating *Encephalartos* embryo regeneration

As outlined above some seeds selected for experimentation did not have embryos. In *E. altensteinii* 417 of 1205 seeds contained embryos whereas in *E. manikensis* 261 embryos were obtained from 763 seeds. Due to the shortage of embryos, some treatments were not carried out for *E. altensteinii* (30 g/L sucrose in the Kinetin treatment and 0 g/L sucrose in the 6-BAP treatment) and for *E. manikensis* ((0, 15 and 30) g/L and (15 and 30) g/L in the Kinetin and 6-BAP treatments respectively (Table 2.2). As for the control, embryos were plated on sucrose and PGR free media. After sterile excision embryos were plated on the various media outlined above (Table 2.2) and were maintained in two different growth chambers, one in constant dark, and the other in constant light for 24h a day, for a period of six weeks. Both growth chambers were maintained at room temperature (26 ± 1 °C) at a low photosynthetic photon flux density of 4 µmol $m^{-2}.s^{-1}$. There were seven embryos per treatment, and each treatment was replicated three times.

2.2.1.2. Maintenance, growth and development of *Encephalartos* embryos in culture medium

Contamination-free embryos that survived the initial six weeks of culture, having either roots, shoots, callus or showed no changes were subcultured onto fresh plant growth media. Here, the media was similar to that outlined above, half strength MS medium was supplemented with 15 g/L sucrose and in each instance, embryos were transferred to same concentrations of PGRs (or not, in the case of controls) outlined above (Table 2.3). All cultures were maintained in a growth chamber with a 16h photoperiod at room temperature (26 ± 1 °C) at a photosynthetic photon flux density of 4 µmol m^{-2} s^{-1}. The responses of the *E. altensteinii* embryos that were initially in the

growth chamber with 24h constant of darkness were compared with the embryos that were initially in the growth chamber with 24h constant of light. For *E. manikensis* embryos, the differences in responses to the different PGR concentrations were compared. There were ten embryos per treatment, and each treatment was replicated three times.

Observations: Recording data

Cultured embryos were observed once a week over a six week period, during which changes in embryo colour, degree of callusing, browning and death was recorded. Germination was recorded as embryos that developed shoots and/or roots. Proportions of embryos that showed germination, callusing, necrosis and contamination (as shown in Figure 2.2) were recorded as categorical variable of either Yes or No.

Effects of wounding treatments on *in vitro* growth, callus rescue and regeneration of *E. altensteinii*

Wounding of plant tissue can sometimes improve cell differentiation, this differentiation is achieved when endogenous hormones are transferred to the location of the wound, thus causing production of callus (Xu et al*.,* 2011). This part of the experiment was conducted to examine the effects of wounding then treating embryo-derived *in vitro* plantlets with PGR, in order to enhance *in vitro* growth induction. Furthermore callus was harvested and subcultured in media with varying PGRs to enhance *in vitro* growth induction. Embryo-derived *in vitro* plantlets of *E. altensteinii* were used in this experiment. After a 12 week incubation period plantlets that had regenerated roots and shoots or ones that did not have both roots and shoots were subjected to

1031 wounding, by cross sectioning the apical meristem. There were three treatments,
1032 control (no wounding and no application of PGR) and application of PGRs in the
1033 wounded parts, one hour and 4 h immersion in PGR.

1034 Embryo-derived *in vitro* seedlings of *E. altensteinii* were treated with different
1035 PGRs. *In vitro* plantlets that had regenerated roots, callus and no adventitious roots
1036 or shoots were wounded and treated in a PGR mixture of 0.5 mg/L Kinetin + 0.5 mg/L
1037 6-BAP and transferred to ½ MS basal culture medium with vitamins and macro- and
1038 microelements (Murashige & Skoog, 1962), 15 g/L sucrose, 10 g/L agar and
1039 supplemented with Kinetin + 0.5 mg/L 6-BAP for shoot induction. *In vitro* seedlings
1040 that had regenerated shoots were wounded and treated in 0.5 mg/L IBA and
1041 transferred to ½ MS basal culture medium with vitamins and macro- and
1042 microelements (Murashige & Skoog, 1962), 15 g/L sucrose, 10 g/L agar and
1043 supplemented with 0.5 mg/L IBA for root induction. Sample sizes varied from (n =7) to
1044 (n = 9).

1045 ***Ex vitro* hardening and acclimatization of** *E. altensteinii* **and** *E. manikensis*

1046 *In vitro* derived plantlets with well-developed shoot and root systems were removed
1047 from the culture, washed with distilled water to remove traces of excess media,
1048 transplanted into plastic pots filled with perlite and vermiculite (1:1 ratio) and covered
1049 with transparent polyethylene bags to ensure high humidity. Plantlets were placed in
1050 a walk-in culture room maintained at 16h photoperiod, 26 ± 1 °C at a low
1051 photosynthetic photon flux density of 4 µmol $m^{-2}.s^{-1}$ for 2 weeks (Naderi et al., 2015).
1052 Thereafter, the plantlets were placed in a mist house, maintained at ambient
1053 temperature with water sprinklers every 30 min, for two weeks. *In vitro* derived
1054 plantlets were then transferred to a greenhouse with natural temperature and natural

photoperiodic conditions for a period of 300 days after initial culture, after which the experiments were suspended. Plantlets were monitored weekly and watered with tap water when necessary.

Statistical analyses

Equation 1 was used to calculate the proportion of rooting embryos in culture as:

$$R_p = \frac{R}{Te}$$

Where R_p is the rooting proportion, R is the number of rooting embryos and Te is the total number of embryos in culture.

Equation 2

$$S_p = \frac{S}{Te}$$

Equation 3

$$Ca_p = \frac{Ca}{Te}$$

Equation 4

$$D_p = \frac{D}{Te}$$

Equation 5

$$Co_p = \frac{Co}{Te}$$

Equation 2, 3, 4 and 5 were used to calculate the proportions of shooting, callusing, death due to browning and contamination of the embryos in culture. Where S_p, Ca_p, D_p and Co_p are the proportions of shooting, callusing, death due to browning and contamination of the embryos in culture. *S, Ca, D* and *Co* are the numbers of shooting,

1075 callusing, dead due to browning and contaminated embryos in culture, and *Te* is the
1076 total number of embryos in culture. These proportions were calculated in order to
1077 determine the responses of the embryos in culture.

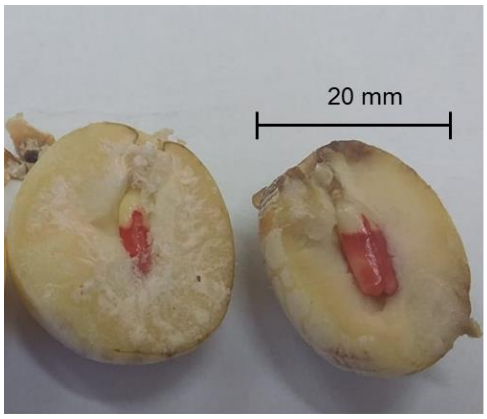
1078 To determine the conditions that allow germination of cycad embryos in *in vitro*
1079 tissue culture, generalised linear models with beta distribution for proportional data
1080 were employed using the *betareg* package in R (Zeileis et al., 2016). Proportions of
1081 germinating, rooting, shooting, callusing and dead embryos were modelled separately
1082 as the response variables, with sucrose concentration, PGR and light were used as
1083 fixed variables. A series of models were constructed and the Akaike information
1084 criterion (AIC) was used to select the best model (Akaike, 1973). A simpler model with
1085 the lowest AIC and a difference of more than two was selected as the best model.

1086

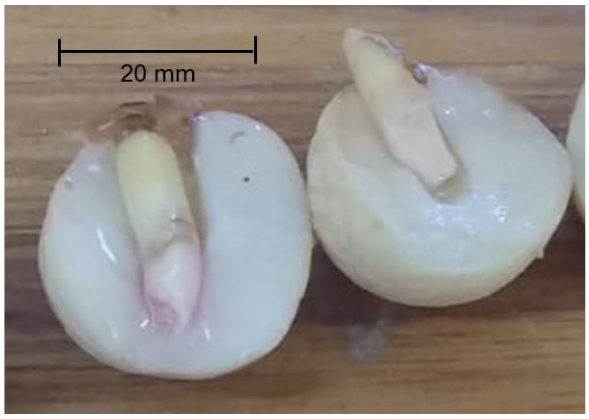
2.3. Results

Embryo viability

All the treated embryos of both *E. altensteinii* and *E. manikensis* stained red, within 15 to 30 min after being placed in TZ indicating their viability (Figure 2.1).



a



b

Figure 2.1: Tetrazolium test (a) viable *E. manikensis* embryos, (b) non-viable and non-stained *E. altensteinii* embryos

Initiating embryo regeneration: Initial six weeks of culture for *Encephalartos altensteinii* embryos

A total of 417 *E. altensteinii* embryos were incubated under different culture conditions to initiate germination, 210 embryos were exposed to constant light for 24h and 207 embryos were exposed to constant darkness for 24h, for a period of six weeks. After the first week of incubation, the most notable change was the colour of the embryos exposed to light 24h, where 100% of embryos turned green in colour (Figure 2.2b & d), regardless of the Plant Growth Regulator (PGR) and sucrose concentrations applied in media. All the embryos exposed to 24h darkness, did not change colour, they retained their original colour as the first day of culture (Figure 2.2a). During the

second week of culture, the most notable change observed was the thickening of the embryos in > 90% of 417 embryos irrespective of the PGR and sucrose treatments or dark/light exposure. Embryos were observed to elongate and develop roots, and at the end of six weeks ~ 59% of 417 embryos had developed roots (Figure 2.2d), regardless of the treatment or light exposure. Shoot formation was evident on the fourth week of incubation, where ~ 14% of 417 embryos had developed shoots (Figure 2.2a). Callus formation on the embryos was evident from the second week in culture, and at the end of six weeks incubation period ~ 19% of 417 embryos had formed callus (Figure 2.2b), *Encephalartos altensteinii* embryos regenerated roots and shoots either by direct or indirect organogenesis from callus. In cases where the embryos formed callus first, the embryos were able to further regenerate shoots, however no root regeneration was observed in the presence of callus.

The main cause of embryo death was contamination and necrosis (Figure 2.2c). Embryo contamination was evident in the second week of incubation and increased as the weeks progressed, with overall contaminated embryos was ~ 28%, at the end of six weeks of incubation. Embryo necrosis was recorded to be ~ 23% embryos at the end of six weeks. There were also embryos that did not develop roots, shoots and callus throughout the duration of the six weeks of incubation, although embryos remained healthy and free of contamination. All the changes noted in all the embryos in culture (except colour changes), were irrespective of the treatments and light exposure.

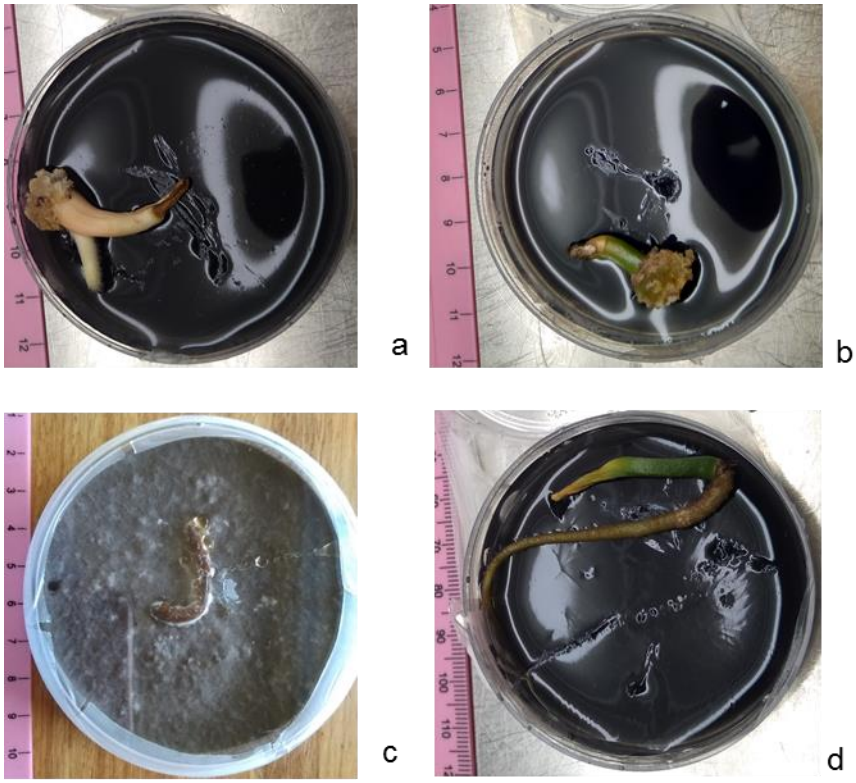


Figure 2.2: *Encephalartos altensteinii* embryo regeneration after six weeks in culture. (a) Embryo with callus and shoot, maintained in a growth chamber kept at constant darkness for 24h, (b) callusing embryo maintained in a growth chamber kept at constant light for 24h, (c) embryo with contamination and necrosis and (d) rooting embryo from a growth chamber kept at constant light for with 24h.

Table 2.4: Results of generalised linear models with beta distribution for proportions of *E. altensteinii* embryo rooting, shooting, callusing, contamination and death due to browning. The change in AICc (ΔAICc) between the best model and the next best and the null model are also given. In cases where there was more than one best possible model, these are reported as “Model1” and “Model2”. Psuedo R^2 measuring the variation explained by the fixed variables, (n = 417).

Embryo Response	Model	AIC (Best model)	ΔAIC (2nd best model)	ΔAIC (Null model)	Pseudo R^2
Rooting	Best Model ~ PGR** Model equation: $Y = e^{-0.66+0.15NP-0.40B+0.31KB+0.62K}$ 2nd Best Model~PGR* + Sucrose Model equation: $Y = e^{-0.66 + 0.004NP - 0.54B + 0.21KB + 0.57K + 0.01S}$	-99.094	1.0	24.59	0.08
Callusing	Best model ~ Env.con:Sucrose** + PGR + Sucrose*** Model equation: $Y=e^{-2.96 + 0.35L - 0.04S - 0.25NP - 0.29B - 0.11KB - 0.35K - 0.3L:S}$ 2nd Best model ~ Env.con + Sucrose*** + PGR Model equation: $Y = e^{-2.76 - 0.72L + 0.02S - 0.21NP – 0.24B - 0.09KB - 0.30K}$	-1312.05	7.89	14.24	0.13
Contamination	Best model ~ PGR*** Model equation: $Y=e^{-1.78 + 0.16NP - 0.68B + 0.13KB + 0.22K}$ 2nd Best model~Env.con + PGR*** + Sucrose Model equation: $Y = e^{-1.77 - 0.03L - 0.02NP -0.84B + 0.03KB + 0.17K + 0.01S}$	-780.36	2.2	36.0	0.19
Necrosis	Best Model ~ PGR* Model equation: $Y=e^{-2.29 + 0.005NP - 0.41B + 0.27KB + 0.16K}$ 2nd Best Model ~ Env.con + PGR* Model equation: $Y = e^{-2.36 + 0.11L + 0.02NP - 0.39B + 0.30KB + 0.17K}$	-1086.52	0.9	16.0	0.13
Shooting	Best model (Null) AIC = -2072.9, Δ AIC 2nd best model = 5.3, Δ AIC 3rd best model = 7.9 Model equation: $Y = e^{-3.86}$				
Abbreviations: NP = No PGR, B = 6-BAP, K = Kinetin, KB = Kinetin+6-BAP, S = Sucrose, L = Light					

1138

Embryo rooting was significantly affected by PGRs. Rooting was significantly higher in treatments with 0.5 mg/L Kinetin (Z = 2.7, P = 0.006) (Figure 2.3 and Table 2.4).

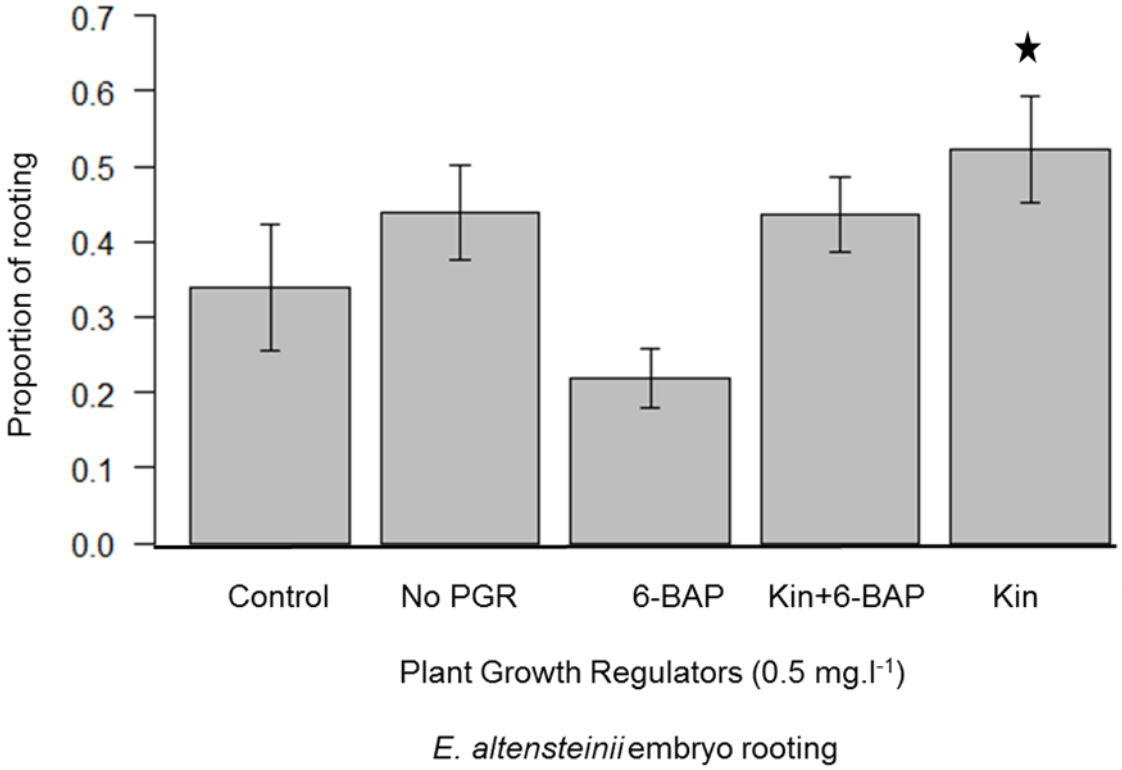


Figure 2.3: Proportion of rooting of *E. altensteinii* embryos in the first six weeks of incubation, obtained from generalised linear models with beta distributions. Embryo rooting was significantly high in 0.5 mg/L Kinetin (P = 0.006), significance is indicated by ★ . n = 7; bars represent Standard Error (±SE).

The fixed variables that were evaluated in this study, which were light(exposure to light or darkness), PGRs and sucrose concentration did not have any significant influence on the formation of shoots on *E. altensteinii* embryos. The best model for embryo shooting was indicated by the Null model (Table 2.4).

Embryo callusing in *E. altensteinii* was affected by lightand sucrose. In both growth chambers callusing significantly increased with an increase in sucrose concentration

(*Z* = 5.4 *P* = 0.0001), however this increase was lower in the growth chamber with light, compared to the growth chamber with darkness (*Z* = -3.24, *P* = 0.001) (Figure 2.4 and Table 2.4).

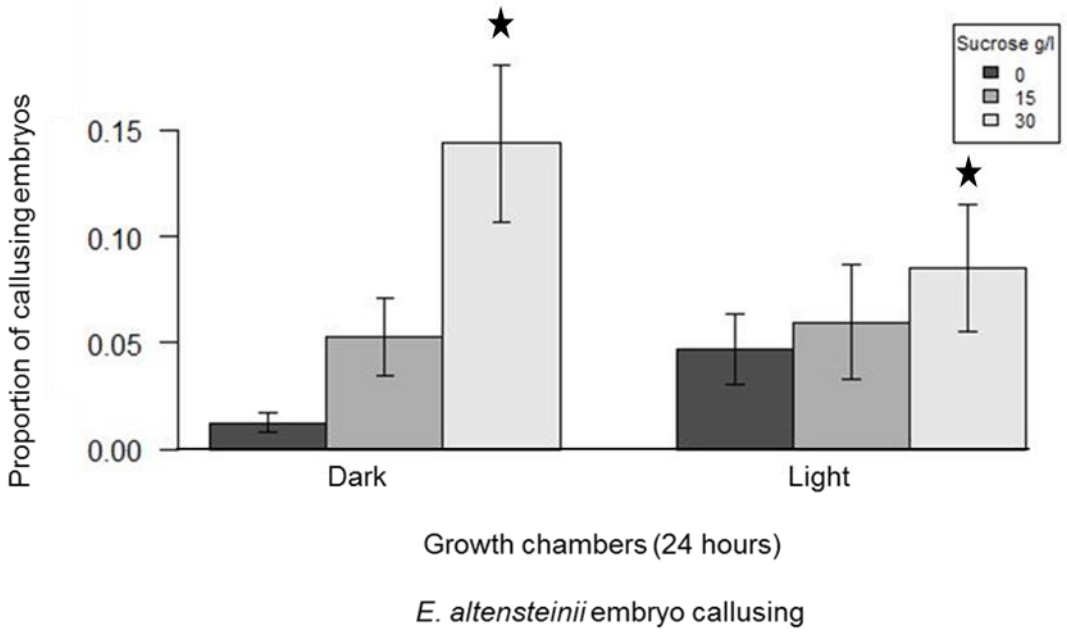
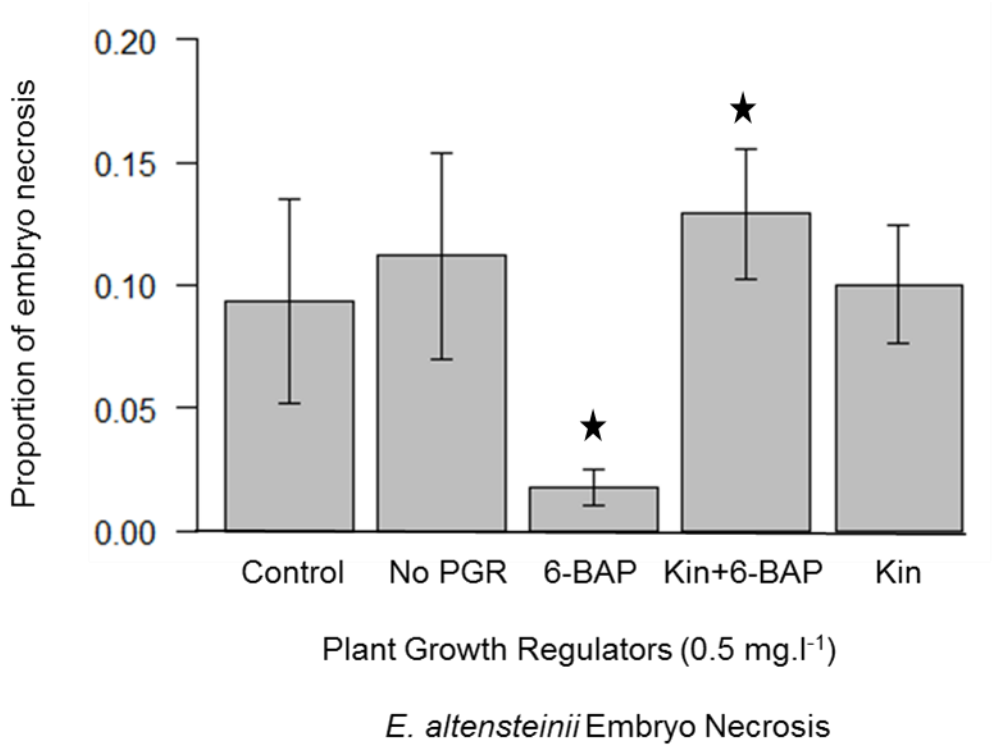


Figure 2.4: Proportion of callusing *E. altensteinii* embryos in the first six weeks of incubation, obtained from generalised linear models with beta distributions affected by light and sucrose. Embryo callusing increased sucrose concentration (*P* = 0.0001), significance is indicated by ★. *n* = 7; bars represent Standard Error (±SE).

Embryo necrosis was significantly affected by PGRs (Table 2.4). It was significantly lower in treatments with 0.5 mg/L 6-BAP (*Z* = -2.8 *P* = 0.005), with less than 5% dead embryos, and significantly higher in treatments with 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP (Z = 2.1 P = 0.04) with ~13% dead embryos (Figure 2.5; Table 2.4).

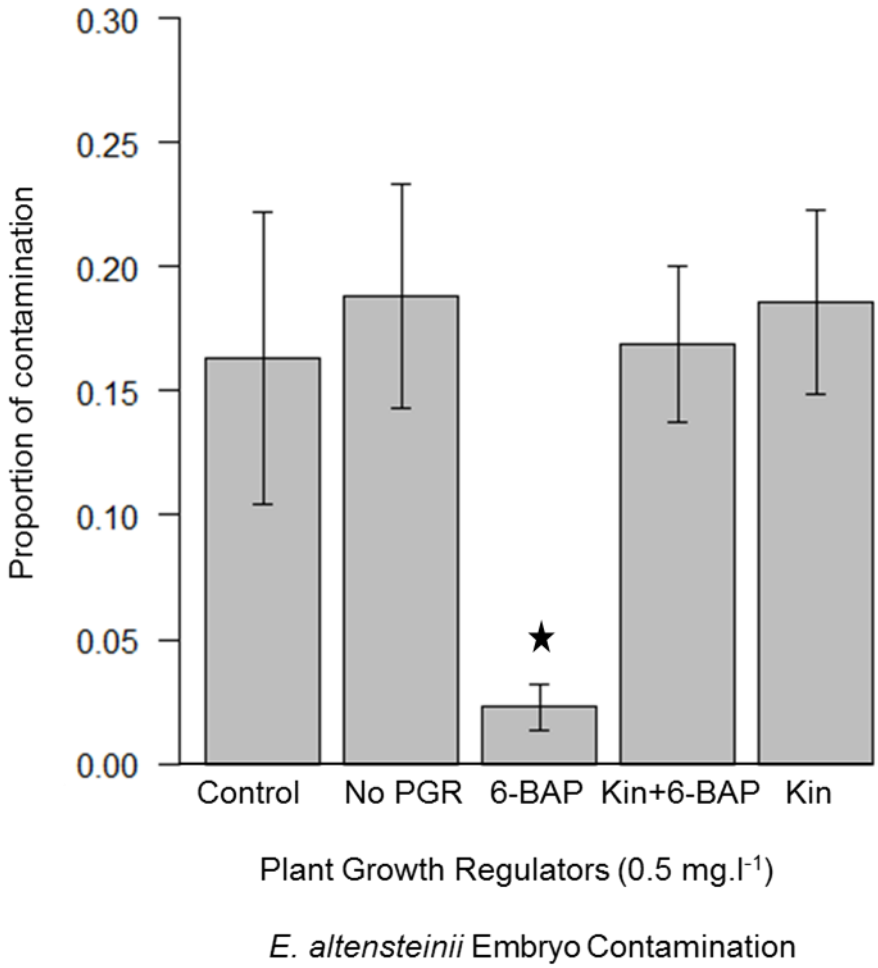


1165

1166 Figure 2.5: Proportion of *E. altensteinii* embryos displaying necrosis in the first six
1167 weeks of incubation, obtained from generalised linear models with beta distributions.
1168 Embryo necrosis was significantly low in 0.5 mg/L 6-BAP (P = 0.005), and significantly
1169 high in 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP (*P* = 0.04), significance is indicated by ★.
1170 *n* = 7; bars represent Standard Error (±SE).

1171

1172 The results further show that *E. altensteinii* embryo contamination was not significantly
1173 affected by light and sucrose concentrations (Table 2.4). In contrast, embryo
1174 contamination was significantly affected by PGRs. The treatments with 0.5 mg/L 6-
1175 BAP had significantly lower embryo contamination (*Z* = -3.40, *P* = 0.0007), compared
1176 to the other treatments (Figure 2.6).



1177

1178 Figure 2.6: Proportion of contaminated *E. altensteinii* embryos in the first six weeks of
1179 incubation obtained from generalised linear models with beta distributions.
1180 Contamination was significantly low in 0.5 mg/L 6-BAP, significance is indicated by ★
1181 . *n* = 7; bars represent Standard Error (±SE).

1182

1183 **Growth and development of *E. altensteinii* embryos: After subculture, (Overall**
1184 **12 weeks embryo incubation).**

1185 After the initial six weeks of culture, ~ 177 (42.4%) of the 417 *E. altensteinii* embryos
1186 were discarded due to necrosis and contamination. All embryos that were not
1187 contaminated, composed of embryos that had regenerated roots, shoots, callus or had

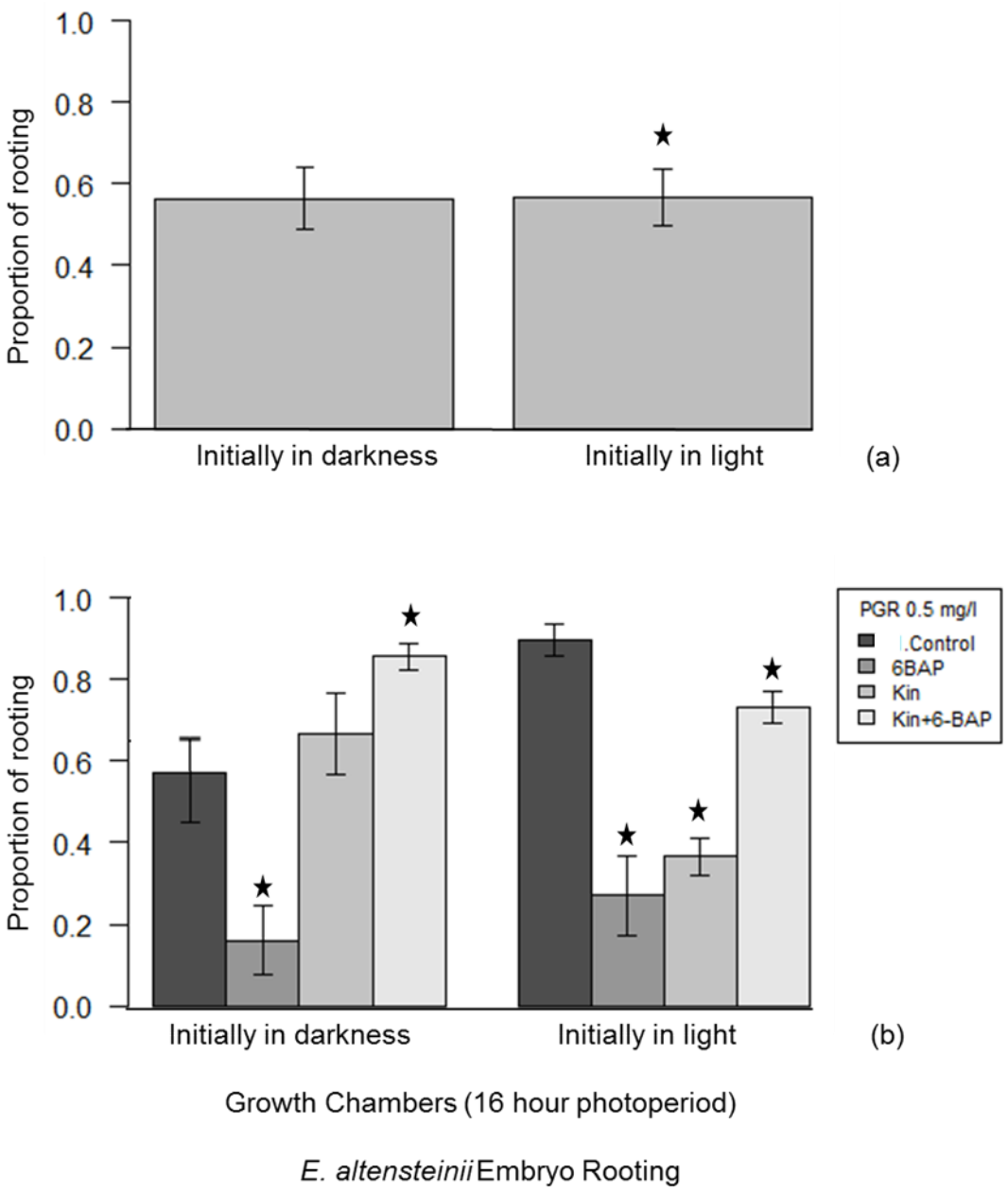
not regenerated any roots shoots nor callus, obtained across all the treatments in Table 2.1. These were subcultured onto fresh media as per Table 2.2, and maintained for a further six weeks. At the end of this incubation period (total 12 weeks), 103 of the 240 subcultured embryos were lost due to necrosis and infection. The surviving 137 of 240 embryos had neither shoots, root nor callus (30) or had regenerated roots only (65), shoots only (10), callus only (8), a combination of roots and shoots (9) and lastly shoots and callus (15). These embryos were used for the wounding experiments. The results below were obtained after subculture; after 12 weeks of initial incubation.

Table 2.5: Results of generalised linear models with beta distribution for proportions of *E. altensteinii* embryo germination, callusing and dead after subculture. The change in AICc (ΔAICc) between the best model and the next best and the null model are also given. In cases where there was more than one best possible model, these are reported as “Model1” and “Model2”. Psuedo R^2 measuring the variation explained by the fixed variables. Sample size (*n* = 240).

Response	Model	AIC (Best model)	ΔAIC (2nd best model)	ΔAIC (Null model)	Pseudo R^2
Rooting	Best Model ~ Env.con:PGR *** Model equation: $Y = e^{0.26 + 1.71L - 2.20B + 0.46K + 1.19KB - 1.19L:B - 2.91L:K - 2.31L:KB}$ 2nd Best Model~Env.con + PGR*** Model equation: $Y = e^{1.06 + 0.05L - 0\ 2.61B - 0.97K - 0.03KB}$	-159.9	48.1	156.9	0.65
Shooting	Best Model ~ Env.con:PGR *** Model equation: $Y = e^{-0.66 - 0.62L - 2.25B - 1.47K - 1.18KB + 1.70L:B + 0.49L:K\ 1.33L:KB}$ 2nd Best Model~Env.con + PGR*** Model equation: $Y = e^{-1.06 + 0.18L - 1.36B - 1.16K - 0.48KB}$	-295.9	17.4	60.5	0.42
Callusing	Best Model ~ Env.con:PGR *** Model equation: $Y = e^{-2.22 - 0.8L + 1.45B + 0.07K + 0.32KB + 0.06L:B + 0.41L:K + 1.82L:KB}$ 2nd Best Model~Env.con + PGR*** Model equation: $Y = e^{-2.4 - 0.25L + 1.41B + 0.25K + 0.47KB}$	-374.2	30.2	83.4	0.41
Contamination	Best Model ~ Env.con:PGR *** Model equation: $Y = e^{-1.78 - 1.03L - 1.3B - 1.03K - 1.03KB + 1.3L:B + 0.76L:K + 1.13L:B}$ 2nd Best Model ~ Env.con + PGR** Model equation: $Y = e^{-2.14 - 0.26L - 0.64B - 0.61K - 0.45KB}$	-552.6	4.8	7.8	0.27
Necrosis	Best Model ~ Env.con:PGR ** Model equation: $Y = e^{-0.41 - 1.06L - 0.93B + 0.04K - 037KB + 1.53L:B + 0.42L:K + 0.83L:KB}$ 2nd Best Model~Env.con + PGR* Model equation: $Y = e^{-0.75 - 0.36L - 0.18B + 0.26K - 0.06KB}$	-84	4.8	3.9	0.11
Abbreviations: NP = No PGR, B = 6-BAP, K = Kinetin, KB = Kinetin + 6-BAP, S = Sucrose, L = Light					

1203

Table 2.5 shows that *E. altensteinii* embryo rooting was significantly affected by light and PGRs. Embryo rooting was significantly higher in the embryos that were initially maintained in the growth chamber with 24h of light ($Z = 5.9$, $P < 0.0001$), compared to the embryos that were initially maintained in the growth chamber with 24h of darkness (Figure 2.7a). The treatments with 0.5 mg/L 6-BAP had significantly lower rooting ($Z = -7.4$, $P < 0.0001$), compared to the other treatments. However, with the 0.5 mg/L 6-BAP treatments, rooting was much lower (< 20%) in the embryos that were initially maintained in the growth chamber with 24h of darkness ($Z = -2.9$, $P = 0.004$), compared to those initially maintained in 24h of light, ~ 30% (Figure 2.7b). Furthermore, rooting was significantly higher in the treatments with 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP ($Z = 4.4$, $P < 0.0001$), with the embryos that were initially maintained in the growth chamber with 24h of light having significantly lower rooting ($Z = -7.4$, $P < 0.0001$) compared to the embryos that were initially maintained in the growth chamber with 24h of darkness. In the treatments with 0.5 mg/L Kinetin, embryo rooting was significantly lower in the embryos that were initially maintained in light ($Z = -5.7$, $P < 0.0001$) compared to those that were initially in darkness (Figure 2.7b).

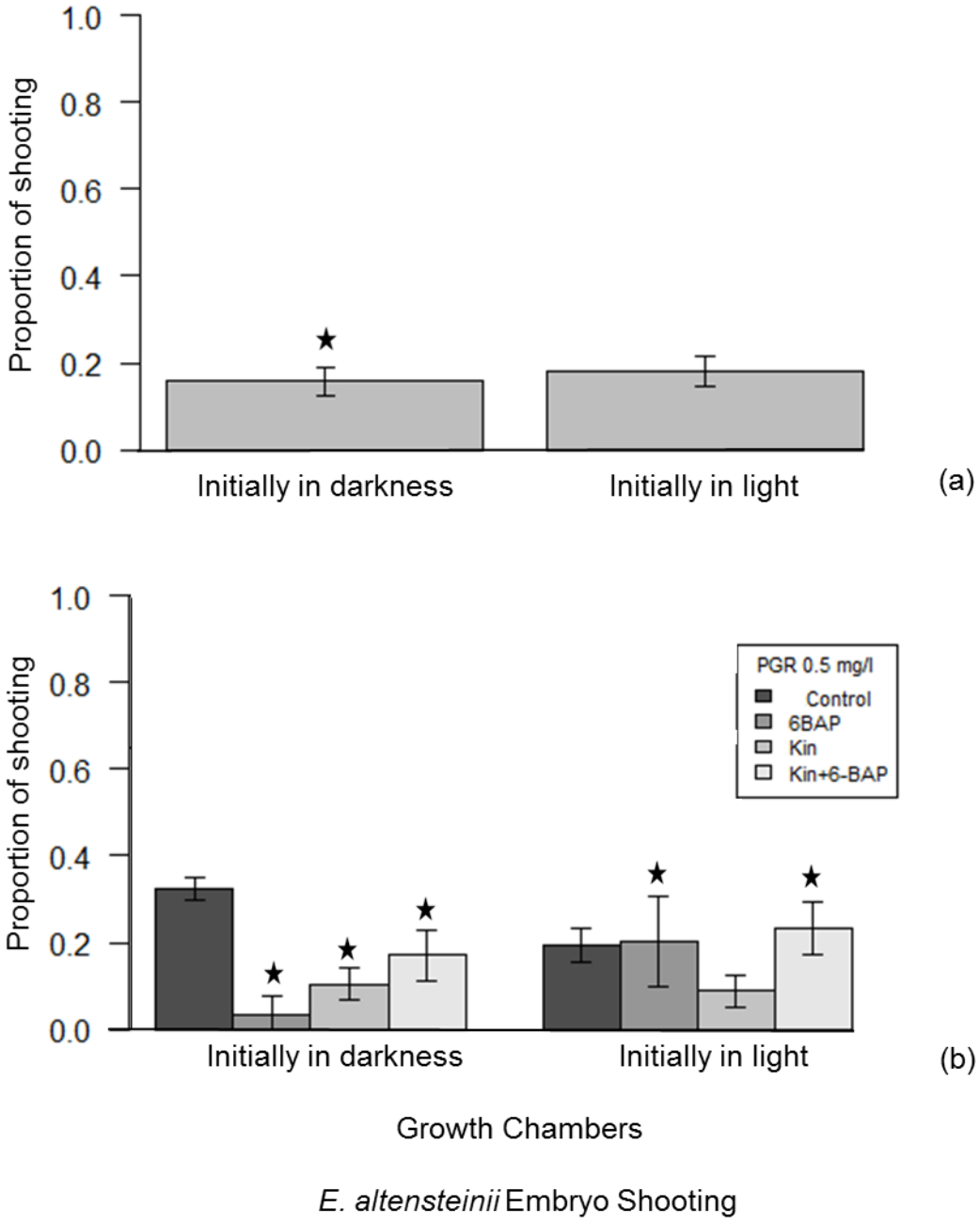


1220

1221 Figure 2.7: Proportion of rooting *E. altensteinii* embryos after subculture, 12 weeks of
1222 incubation obtained from generalised linear models with beta distributions (a), rooting
1223 was significantly higher in light ($P < 0.0001$) and (b) rooting was affected by light and
1224 PGRs, with significantly lower rooting in 0.5 mg/L 6-BAP ($P < 0.0001$), significance is
1225 indicated by ★. $n = 10$; bars represent Standard Error (±SE).

1226

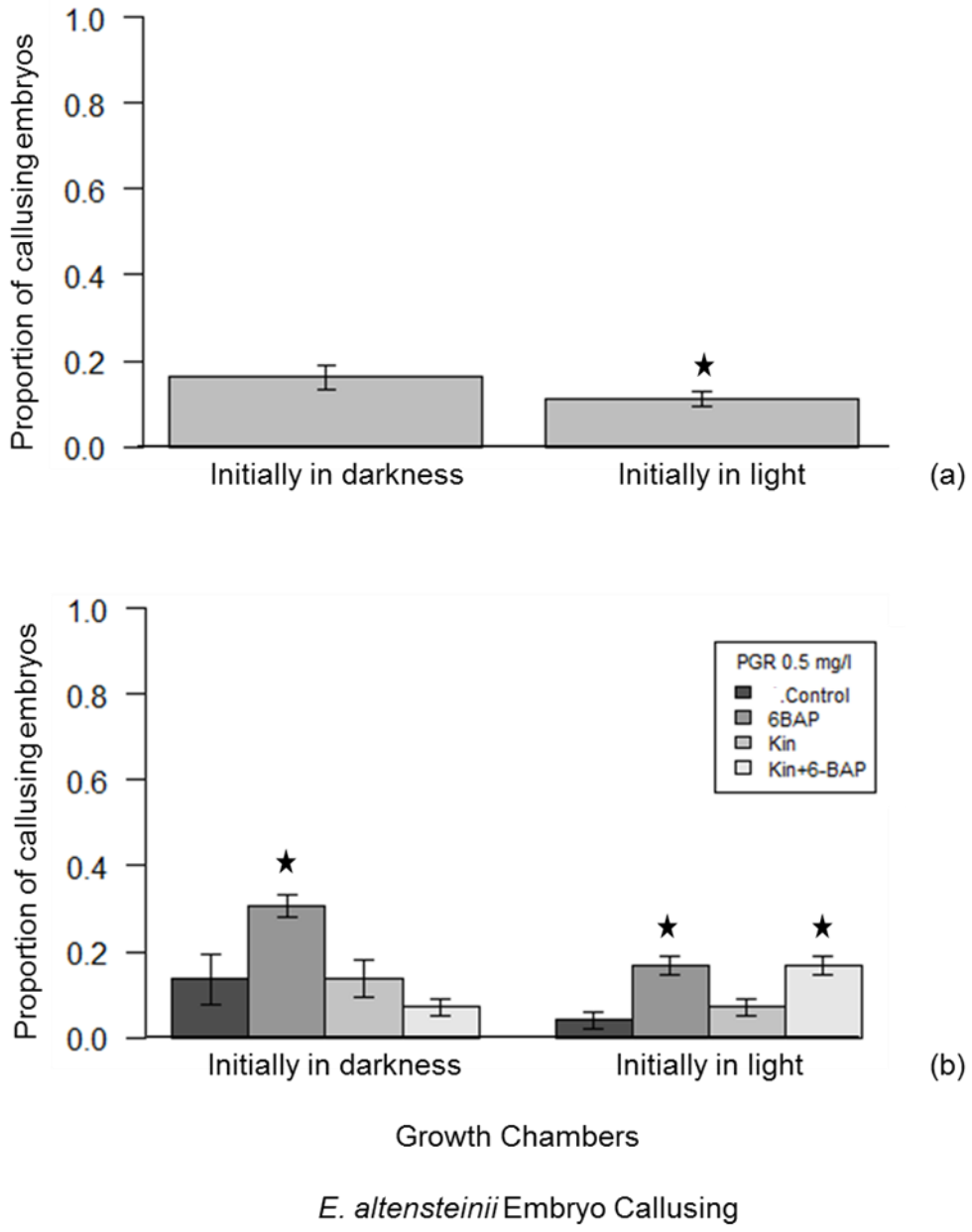
Shoot formation was significantly lower (by ~ 2%) in the embryos that were initially maintained under 24h of darkness (Z = -2.6, P = 0.009), compared to the embryos initially maintained in 24h of light (Figure 2.8a). Shoot formation was significantly lower in treatments with 0.5 mg/L 6-BAP (Z = -7.9, P < 0.0001), 0.5 mg/L Kinetin (Z = -5.5, P < 0.0001) and 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP (Z = -4.6, P < 0.0001) compared to the control in the embryos that were initially maintained in 24h of darkness. However, in the embryos that were initially maintained in 24h of light the treatments with 0.5 mg/L 6-BAP and 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP shoot formation was significantly higher (Z = 4.4, P < 0.0001) and (Z = 3.7, P = 0.0002) respectively, compared to the control (Figure 2.8b, Table 2.5).



1237

1238 Figure 2.8: Proportion of shooting *E. altensteinii* embryos after subculture, 12 weeks
1239 of incubation obtained from generalised linear models with beta distributions (a),
1240 shooting was significantly low in darkness (P = 0.009) and (b) shooting was affected
1241 by PGRs with significantly low shooting in 0.5 mg/L Kinetin (P < 0.0001), significance
1242 is indicated by ★. n = 10; bars represent Standard Error (±SE).

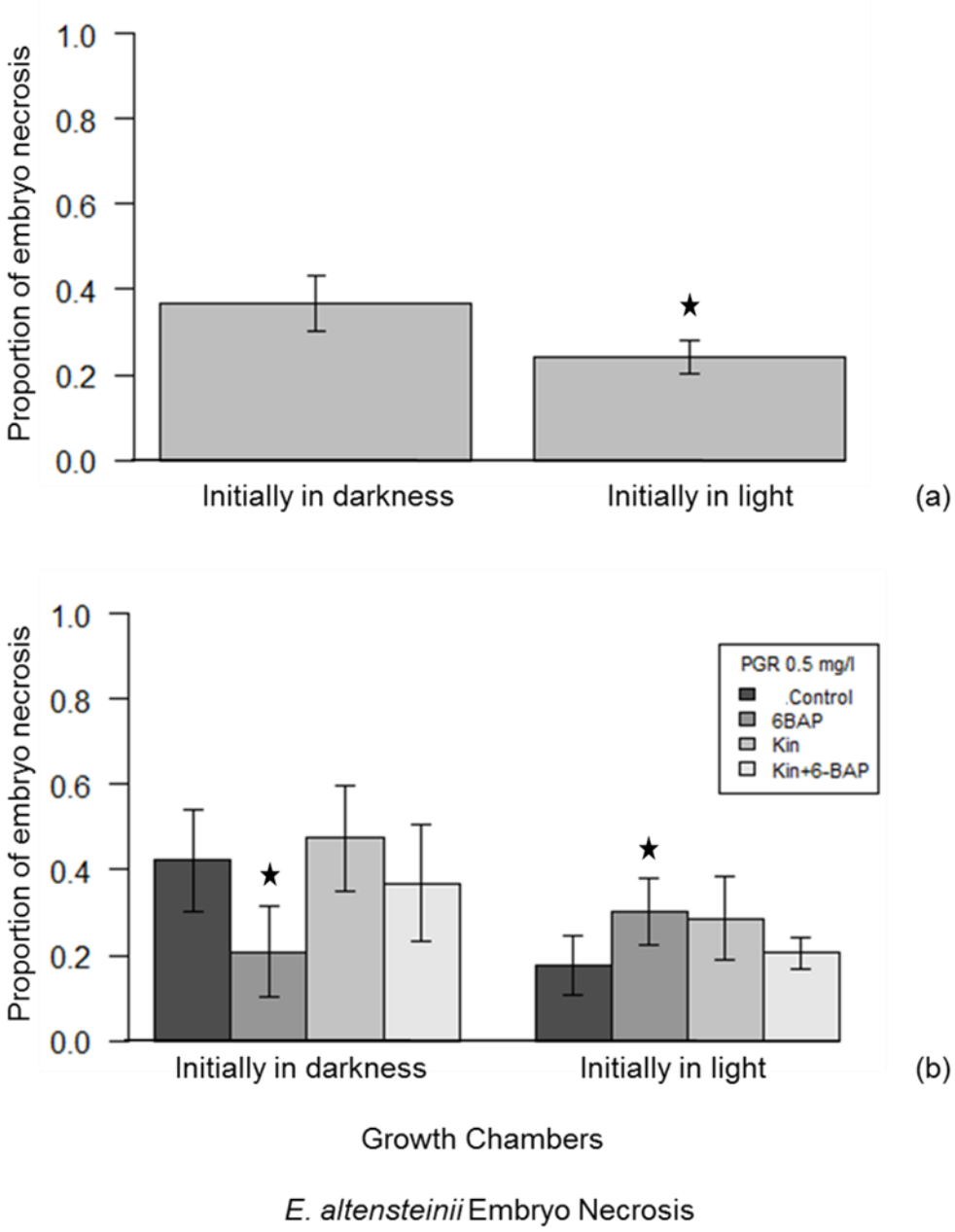
Embryo callusing was significantly lower (by ~3%) in the embryos that were initially maintained in the growth chamber with 24h of light (Z = -2.9, P = 0.003), compared to the embryos initially maintained in 24h of darkness (Figure 2.9a and Table 2.5). Embryo callusing was significantly higher in the treatments with 0.5 mg/L 6-BAP (Z = 6.8, P < 0.0001). However, in the embryos initially maintained in the growth chamber with 24h of light, embryo callusing was significantly higher in the treatments with 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP, ~ 18% (Z = 5.1, P < 0.0001) (Figure 2.9b and Table 2.5).



1251

1252 Figure 2.9: Proportion of callusing *E. altensteinii* embryos after subculture, 12 weeks
1253 of incubation obtained from generalised linear models with beta distributions (a)
1254 callusing was significantly low in light (P = 0.003) and (b) callusing was significantly
1255 high in 0.5 mg/L 6-BAP (P < 0.0001) and (c) callusing was significantly high in 0.5
1256 mg/L Kinetin + 0.5 mg/L 6-BAP (P < 0.0001), significance is indicated by ★. n = 10;
1257 bars represent Standard Error (±SE).

Embryo necrosis was significantly lower (by ~12%) in the embryos that were initially incubated in the growth chamber with 24h of light compared to the embryos that were initially in 24h of darkness (Z = -3.1, P = 0.002) (Figure 2.10a; Table 2.5). Embryo necrosis was also significantly lower in treatments with 0.5 mg/L 6-BAP, ~20% (Z = -2.8, P < 0.006) compared to the control (~42%), in the embryos that were initially in darkness (Figure 2.10b; Table 2.5). In the embryos that were initially in the growth chamber with light, there was significantly higher necrosis in the treatments with 0.5 mg/L 6-BAP, ~30% (Z = 3.2, P < 0.001) compared to the control which was just below 20%.

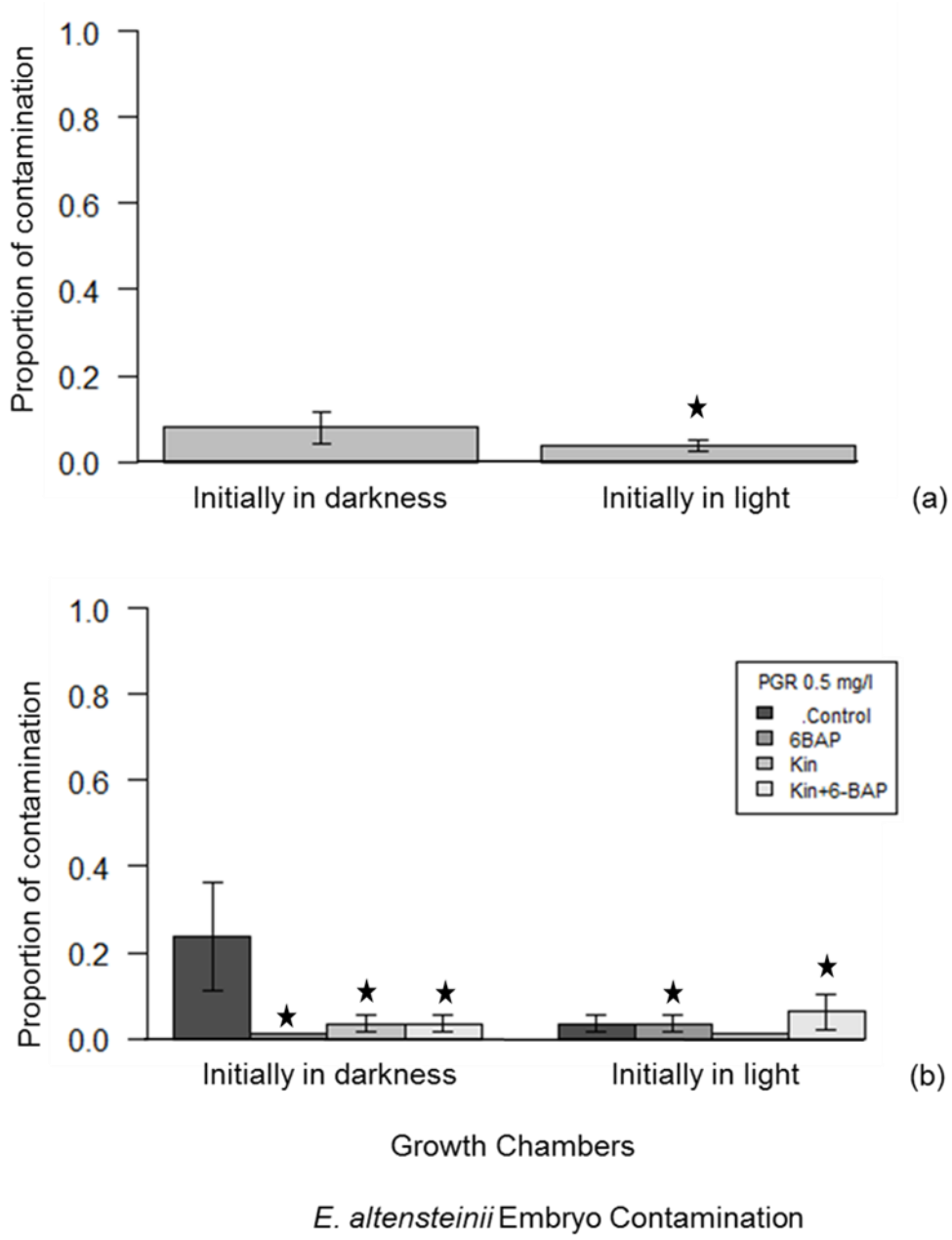


1267

1268 Figure 2.10: Proportion of *E. altensteinii* embryo necrosis after subculture, 12 weeks
1269 of incubation obtained from generalised linear models with beta distributions (a)
1270 necrosis was significantly low in light (P = 0.002) and (b) significantly low in 0.5 mg/L
1271 6-BAP (P < 0.006) in the embryos that were initially in darkness, significance is
1272 indicated by ★. n = 10; bars represent Standard Error (±SE).

1273 Embryo contamination was significantly lower (by ~15%) in embryos that were initially
1274 incubated in the growth chamber with light (Z = -3.5, P = 0.0004) compared to the

embryos that were initially in the growth chamber with darkness (Figure 2.11a; Table 2.5). In the embryos initially maintained in darkness, contamination was significantly lower in 0.5 mg/L 6-BAP (Z = -4.4, P < 0.0001), 0.5 mg/L Kinetin (Z = -3.5, P = 0.004) and 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP (Z = -3.5, P < 0.004) compared to the control (Figure 2.11b; Table 2.5). However, in the embryos that were initially in light, contamination was significantly higher in treatments with 0.5 mg/L 6-BAP Z = 3, P = 0.002) and 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP, ~30% (Z = 2.7, P = 0.007), compared to the control which was ~20%.



1283

1284 Figure 2.11: Proportion of contamination *E. altensteinii* embryos (*n* = 10) after
1285 subculture, 12 weeks of incubation obtained from generalised linear models with beta
1286 distributions (a) contamination was significantly low in light (*P* = 0.0004) and (b)
1287 contamination was significantly low in 0.5 mg/L 6-BAP (*P* < 0.0001), significance is
1288 indicated by ★. The bars represent Standard Error (±SE). *n* = 10; bars represent
1289 Standard Error (±SE).

1290

Effects of wounding treatments on *in vitro* growth, callus rescue and regeneration of *E. altensteinii*

All the *E. altenstinii* embryos/plantlets that survived an overall 12 weeks incubation period were wounded and treated with PGRs (See Section 2.2.8). All treated embryos/plantlets were lost due to necrosis and contamination within a week after wounding and PGR treatment.

Ex vitro hardening and acclimatization of *E. altensteinii*

In vitro embryo-derived plantlets of *E. altensteinii* were subjected to primary hardening for two weeks. The plantlets dried out and died after ~ 84 - 98 days from initial incubation. As a result, no acclimatization for *E. altensteinii* was achieved.

Initiating embryo regeneration: Initial six weeks of culture for *Encephalartos manikensis* embryos

A total of 261 *E. manikensis* embryos were incubated under different culture conditions to initiate germination, 128 embryos were exposed to light for 24h and 133 exposed to darkness for 24h, for a period of six weeks. After the first week of incubation, the most notable change was the colour of the embryos exposed to light for 24h, where 100% embryos turned green in colour (Figure 2.12b, d & f), this was regardless of the PGRs and sucrose concentration in the plant growth media. All embryos exposed to 24h darkness, did not change colour, remaining the original colour (Figure 2.12a, c & e). During the second week of culture, the most notable change observed was the thickening of the embryos, irrespective of the treatments. Embryos were observed to elongate and develop roots, and at the end of six weeks ~42% of 261 embryos had developed roots (Figure 2.12a, b & c), regardless of the treatment or light exposure. Shoots formation was evident on the fourth week of incubation, ~20% of 261 embryos had developed shoots (Figure 2.12d). Callus formation on the embryos was evident from the second week in culture, and at the end of six weeks incubation period ~3% of 261 embryos had formed callus. *Encephalartos manikensis* embryos regenerated roots and shoots either by direct or indirect organogenesis from callus. In cases where the embryos formed callus first, the embryos were able to further regenerate shoots, however roots would not regenerate in the presence of callus (Figure 2.16).

The main cause of embryo death was necrosis and contamination. Embryo contamination was evident in the second week of incubation and increased as the weeks progressed, with overall contamination ~62% of 261 (Figure 2.12e & f), at the end of six weeks of incubation. Embryo necrosis was recorded to be ~14% of 261 embryos at the end of six weeks. There were also embryos that did not develop roots,

shoots and callus throughout the duration of the six weeks of incubation, although embryos remained healthy and free of contamination. All the changes noted in all the embryos in culture, were irrespective of the treatments and light exposure.

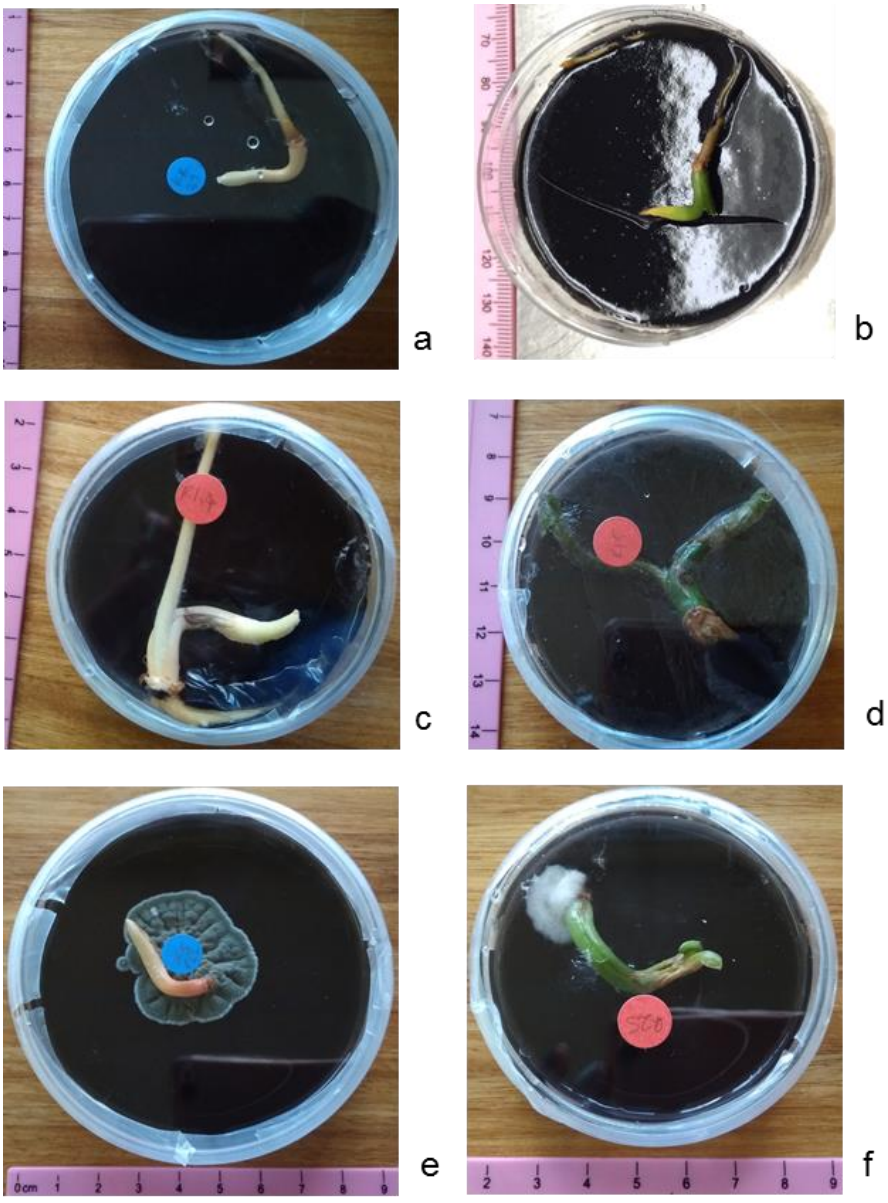
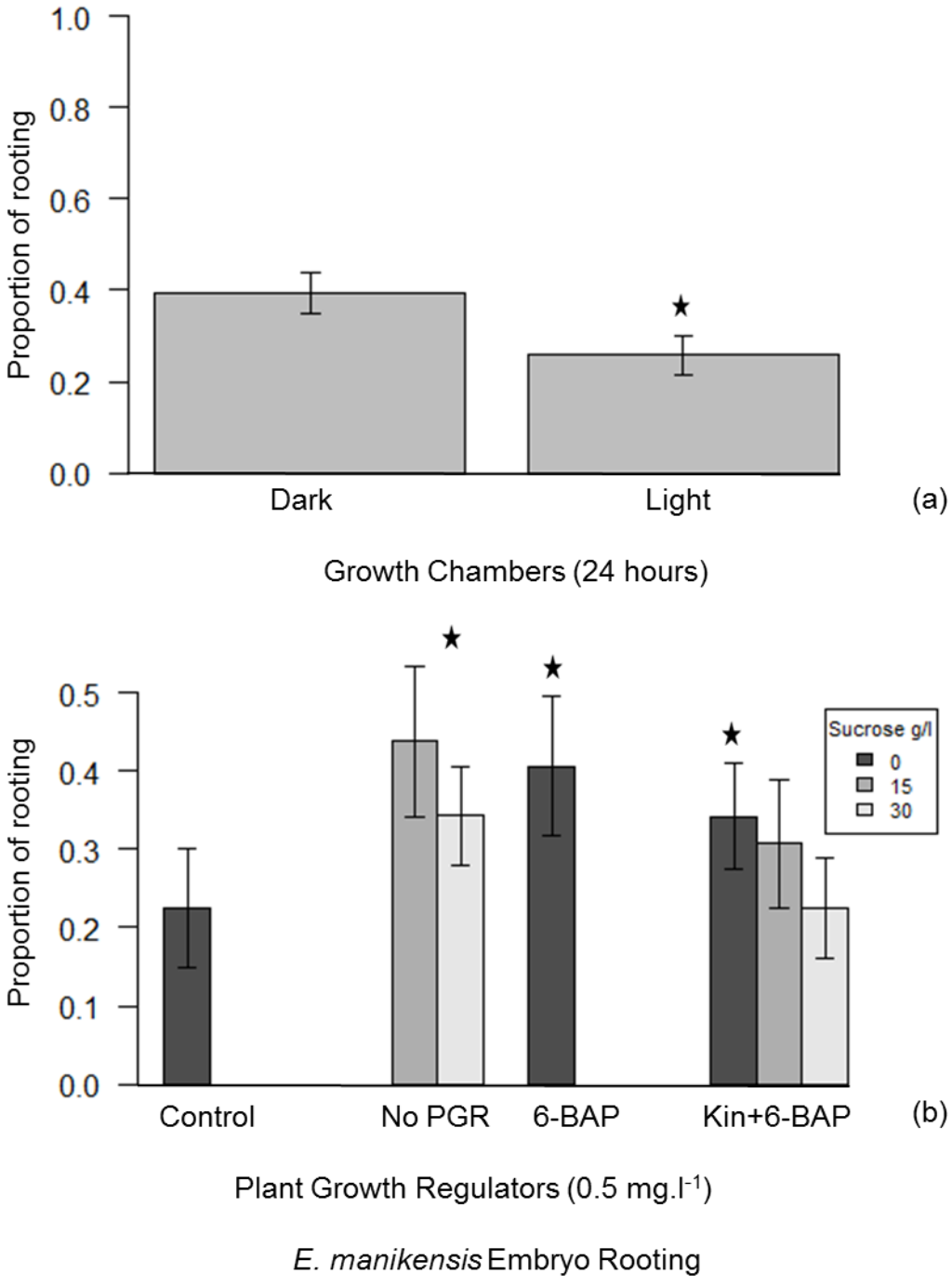


Figure 2.12: *Encephalartos manikensis* embryo regeneration after six weeks in culture, maintained in growth chambers with 24h of darkness (a, c & e) and 24h of light (b, d & f). (a & b) Embryo rooting, (c) rooting and shooting embryo, (d) contaminated embryos maintained in their respective growth chambers.

Table 2.6: Results of generalised linear models with beta distribution for proportions of rooting, shooting, callus, contamination and dead *E. manikensis* embryos. The change in AICc (ΔAICc) between the best model and the next best and the null model are also given. In cases where there was more than one best possible model, these are reported as "Model1" and "Model2". Psuedo R^2 measuring the variation explained by the fixed variables, sample size (*n* = 261).

Response	Model	AIC (Best model)	∆AIC (2nd Best model)	∆AIC (Null model)	Pseudo R^2
Rooting	Best Model ~ Env.con*** + PGR*** + Sucrose* Model equation: $Y = e^{-1.08 - 0.47L + 1.14NP + 0.76B + 0.68KB - 0.02S}$ 2nd Best Model ~ Env.con:PGR + Sucrose* Model equation: $Y = e^{-0.83 - 0.96L + 0.88NP + 0.47B + 0.37KB - 0.02S + 0.52L:NP + 0.57L:B + 0.59L:KB}$	-139.30	3.5	23.1	0.13
Contamination	Best Model ~ PGR + Env.con:Sucrose** Model equation: $Y = e^{-0.63 - 0.49NP - 0.26B - 0.14KB - 0.42L + 0.01S + 0.03L:S}$ 2nd Best Model ~ Env.con:PGR + Env.con:Sucrose* Model equation: $Y = e^{-0.68 - 033L - 0.56NP - 0.29B + 0.04KB + 0.01S + 0.17L:NP + 0.08L:B - 0.32L:KB + 0.03L:S}$	-122.08	3.6	12.3	0.10
Necrosis	Best Model ~ Env.con:PGR* + Env.con:Sucrose** Model equation: $Y = e^{-2.39 - 0.68L - 0.30NP - 0.56B - 0.16KB - 0.01S - 0.06L:NP - 0.68L:B - 0.88L:KB + 0.04L:S}$ 2nd Best Model ~ Env.con:PGR** + Sucrose Model equation: $Y = e^{-2.37 + 0.67L - 0.79NB - 0.56B - 0.53KB + 0.01S + 0.88L:NP - 0.67L:B - 0.26L:KB}$	-801.41	8.11	63.19	0.46
Shooting	Best model (Null) : AIC (Null) = -770.44, ∆AIC (2nd Best model) = 0.65, ∆AIC (3rd Best model) = 3.1 Model equation: $Y = e^{-2.29}$				
Callusing	Best model (Null) : AIC (Null) = -1350.98, ∆AIC (2nd Best model) = 2.0, ∆AIC (3rd Best model) = 2.87 Model equation: $Y = e^{-3.79}$				
Abbreviations: NP = No PGR, B = 6-BAP, K = Kinetin, KB = Kinetin + 6-BAP, S = Sucrose, L = Light					

The results show that *E. manikensis* embryo rooting was significantly affected by all the variables tested in this study. Embryo rooting was significantly lower by ~18% in the growth chamber with 24h of light (Z = -3.6, P = 0.0003), compared to the growth chamber in darkness (Figure 2.13a; Table 2.6). Embryo rooting was significantly higher in the treatments with no PGR (Z = 4.3, P = 0.0001) (Figure 2.13b). Furthermore, rooting was significantly higher in the treatments with 0.5 mg/L 6-BAP at ~40% (Z = 2.8, P = 0.005) and 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP at ~35% (Z = 2.9, P = 0.003), where the sucrose concentration is 0 g/L (Z = -2.4, P = 0.02), compared to the control (Figure 2.13b; Table 2.6).

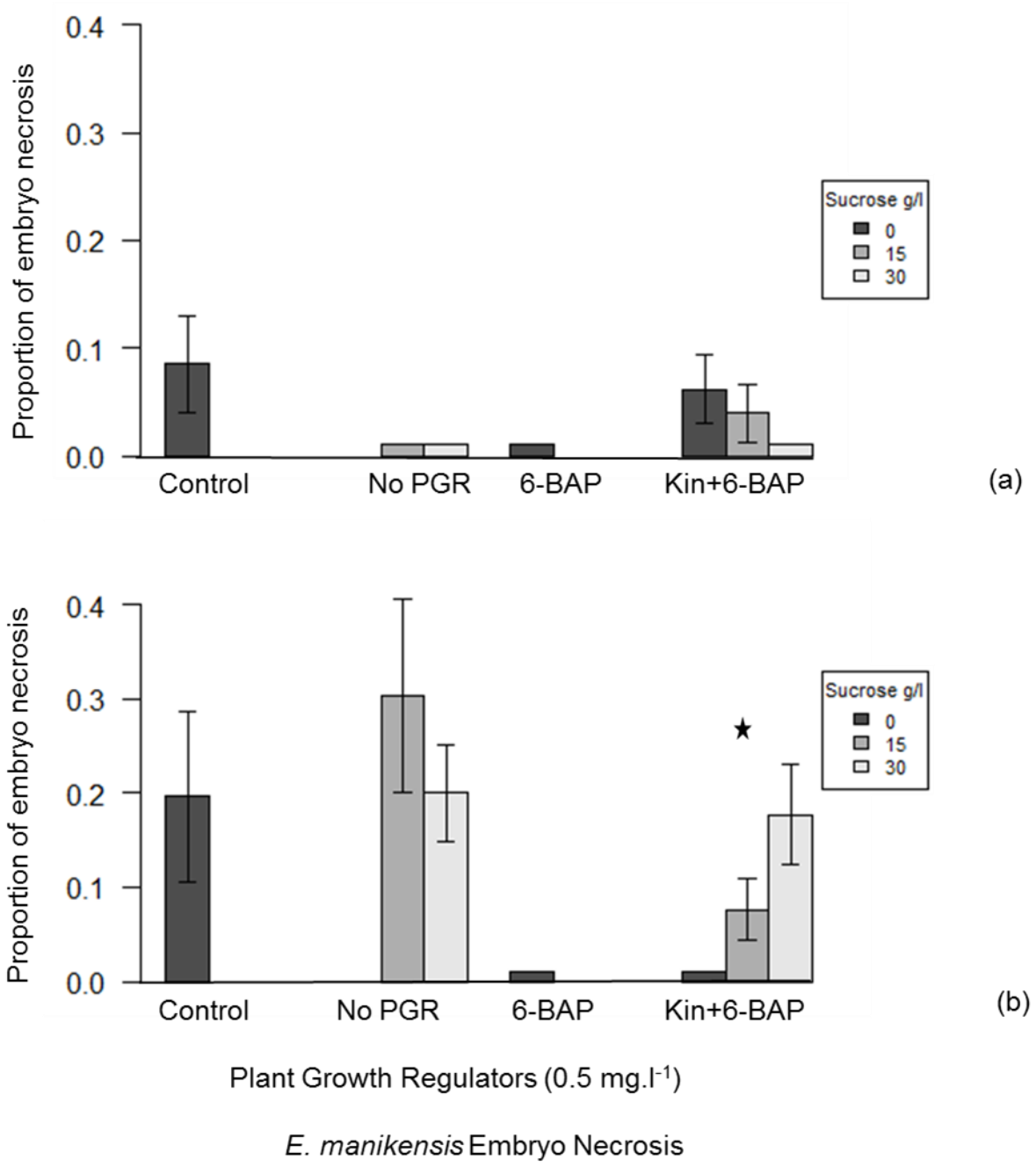


1348

1349 Figure 2.13: Proportion of *E. manikensis* embryos rooting in the first six weeks of
1350 incubation, obtained from generalised linear models with beta distributions.
1351 Germination is significantly low in embryos maintained in light (P = 0.0004),
1352 significance is indicated by ★. n = 7; bars represent Standard Error (±SE).

The fixed variables that were evaluated in this study, which were light(exposer to light or darkness), PGRs and sucrose concentration did not have any significant influence on the formation of shoots, as well as the formation of callus on *E. manikensis* embryos. In both cases, the best model was indicated by the lowest AIC which was the Null model (Table 2.6).

Embryo necrosis was significantly affected by light, sucrose concentration and PGRs (Table, 2.6). Embryo necrosis was higher in the growth chamber with 24h of light (Z = 2.3, P = 0.02; Figure 2.14b; Table 2.6). However, the interaction between the light and PGR showed that in the presence of light, embryo necrosis was lower in the treatment with 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP (Z = -2.2, P = 0.03) compared to the PGR free treatments. Furthermore, in the growth chamber with light, embryo necrosis increased with increasing sucrose concentration, with significantly high embryo necrosis where sucrose is 30 g/L (Z = 3.2, P = 0.001) (Figure 2.14b; Table 2.6).



1368

1369 Figure 2.14: Proportion of *E. manikensis* embryos with necrosis in the first six weeks
1370 of incubation, obtained from generalised linear models with beta distributions. Embryo
1371 necrosis in the growth chamber with 24h (a) darkness and (b) light**,** significance is
1372 indicated by ★. *n* = 7; bars represent Standard Error (±SE).

1373

Contamination was significantly affected by the combination of light conditions and sucrose concentration (Table 2.6). *Encephalartos manikensis* embryo contamination was significantly lower in the embryos maintained in the growth chamber with 24h of light (Z = -2.02, P = 0.04) however, contamination increased with increasing sucrose concentration (Z = 2.8, P = 0.005) (Figure 2.15; Table 2.6).

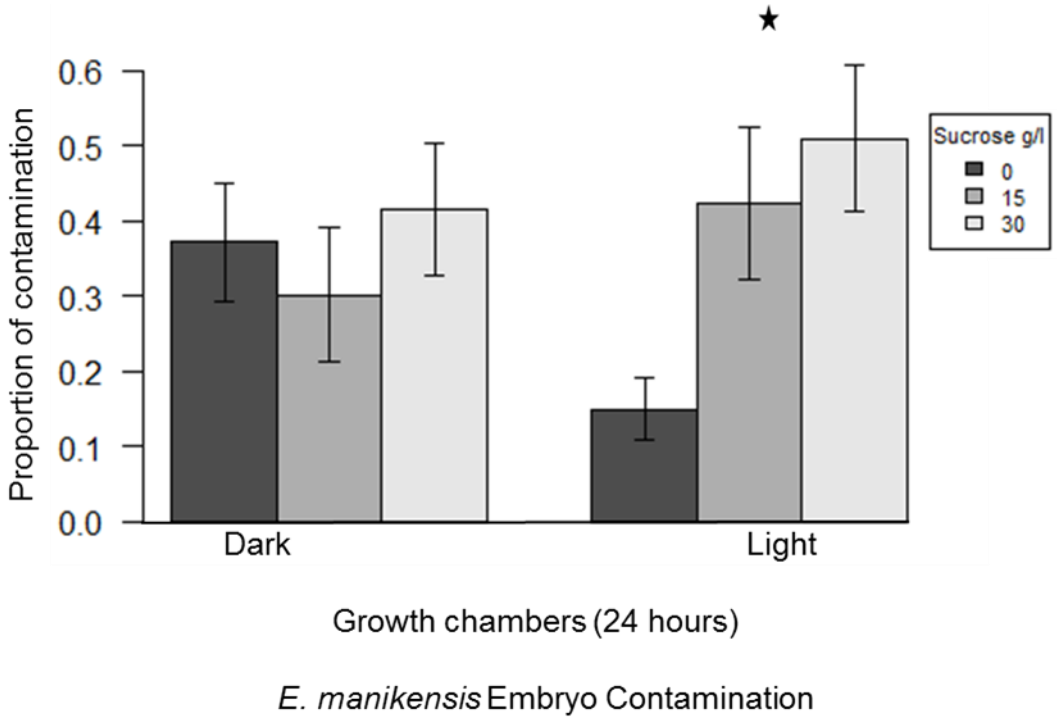


Figure 2.15: Proportion of *E. manikensis* embryos with contamination in the first six weeks of incubation, obtained from generalised linear models with beta distributions. Embryo contamination was significantly low in the growth chamber with light, with increasing sucrose concentration (P = 0.005), significance is indicated by ★. n = 7; bars represent Standard Error (±SE).

Growth and development of *E. manikensis* embryos: After subculture, (Overall 12 weeks embryo incubation).

A total of 90 of the 261 (34.5%) embryos survived the initial six weeks of culture. These healthy embryos were subcultured and maintained in growth chambers with 16h photoperiod at 26 ºC as per Table 2.2. At the end of the six weeks incubation period (overall 12 weeks embryo incubation) out of the 90 subcultured embryos/plantlets, 42 (41%) were lost due to infection (23), necrosis (6) or the combination of the two (13). Across the three treatments, there were 48 (59%) healthy embryos that either had roots only (13), shoots only (1) callus only (3), a combination of shoots and callus (4), or shoots and roots (27) that survived the six weeks incubation period. The results from these experiments are given below (Table 2.7; Figure 2.16). Following this, surviving plantlets were subjected to hardening and acclimatization, while the rest were subcultured onto fresh media every six weeks. There were however no further developments on these embryos/plantlets, after which the experiments were suspended.

a

b

c

Figure 2.16: *Encephalartos manikensis* (a) at the end of six weeks incubation period, with callus and a newly merged shoot, (b & c) embryo-derived plantlet without a root at the end of 12 week incubation.

Table 2.6: Results of generalised linear models with beta distribution for proportions of germination, callus, necrosis and contamination of *E. manikensis* embryos. The change in AICc (ΔAICc) between the best model and the next best and the null model are also given. In cases where there was more than one best possible model, these are reported as "Model1" and "Model2". Psuedo R^2 measuring the variation explained by the fixed variables. Sample size (*n* = 90).

Response	Model	AIC (Best model)	∆AIC (Null)	Pseudo R^2
Rooting	Best Model ~ PGR*** Model equation: $Y = e^{-0.66 - 0.78KB(a) + 0.05KB(b)}$	-51.5	10.22	0.17
Shooting	Best Model ~ PGR* Model equation: $Y = e^{-0.88 + 0.73KB(a) + 0.45(b)}$	-52.05	13.82	0.17
Callusing	Best Model ~ PGR* Model equation: $Y = e^{-2.27 – 0.17KB(a) - 0.55KB(b)}$	-235.67	1.42	0.09
Contamination	Best Model ~ PGR*** Model equation: $Y = e^{0.08 – 1.26KB(a) + 0.89KB(b)}$	-37.15	33.49	0.4
Necrosis	Best Model ~ PGR* Model equation: $Y = e^{-1.95 + 0.15KB(a) + 0.46KB(b)}$	-127.51	0.61	0.06
NP=No PGR, B=6-BAP, K=Kinetin, KB(b)=0.5mg/L(Kinetin+6-BAP), KB(a)= 1.0mg/L(Kinetin+6-BAP), S=Sucrose, L=Light				

The results following subculturing (Table 2.7) show that embryo rooting and shooting were significantly higher in the treatment with 1.0 mg/L Kinetin +1.0 mg/L 6-BAP,

compared to the control and the treatment with 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP (Figure 2.17 and Figure 2.18). *Encephalartos manikensis* embryo rooting was significantly high (~60%) (Z = 3.4, P = 0.0007), in the treatments with 1.0 mg/L Kinetin + 1.0 mg/L 6-BAP, compared to the control ~38% and the treatment with 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP at ~41% (Figure 2.17 and Table 2.8). Similarly embryo shooting was significantly high in the treatments with 1.0 mg/L Kinetin + 1.0 mg/L 6-BAP (~50%) (Z = 2.5, P = 0.01) compared to the control and the treatment with 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP, both at ~25% (Figure 2.18 and Table 2.7).

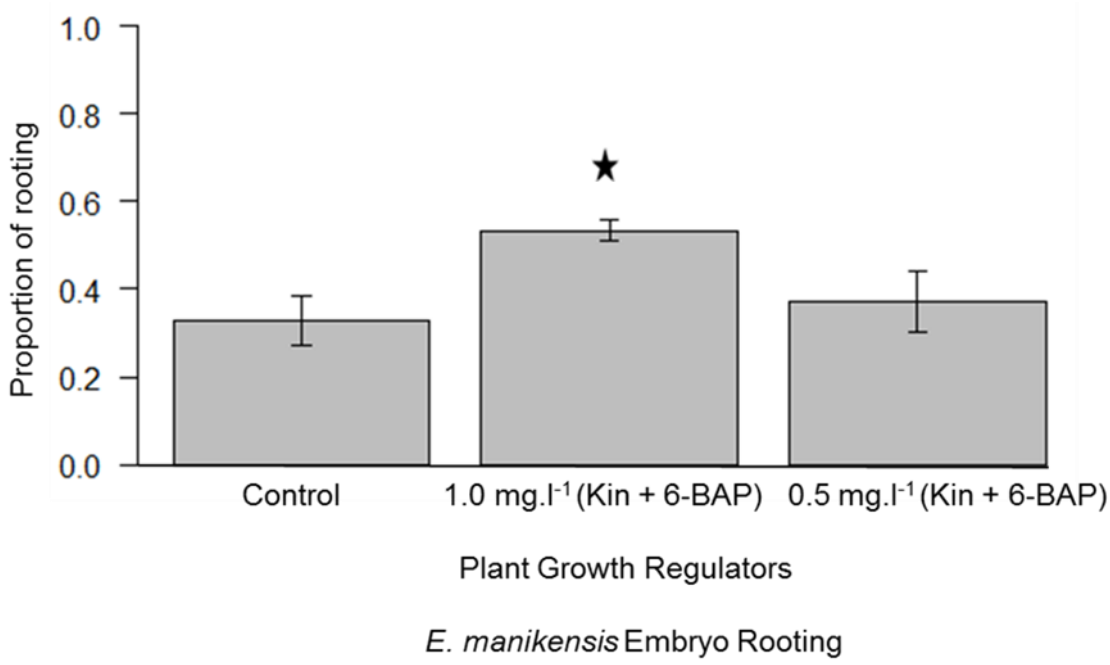
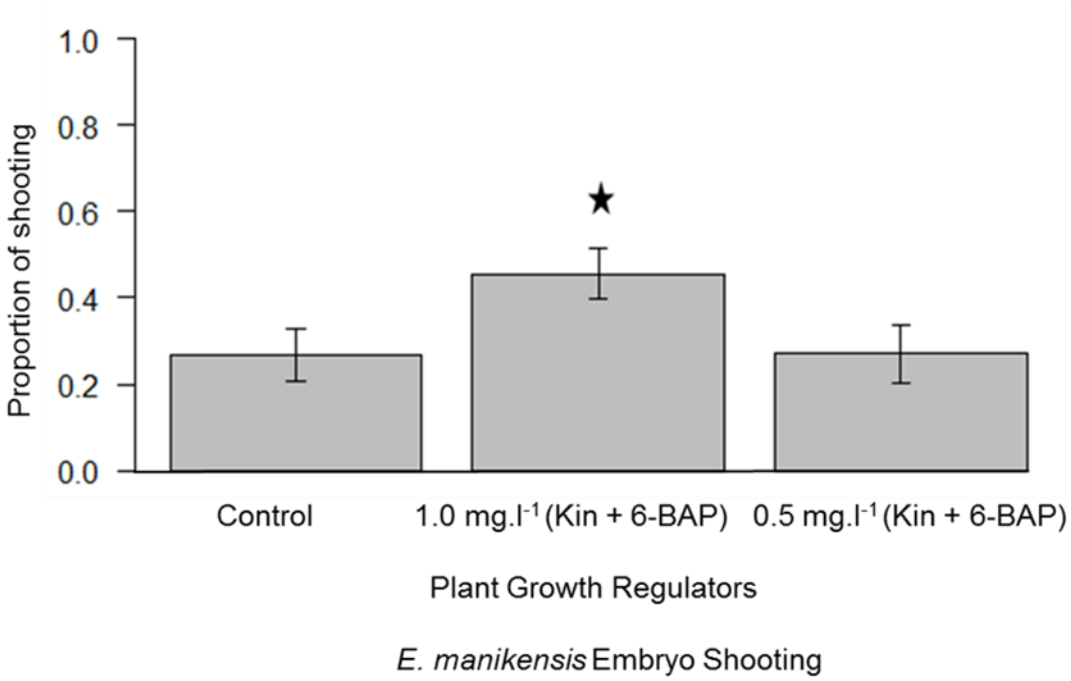


Figure 2.17: Proportion of *E. manikensis* embryos with rooting after subculture, 12 weeks of incubation obtained from generalised linear models with beta distributions. Rooting was significantly high in 1.0 mg/L Kinetin + 1.0 mg/L 6-BAP (P = 0.006), significance is indicated by ★. n = 10; bars represent Standard Error (±SE).



1429

1430 Figure 2.18: Proportion of *E. manikensis* embryos with shoots after subculture, 12
1431 weeks of incubation obtained from generalised linear models with beta distributions.
1432 Shooting was significantly high in 1.0 mg/L Kinetin + 1.0 mg/L 6-BAP (P = 0.01),
1433 significance is indicated by ★. n = 10; bars represent Standard Error (±SE).

1434

1435 According to the results, *E. manikensis* embryo callusing was significantly lower in the
1436 treatment with 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP (Z = -2.3, P = 0.02) compared to the
1437 control and treatment with 1.0 mg/L Kinetin + 1.0 mg/L 6-BAP (Figure 2.19 and Table
1438 2.7).

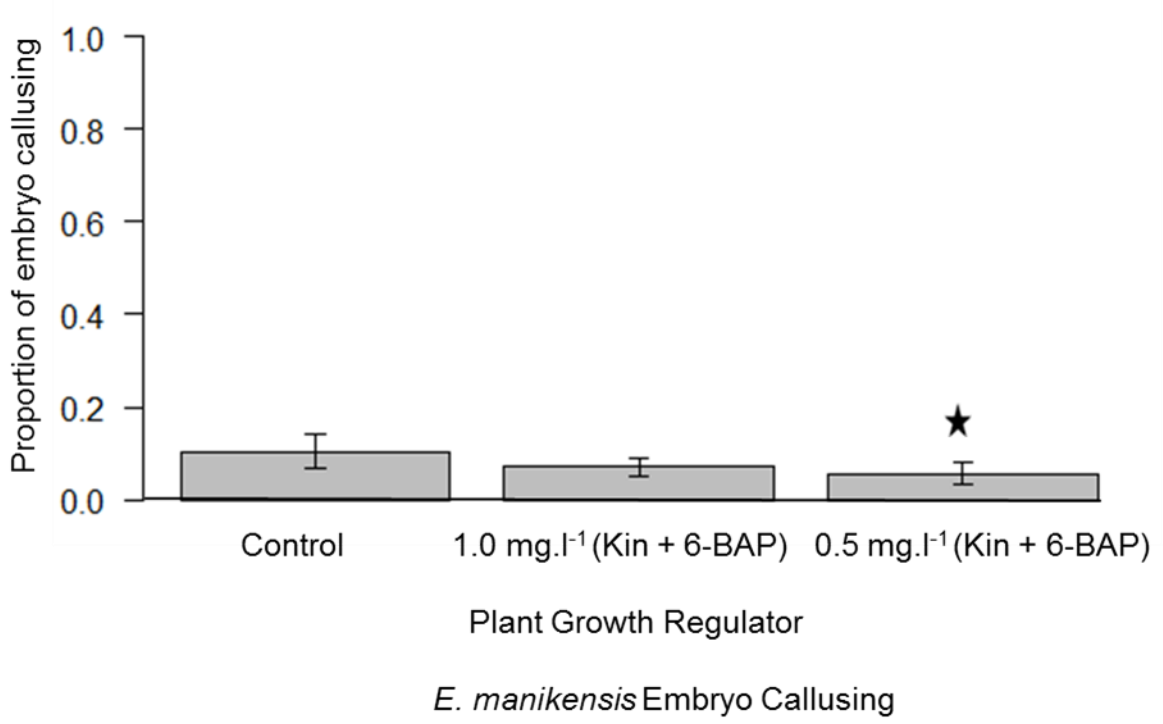
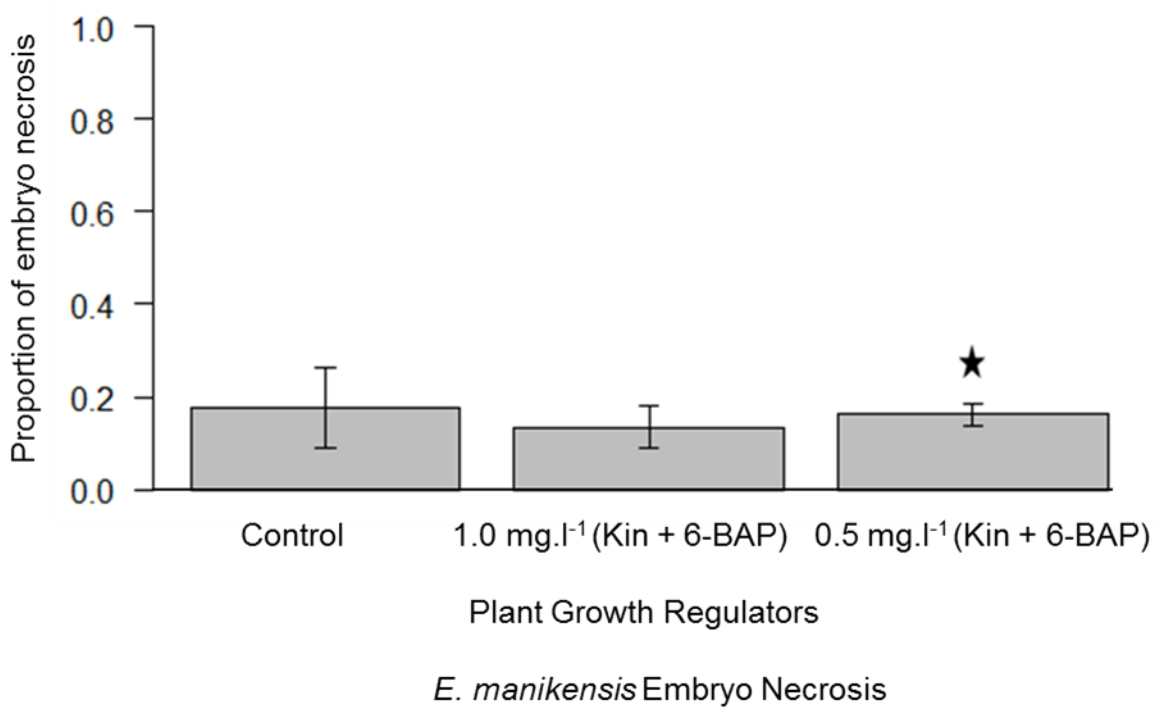


Figure 2.19: Proportion of *E. manikensis* embryos callusing after subculture, 12 weeks of incubation obtained from generalised linear models with beta distributions. Embryo callusing was significantly low in 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP (P = 0.002), significance is indicated by ★. n = 10; bars represent Standard Error (±SE).

According to the results, after subculture embryo necrosis was significantly high in the treatment with 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP, ~20% (Z = 2, P = 0.05), compared to the control and the treatment with 1.0 mg/L Kinetin + 1.0 mg/L 6-BAP, which are both below 20% (Figure 2.20 and Table 2.7).



1449

1450 Figure 2.20: Proportion of *E. manikensis* embryos showing necrosis after subculture,
1451 12 weeks of incubation obtained from generalised linear models with beta
1452 distributions. Embryo necrosis was significantly high in 0.5 mg/L Kinetin + 0.5 mg/L 6-
1453 BAP (P = 0.05), significance is indicated by ★. n = 10; bars represent Standard Error
1454 (±SE).

1455

1456 Embryo contamination was significantly low in the treatment supplemented with 1.0
1457 mg/L Kinetin + 1.0 mg/L 6-BAP, ~18% (Z = -3.6, P = 0.0004) compared to control and
1458 the treatment with 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP at ~57 and ~61% respectively
1459 and significantly high in 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP (Z = 3, P = 0.003)
1460 compared to the control (Figure 2.21 and Table 2.7).

1461

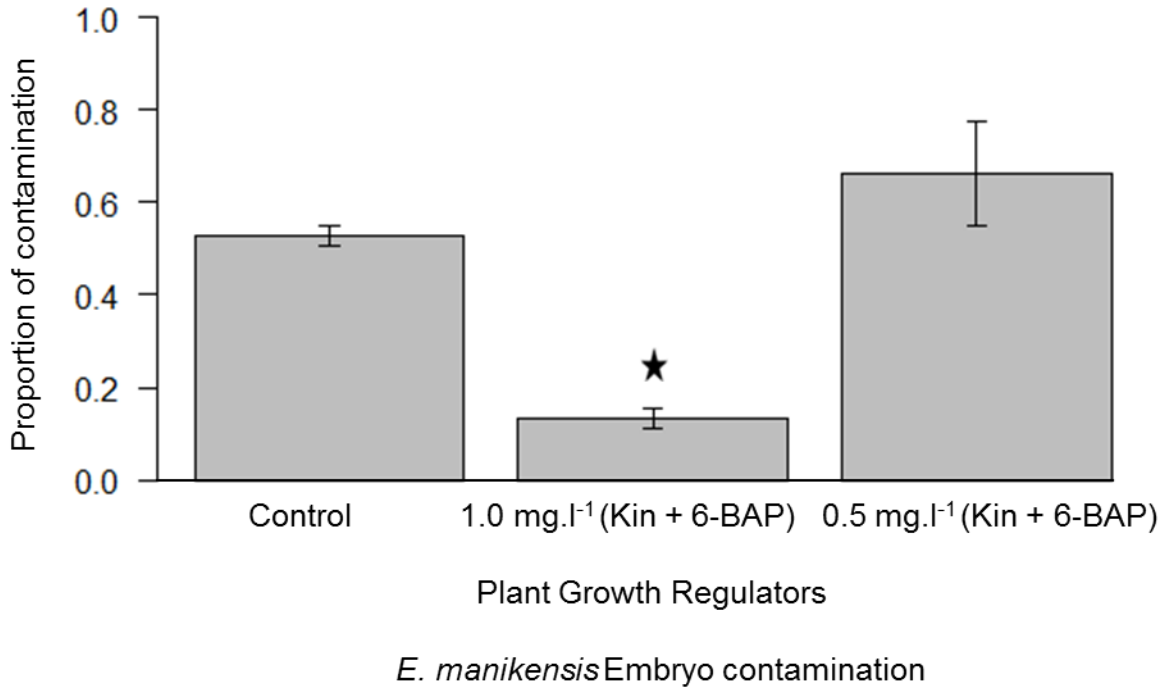


Figure 2.21: Proportion of *E. manikensis* embryos with contamination after subculture, 12 weeks of incubation obtained from generalised linear models with beta distributions. Embryo contamination was significantly low in 1.0 mg/L Kinetin + 1.0 mg/L 6-BAP (P = 0.0004), significance is indicated by ★. n = 10; bars represent Standard Error (±SE).

Encephalartos manikensis **hardening and acclimatization**

At the end of the 12 week incubation period, 24 out of the 261 (9.2%) embryos had regenerated both roots and shoots. These plantlets were subjected to primary hardening for two weeks, 10 (3.8%) of which died after ~98 days from initial incubation. The surviving 14 (5.4%) plantlets were placed in a lesser controlled environment (outside enclosed growth chambers) under ambient temperatures for secondary hardening for a further two weeks, 5 (1.9%) died. After 112 days after initial culture 9 (3.4%) plantlets of the 261 embryos were potted in 1:1 ration bark and potting soil and

placed outdoors. These plantlets survived acclimatization for over 300 days, after which the experiments were halted (Figure 2.22).

Figure 2.22: Hardening and acclimatization of *E. manikensis* embryo-derived plantlets. (a) Plantlet with both root and shoot 84 days after the beginning of culture. (b) Plantlet during primary hardening in perlite and vermiculite substrate, 112 days after initial culture. (c) Hardening plantlet covered with a plastic bag to maintain high relative humidity. (d) Elongated shoots (pinnate leaves) and primary root with small secondary roots, between 112-150 days after initial culture. (e) Acclimatised plantlet.

2.4. Discussion

The primary aim of this study was to establish an efficient *in vitro* regeneration protocol for *Encephalartos altensteinii and E. manikensis* embryos. This study looked at three variables, namely; light conditions, the presence of exogenous plant growth regulators (PGRs) Kinetin and 6-BAP (alone and in combination) and different sucrose concentrations (15 and 30) g/L. This study was set-up to test whether any of these variables would have an effect on the regeneration of *E. altensteinii* and *E. manikensis* embryos *in vitro*. Within the first six weeks of culture, both species were able to regenerate roots and shoots, either by direct or indirect organogenesis from callus. Embryos regenerated roots or formed callus first before the emergence of shoots. During this stage of culture, under the same experimental conditions, embryos from both species were able to regenerate roots and shoots across all treatments, with varying combinations of PGRs, sucrose concentrations and with either 24h of light or darkness. Roots and shoots were also regenerated on PGR free media, however this was not significant. This may however suggests that some of the embryos used in the current study may have already matured and were ready for germination, therefore not needing PGRs in plant growth media. The differences in genetic make-up and the presence of different endogenous phytohormones in seeds of *E. altensteinii* and *E. manikensis* are most likely to be probable factors which resulted in different effects/outcomes on embryo regeneration and seedling formation of these two species.

The effect of sucrose on embryo regeneration of *E. altensteinii* **and** *E. manikensis*

Different types and concentrations of sugars are often used to provide explants with energy, with sucrose as the most frequently used sugar in many studies (Gaj, 2004), including cycad *in vitro* studies. In the initial six weeks of culture of this study, different sucrose concentrations (15 and 30 g/L, and a control with no sucrose) were used and they affected *E. altensteinii* and *E. manikensis in vitro* embryo regrowth differently. Sucrose was found to have no influence on *E. altensteinii* embryo root and shoot regeneration, necrosis and contamination. However, it showed a significant positive effect on callus induction, where callus formation increased with increase in sucrose concentration in both light and darkness. This increase was significantly higher in darkness compared to light treatments. The increase in callus suggests that having precise concentration of sucrose is essential for *E. altensteinii* regeneration, as callus has the potential to differentiate to either roots or shoots (Haberlandt, 1902). In this study, in cases where the embryos that developed callus first, some of the embryos were able to further regenerate shoots; however, no roots were formed.

Sucrose had a negative influence on *E. manikensis* rooting, with a significant decrease in rooting as sucrose concentration increased. Increase in sucrose concentration also increased necrosis and contamination. It is thus apparent that higher sucrose concentration had adverse effects on *E. manikensis* regeneration. Hence, it may be beneficial either to use moderate concentrations (15 g/L) or remove sucrose altogether. Furthermore under 24h of light, increasing sucrose concentrations resulted in significantly higher embryo necrosis and contamination. A possible reason for higher necrosis and contamination may be a result of microbes that are well suited

to thrive in light conditions in media with high sucrose concentration; as it has been suggested that plant growth media is also a good source for microbial growth (Sood et al., 2012). Microbes tend to outcompete explants for nutrients in media, resulting in increased explant necrosis and contamination (Odutayo et al., 2007a). This may have further affected the species regeneration. Based on these observations, sucrose concentration was set at a constant 15 g/L with a 16h photoperiod in the subsequent subculture (see also Naderi et al., 2015) with postulations that this would limit *E. altensteinii* callus formation as well as decrease the rates of necrosis and contamination on *E. manikensis*.

The effects of light conditions and PGRs on the regeneration of *E. altensteinii* roots, shoots and callus

In the initial stages of *E. altensteinii* culture, the treatment with 0.5 mg/L Kinetin induced rooting in over 50% of the embryos, this being significantly higher than the other treatments. This treatment resulted in the induction of more roots while 0.5 mg/L 6-BAP seemed to have a negative effect on both the regeneration of roots and shoots of *E. altensteinii* embryos. Kinetin and 6-BAP belong to a class of PGRs classified as cytokinins which predominantly play a role in cell division and shoot induction (Singh, 2018). This explains the low rooting in treatments with 6-BAP. However, this does not explain the high rooting in Kinetin treatments and the low shooting in 6-BAP treatments. The treatments that were supplemented with 0.5 mg/L 6-BAP resulted in the lowest number of roots and shoots induced in the initial stages of culture. A possible reason for this could be the use of activated charcoal (AC) as a supplement in the initial six weeks of culture.

Activated charcoal has properties that adsorb unwanted substances, such as phenolics which results in browning of explants in culture (Pan & Van Staden, 1999). However, the presence of AC in the media has also been suggested to be capable of adsorbing the much needed PGRs and other nutrients (Thomas, 2008). This results in a decrease in PGRs concentrations, leaving unknown PGR quantities available for plant tissue. Activated charcoal was not evaluated amongst the variables which may have had an impact on embryo regeneration. As such, AC could have had an influential role on the regeneration of the embryos. It may be crucial to assess the actual quantities of PGRs that may be adsorbed by AC and if this could nullify the application of PGRs.

After subculture, treatments with 0.5 mg/L Kinetin resulted in much higher root and shoot regeneration, compared to those of 0.5 mg/L 6-BAP. This was however an exception to shoot regeneration of the embryos that were initially maintained in 24h light, which had significantly higher shoot induced in the treatment with 0.5 mg/L 6-BAP. Moreover, the combination of the Kinetin and 6-BAP (0.5 mg/L Kinetin + 0.5 mg/L 6-BAP) resulted in improved root and shoot regeneration than when the PGRs were applied independently. Root and shoot regeneration were significantly high in the treatments with this combination. This may suggest that the combination of these two cytokinins work better together in promoting *E. altensteinii* root and shoot regeneration, rather than when they are used individually; more so in an environment with both light and darkness (16h photoperiod) after subculture, rather than constant light or darkness (as observed in the initial six weeks of culture). These results suggest that these cytokinins may be sensitive to excessive light and thus requires an alternation between light and darkness. Another possible reason for the increased root

and shoot regeneration after subculture may be the absence of AC, indicating that AC may have been negating the function of the PGRs in the first six weeks of culture.

Moreover, rooting was lower in the embryos that were initially maintained at 24h light and high in those that were in darkness. While shoot regeneration was higher in the embryos that were initially maintained in 24h light, compared to those that were in darkness. After subculture, when the embryos were maintained in a 16h photoperiod, it became evident that light conditions had a significant effect on the regeneration of both roots and shoots of *E. altensteinii*. Embryos that were initially maintained in the growth chamber with 24h of darkness regenerated more roots than those that were initially in light. However, the opposite effect was observed with regards to shoot regeneration. This is in agreement with what happens in nature, roots generally grow in a dark environment, downward into the soil, away from light (positive gravitropic response) while shoots grow in response towards light (negative gravitropic response) (Silva-Navas et al., 2015; Hangarter, 1997).

In addition to *E. altensteinii* root and shoot regeneration, callus was also induced, affected by lightand sucrose in the first six weeks of culture (as discussed in section 4.2). After subculture, the embryos that had been maintained in darkness in the treatment with 0.5 mg/L 6-BAP resulted in the highest callus induction, suggesting that this cytokinin together with a dark environment promotes callus induction of *E. altensteinii*. Similarly, in *C. revoluta*, darkness was reported to promote callus formation (Rinaldi, 1999). The effect of 6-BAP on callus formation was in contrast to its effect on rooting and shooting (described above). It is possible that this cytokinin is more efficient at promoting undifferentiated cell division.

Callusing was also affected by an interaction of light and PGRs. The treatments with Kinetin + 6-BAP had lower callus induced compared to 6-BAP, although this was not significant. However, in the in the embryos that were initially maintained in the light, Kinetin + 6-BAP caused significantly more callus induction compared to those that were initially maintained in the dark. The response of *E. altensteinii* with regards to callus induction, suggests that light could be an inhibiting factor. Considering that in both the initial culture and subculture, darkness promoted greater induction of callus. All the callus induced in *E. altenstinii* was harvested and further subcultured onto fresh media with various PGRs, however the callus did not proliferate nor regenerate any morphological organs.

The results obtained, with regards to the regeneration of *E. altensteinii* embryos demonstrate that embryos were able to regenerate in PGR free media. While Kinetin and 6-BAP, individually were also able to regenerate roots and shoots, the treatments with 6-BAP alone resulted in low root and shoot regeneration and enhanced callus formation (Appendix 1, 2 and 3). The combination of Kinetin and 6-BAP was the most effective treatment in regenerating roots and shoots (Appendix 1 and 2). Light conditions were also important for the regeneration of *E. altensteinii* embryos. Regeneration of roots was favoured by 24h of darkness in the initial culture, while shoot regeneration was significantly better in the embryos that were initially maintained in 24h of light. After subculture both roots and shoots were favoured by a combination of light and darkness.

The effects of light and PGRs on the regeneration of *E. manikensis* roots, shoots and callus

Shoots and callus of the *E. manikensis* were induced in the initial six weeks of culture; however, the induction of both was not explained by neither of the variables assessed in the initial culture. Nevertheless, all of the three assessed variables were significantly influential in *E. manikensis* root regeneration. Rooting was greater in darkness during this period. These findings, like with *E. altensteinii*, were not surprising as this reflects what happens in nature. Regeneration of roots was also significantly affected by PGR, although the treatment with no PGR showed greater rooting rates. There are three possible reasons for this; 1) the applied AC supplement may have adsorbed the PGRs; 2) *E. manikensis* had endogenous phytohormones that enabled the embryos to root without additional PGR in the media; 3) the concentration of the PGR used in this experiment (0.5 g/L) may be too low to improve the regeneration of these embryos (Dehgan & Schutzman, 1989). To test the third potential reason above; upon subculture, the effects of PGRs on embryo regeneration were tested with the original concentration of Kinetin + 6-BAP (0.5 g/L) and a treatment with increased concentration (i.e. 1.0 g/L as per Table 2.2). As expected, regeneration of roots and shoots was significantly higher in the treatment with the increased concentration of Kinetin + 6-BAP compared to both the treatment with no PGR and the treatment with the original Kinetin + 6-BAP concentration. In addition, embryo callusing was also significantly lower in the original Kinetin + 6-BAP treatment compared to the treatment with the increased concentration. These findings suggest that, in addition to initiating embryo regeneration in a dark environment, increasing the concentration of Kinetin + 6-BAP was also beneficial to the regeneration of *E. manikensis*.

Embryo necrosis and contamination in *E. altensteinii* and *E. manikensis*

A staggering 67% of *E. altensteinii* and 79% of *E. manikensis* embryos were lost as a result of necrosis and contamination by the end of the 12 weeks incubation period. Contamination contributed more to this embryo death than necrosis. During initial culture, both embryo necrosis and contamination of *E. altensteinii* were significantly lower in the media supplemented by with 0.5 mg/L 6-BAP, compared to the other treatments. Following subculture, necrosis and contamination were both affected by an interaction between environmental condition and PGR, resulting in lower embryo necrosis and contamination in treatments that were initially maintained in light compared to darkness. However, in the light, necrosis increased significantly in the treatments with 0.5 mg/L 6-BAP.

Both necrosis and contamination of *E. manikensis* embryos were significantly lower in the light in the first six weeks. However, as mentioned above, this increased with increase in sucrose concentration. During subculture, *E. manikensis* embryos were subjected to a constant 15 g/L sucrose and varied concentrations of the kinetin and 6-BAP combination (original concentration: 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP and Increased concentration: 1.0 mg/L Kinetin + 1.0 mg/L 6-BAP). While using a moderate sucrose concentration may have reduced necrosis and contamination, both these variables were higher in the original concentrations. Moreover, contamination was significantly lower in the increased concentrations.

Contamination has been reported to be a common problem in *in vitro* tissues culture, and this was no different in this study, with both species. After stringent measures were taken to eliminate epiphytic microbes in the *Encephalartos* seeds,

contamination persisted. At the point, it is difficult to speculate at the possible causes of this contamination. However, this may be an indication that *Encephalartos* embryos or seeds in general may have endophytic microbes (bacteria or fungi). Endophytic microbial contamination in tissue culture is one of the major problems in many plant species, which cannot be eliminated during surface sterilization (Singh, 2018). Endophytic microbes are bacterial or fungal microorganisms that colonize healthy plant tissue intercellularly and/or intracellularly without causing any apparent symptoms of disease (Wilson, 1995). There are complex interaction between the plant tissues and the endophytes within the tissue that are mutualistic and antagonistic, which result in the maintenance of a stable symbiosis between the two (Nair & Padmavathy, 2014).

Reports of endophytes on various plant tissues i.e. seeds, ovules, roots, tubers, stem, leaves and fruits dates back to the 1940's where endophytic contaminants were suspected to result from poor disinfection protocols or latent pathogens (Hallmann et al., 2010). However, over the years research has demonstrated some positive attributes towards endophytes, such as improved plant growth and reduction in disease symptoms caused by other plant pathogens that enable plants to be well adapted to the environment (Nair & Padmavathy, 2014).

At this point it is unknown whether *E. altensteinii* and *E. manikensis* embryos have bacterial or fungal contaminants, and whether these could be endophytic. However, research has shown that cycads do have a symbiotic relationship with endophytes (Milindasuta, 1975). Endophytic blue-green algae have been found in cycad species and identified as species of *Anabaena* or *Nostoc,* found in the coralloid roots which only occurs in cycads (Spratt, 1915). Cycads are the only known gymnosperms to form symbiotic relationships with nitrogen-fixing endophytic

organisms (*Anabaena* and *Nostoc*) (Milindasuta, 1975). Together the coralloid roots and nitrogen-fixing organisms mutually benefit each other (Das and Varma, 2009). Based on the presence of endophytes in the coralloid roots and the contaminants found in this study, the presence of endophytes in the embryos may be a plausible explanation. However, this needs further investigations in order to make final conclusions.

The effects of wounding *E. altensteinii* embryo-derived *in vitro* plantlets

All the healthy embryo-derived *in vitro* plantlets that survived an overall 12 weeks incubation period, which had developed roots, shoots, callus or neither underwent wounding experiments. While it is well known that wounding can improve dedifferentiation in explants, which may result in stimulation of organ proliferation (Haberlandt, 1902), wounding embryo-derived *in vitro* plantlets of *E. altensteinii* did not stimulate any organs nor callus. All wounded plantlets died as a result of necrosis and/or contamination. The former was likely to be a result of phenolic exudation in the wounded parts of the embryo-derived *in vitro* plantlets. Wounding exposes fresh tissue to air; this, in phenolic rich plants causes a release of phenolic compounds in the wounded part, which in turn results in the oxidation reaction that attacks the remaining tissues (Bhat & Chandel, 1991) causing them to brown. Lethal browning due wounding in *in vitro* experiments is said to be a common phenomenon in woody plants (Bhat & Chandel, 1991). Since cycads are characterised by their woody trunks, it may be safe to assume that they are phenolic rich plant species. Furthermore the lethal browning observed in wounded *E. altensteinii* embryo-derived *in vitro* plantlets as well as in the embryo explants during the 12 weeks of culture may have been due to phenolic exudation. As for contamination, this may have been a result of failure to sterilize

PGRs used to treat the wounded plantlets. Embryo-derived *in vitro* plantlets which had both roots and shoots succumbed to drying when transferred from *in vitro* to *ex vitro* during primary hardening. This may have been caused by the shift of embryo-derived *in vitro* plantlets from sterile and fully controlled environmental conditions to lesser controlled environments, shocking the plantlets and therefore drying. As a result, no acclimatization was achieved with *E. altensteinii* embryo-derived *in vitro* plantlets.

Hardening and acclimatization of *E. manikensis* embryo-derived *in vitro* plantlets

After an overall 12 week incubation period, there were 24 *E. manikensis* embryo-derived *in vitro* plantlets (from the 261 embryos in the beginning of culture) which had developed both roots and shoots. While the plantlets were maintained *in vitro*, they were in 100% relative humidity and received constant supply of nutrients and sucrose from the media. These plantlets were subjected to hardening, and potted on 1:1 ratio perlite and vermiculite, which is a crucial step prior to plantlet transplantation to actual soil. During the process of weaning, the potted plantlets were covered under a plastic bags in order to maintain a high relative humidity and maintained in a growth chamber with similar conditions as in culture for one week. In the second week, the plastic bags were opened up gradually to lower the relative humidity. During this period other plantlets developed stronger roots, build up new leaflets and became photosynthetically active with others succumbing to death.

The transfer of these plantlets from *in vitro* to *ex vitro* led to the drying out and death of 15 plantlets, having survived 98-111 days from initial culture. This may have been caused by changes in environmental conditions during the transfer from *in vitro* to *ex vitro* which resulted to plantlet stress. The remaining nine plantlets were transferred to bark and potting soil (1:1 ratio), the hardened plantlets had well-

developed roots with secondary roots and strong leaflets. These plantlets were successfully acclimatized and survived over 300 days from initial culture. All the successfully acclimatized embryo-derived *in vitro E. manikensis* plantlets look morphologically similar to seed-derived plants, having one, two or three leaflets. Although this study resulted in a low success rate of ~3.5% successfully acclimatized plants, it has demonstrated a step toward the right in the direction in terms of using plant tissue culture as an additional conservation method for threatened cycad species.

In vitro **regeneration growth patterns between** *Encephalartos* **species and other cycad species**

This study has made it evident that the regeneration *E. altensteinii* and *E. manikensis* cycad embryos from *in vitro* tissue culture is possible. Moreover, this study further shows that when different species of the same genus are treated the same (i.e. the first six week of culture for *E. altensteinii* and *E. manikensis*), the outcomes in regeneration growth patterns differs from species to species. For instance, lightdid not have an influence in rooting of *E. altensteinii*, and the presence of Kinetin induced more roots than other treatments. While *E. manikensis* rooting was affected by both light and PGRs, resulting in more root induction in darkness and in PGR free media. Regeneration pattern differ even more tremendously in cycads of different families or genera. Naderi et al. (2015) reported that in *C. revoluta* shoots were induced in media supplemented with 0.5 mg/L 6-BAP within 35 days of culture and all other combinations of PGRs (including 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP) were reported to result in the formation of callus. Roots were further induced on shoots by continuous culture on rooting medium (½ MS, 0.1 mg/L NAA) within 4-5 weeks (Naderi *et al.*,

2015). In this study for both *E. altensteinii* and *E. manikensis* roots and shoots were induced in the same media, within 6 weeks in culture under similar culture conditions as *C. revoluta* (shoot induction media). The former induced more roots and shoots in media supplemented with 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP, while the latter was also able to induce roots and shoots in the same media, it regenerated even more roots and shoots in 1.0 mg/L Kinetin + 1.0 mg/L 6-BAP following subculture. While 0.5 mg/L 6-BAP resulted in low root and shoot regeneration in *E. altensteinii* and *E. manikensis,* it resulted in greater callus formation in *E. altensteinii.*

2.5. Conclusion

This study has revealed that both *E. altensteinii and E. manikensis* cycad embryos are able to regenerate roots, shoots and callus in a period 12 weeks in *in vitro* tissue culture. Embryos of both species were able to regenerate on plant growth media with or without PGRs, in media supplemented with various sucrose concentrations (0, 15 and 30) g/L and maintained under different light conditions (light and darkness). Although *E. altensteinii* was not acclimatized due to wounding experiments, the plantlets that had regenerated both roots and shoots had the potential to be successfully acclimatized. An overall 12% plantlets regenerated from 261 *E. manikensis* embryos were obtained in a period of 84 days. However, the percentage dropped during hardening while trying to determine the optimum conditions for the plantlets *ex vitro*. Well-developed plantlets with a juvenile leaf, with pinnate leaflets were successfully acclimatized and survived over 300 days after culture initiation, resulting in ~3.5% acclimatized *E. manikensis* plantlets.

This study highlighted the interactions between sucrose, light and PGRs, and how the different combinations and interactions could result in different outcomes,

especially with each of the assessed species. The three assessed variables formed complex interactions which either resulted in beneficial or negative effects on the regeneration of *E. altensteinii* and *E. manikensis.* High sucrose seemed to induce necrosis and contamination (*E. manikensis*), however, total elimination of sucrose was not an option as it supplied the explants with much needed carbon sources in culture media for the viable induction and development of the explants-seedlings, and creates the suitable osmotic conditions for *in vitro* cell/tissue growth (Gaj, 2004). Hence moving forward 15 g/L sucrose was used for both species. Furthermore, the combination of PGRs (Kinetin + BAP) was the most preferred for the regeneration of *E. altensteinii* embryo rooting and shooting, while an increased concentration of Kinetin + BAP (1.0 mg/L) was most preferred for *E. manikensis*. The alternation of darkness and light was most preferred for *E. altensteinii* and *E. manikensis* embryo rooting and shooting, however initiation in darkness was most beneficial and this was applicable to both species.

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CHAPTER THREE: Phytohormone profiling of *Encephalartos altensteinii* seeds

Abstract

Phytohormones are a group of naturally occurring signalling molecules, derived from numerous metabolic pathways, which regulate plant growth and all other physiological processes during the life cycle of plant. Phytohormones are categorised into several classes based on their chemical structures and physiological functions. There are also multiple phytohormone interactions that play a role in the growth and development of a plant, one of which is the interplay between Abscisic acid (ABA) and Gibberillins (GAs) which control seed germination. Seed germination is one of the major physiological processes in a plants' life cycle, and the completion of this process results in the growth of a seedling. In this study, a comprehensive phytohormone analysis was performed on mature 8-12 month old *E. altensteinii* seed tissues (embryos and megagametophytes). The predominant phytohormones found were ABA and its metabolites, cytokinins and auxins. ABA and its catabolites were in both the embryos and megagametophytes, moreover, the higher content in the embryos suggests that their function of inhibiting germination was still prevalent. Furthermore, the detected concentrations of auxins and cytokinins in both tissues were much lower than that of ABA. The low concentrations of auxins and cytokinins, coupled with the absence of GAs in both tissue samples suggest that no real germination was taking place in the assessed seeds. Overall, this study suggests that these seeds are either in a dormancy state or, more likely, shows a very slow developmental process where the ABA is simply preventing precocious germination and what was thought to be mature *E. altensteinii* seeds may in fact be immature seeds.

3.1. Introduction

Phytohormones, also known as plant hormones are a group of naturally occurring signalling molecules that regulate plant growth and all other physiological processes during the life cycle of plants (Liu et al., 2013). Phytohormones are categorised into several classes based on their chemical structures and physiological functions (Davies, 1995). The major phytohormone classes include, but are not limited to; auxins (in particular indole-3-acetic acid, IAA), cytokinins, gibberellins, abscisic acid (ABA), salicylic acid (SA), brassinosteroids (BR), ethylene, strigolactone, jasmonates [jasmonic acid (JA) and derivatives 12-oxo-phytodienoic acid (OPDA)] (Davies, 1995; Santner et al., 2009). These phytohormones are derived from numerous important metabolic pathways, and are generally present endogenously in extremely low concentrations i.e. micromolar (μM) or even lower (Chiwocha et al., 2003). The low concentrations either act locally, where they are synthesized, or near the site of synthesis or in distant tissues (Santner et al., 2009). They also play very crucial roles in interactions between plants and other organisms (Pozo et al., 2005; Pieterse et al., 2009; Santner et al., 2009). Physiological processes governed by phytohormones include development, growth, nutrient translocation and distribution, as well as responses to stresses caused by biotic and abiotic factors i.e. temperature fluctuations, water and nutrient imbalance, and pathogens (Peleg & Blumwald, 2011). Therefore, detecting the presence and quantification of phytohormones is crucial in order to understand their functions in plant metabolism and ecological interactions.

In nature, plants interact and respond to a wide array of environmental factors, such as light, temperature and nutrients. The interaction between phytohormones and environmental conditions play a crucial role in the growth and development of plants

(Benková, 2016). Herein, the presence of endogenous phytohormones facilitates the plants to quickly adjust their growth and development under changing environmental conditions (Vanstraelen & Benková, 2012). Moreover, the physiological and biological activities are controlled by phytohormone availability (Vanstraelen & Benková, 2012). While functions of each phytohormone have been made known by genetic and physiological studies, the availability of endogenous levels of each phytohormone is controlled by factors such as; biosynthetic and metabolic rates, cellular and subcellular localization, transport, and responses of the signal perception and transduction pathways (Zhang et al., 2009; Davies, 2010). Each of these process are unique to each phytohormone (Vanstraelen & Benková, 2012), and changes in these processes may result in countless altered effects on physiological and biological activities (Šimura et al., 2018).

Studies have further shown evidence that there are more complex interactions between phytohormones and their functions (Vanstraelen & Benková, 2012). Apart from each individual phytohormone having a certain function and its own specific hormonal pathway, several phytohormones have been associated with having similar biological functions (Vanstraelen & Benková, 2012). Hormonal pathways not only act in a linear fashion, increasing evidence suggests that phytohormone signalling involves intricate connections between all other pathways involved (Vanstraelen & Benková, 2012). Phytohormones and their pathways can be interlinked by a complex network of interactions and feedback circuits, resulting in completely different outcomes compared to the individual phytohormone and its pathway (Vanstraelen & Benková, 2012). This suggests the importance of analysing several phytohormones with one comprehensive analysis, instead of analysing each phytohormone as an individual entity.

Some of the known phytohormone interactions in the regulation of plant development include (i) auxin-cytokinin interaction, shaping the embryonic root pole, (ii) auxin-cytokinin-gibberellin interactions in the control of shoot apical meristem activity, (iii) auxin, cytokinin, and strigolactone interplay in the regulation of shoot branching, and (iv) interplay of ABA and GAs in the control of germination (Vanstraelen & Benková, 2012). The latter, has demonstrated vital roles played on inhibiting and promoting seed germination. ABA is known to induce and maintain seed desiccation tolerance and dormancy, which is crucial for plant survival as it ensures that seeds germinate upon favourable environmental conditions (Liu et al., 2013). Moreover, the difference in the balance between GA and ABA possibly dictates whether the seed will germinate or not (Bewley et al., 2012). Desiccation tolerance is acquired during seed maturation in orthodox seeds and this is mainly controlled by ABA (Gutierrez et al., 2007), while desiccation sensitivity is associated with high GA levels (White et al., 2000) and reduced levels of ABA or the disruption of its signalling pathway (Ooms et al., 1993). Several phytohormones including brassinosteroids, ethylene, and cytokinin have been shown to play certain roles in seed germination (Kucera et al., 2005; Wang et al., 2011), although GAs have been suggested as the main phytohormones that break seed dormancy and promote germination (Bentsinka & Koornneef, 2008; Finkelstein et al., 2008).

Seed germination is one of the major physiological processes in a plants' life cycle, and the completion of this process results in the growth of a seedling (Millar et al., 2006). Germination is a complex physiological process that is driven by both external factors, such as environmental conditions, as well as internal factors such as genetics and physiology (Zhang et al., 2009). Furthermore, the processes involved in seed development and germination vary with the seed types. For example, prior to

germination, desiccation-tolerant seeds end their development process by losing most of their moisture content and enter a quiescent state (Romero-Rodríguez et al., 2018). Desiccation-sensitive seeds on the other hand, often germinate immediately after shedding, while maintaining high moisture contents and maintaining high metabolic activity without going through a quiescent state (Berjak & Pammenter, 2013; Caccere et al., 2013; Parkhey et al., 2014). While hormones involved in the germination of desiccation sensitive seeds (i.e. cytokinins, GA and auxins) are mostly poised for immediate germination, their functions are possibly suppressed by high levels of ABA (Farrant et al., 1993). These contrasting physiological differences may influence variation in the phytohormones content and changes during both development and germination of the seeds. The way in which the ABA/GA ratio regulates seed germination has been of interest in many studies. However, studies have largely focused on desiccation-tolerant seeds, leaving a knowledge gap on the role of ABA/GA ratio on desiccation-sensitive seeds (Romero-Rodríguez et al., 2018). Few studies on desiccation-sensitive seeds have suggested that precocious germination is likely to be a result of low levels of ABA (Farrant et al., 1993; Prewein et al., 2006).

Encephalartos altensteinii has desiccation-sensitive seeds and therefore falls into less studied group of plants. While there are some reported cycad studies using exogenous application of plant growth regulators for improved germination (Dehgan & Johnson, 1983; Dehgan & Schutzman B, 1983) and *in vitro* regeneration (Motohashi et al., 2008; Naderi et al., 2015; Webb, 1981) no study has reported endogenous phytohormone levels. Studying phytohormone profiles and their metabolites is a vital tool for investigating the roles played by phytohormones during cycad seed germination. Therefore acquiring this knowledge for *E. altensteinii* may offer insights into which phytohormones are present or lacking in these seeds and thus, which PGR

may be necessary for improved *in vitro* regeneration and germination. This study was conducted to evaluate phytohormone levels prevalent in mature (8 - 12 months old) *E. altensteinii* seeds (embryos and megagametophytes), and how these phytohormones (or lack of) possibly affect the regeneration of *E. altensteinii* embryos in *in vitro* tissue culture, and germination as a whole. The selected 8 - 12 months old *E. altensteinii* seeds are assumed to be in a mature stage, and thus are expected to be relatively quiescent, if not dormant, awaiting germination.

3.2. Materials and methods

3.2.1. Seed collection and handling

Encephalartos altensteinii seed collection and handling was done as per Chapter Two in section 2.2.1.

3.2.2. Seed preparation

Thirty three *E. altensteinii* seeds were separated into embryos and megagametophytes which were snap-frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle. Two finely ground frozen tissue samples (embryos and megagametophytes) were transferred into separate Eppendorf tubes which were dried under vacuum for 72 h. Due to the mass of tissue required for experimentation and insufficient seed materials there were no replicates.

A CITES export permit was acquired from the Department of Environmental Affairs, Pretoria, South Africa (Permit number: 206177). The permit and samples were inspected and signed off by the Environmental Management Inspector at Cape Nature, Driftsands Nature Reserve, Cape Town, South Africa. Thereafter the material was shipped to the National Research Council of Canada for analysis.

3.2.3. Chemicals and Calibration Curves

A number of compounds namely Dihydrophaseic acid (DPA), Abscisic acid glucose ester (ABA-GE), Phaseic acid (PA), 7'-Hydroxy-abscisic acid (7'- OH-ABA), neo-Phaseic acid (neoPA), trans-Abscisic acid (t-ABA) and N-(Indole-3-yl-acetyl)-glutamic acid (IAA-Glu) were synthesized and prepared at the National Research Council of Canada, Saskatoon, SK, Canada; cis-Abscisic acid (ABA), N-(Indole-3-yl-acetyl)-

leucine (IAA-Leu), N-(Indole-3-yl-acetyl)-alanine (IAA-Ala), N-(Indole-3-yl-acetyl)-aspartic acid (IAA-Asp), Indole-3-acetic acid (IAA), Zeatin (Z), Zeatin riboside (ZR), Isopentenyladenine riboside (iPR), and Isopentenyladenine (iP) were purchased from Sigma–Aldrich; Dihydrozeatin (dhZ), Dihydrozeatin riboside (dhZR), Zeatin-O-glucoside (Z-O-Glu), and Gibberellins (GAs) 1, 3, 4, 7, 8, 9, 19, 20, 24, 29, 44, and 53 were purchased from OlChemim Ltd. (Olomouc, Czech Republic). Deuterated forms of the hormones which were used as internal standards include: d_3-DPA, d_5-ABA-GE, d_3-PA, d_4-7'-OH-ABA, d_3-neoPA, d_4-ABA, d_4-trans-ABA, d_3-IAA-Leu, d_3-IAA-Ala, d_3-IAA-Asp, and d_3-IAA-Glu were synthesized and prepared at NRCC SK according to Abrams et al. (2003), and Zaharia et al. (2005). The d_5-IAA was purchased from Cambridge Isotope Laboratories (Andover, MA); d_3-dhZ, d_3-dhZR, d_5-Z-O-Glu, d_6-iPR, d_6-iP and d_2-GAs 1, 3, 4, 7, 8, 9, 19, 20, 24, 29, 34, 44, 51 and 53 were purchased from OlChemim Ltd. (Olomouc, Czech Republic). The deuterated forms of selected hormones used as recovery (external) standards were prepared and synthesized at NRCC SK. Calibration curves were created for all compounds of interest. Quality control samples (QCs) were run along with the tissue samples.

3.2.4. Instrumentation

Analysis was performed on a UPLC/ESI-MS/MS utilizing a Waters ACQUITY UPLC system, equipped with a binary solvent delivery manager and a sample manager coupled to a Waters Micromass Quattro Premier XE quadrupole tandem mass spectrometer via a Z-spray interface. MassLynx™ and QuanLynx™ (Micromass, Manchester, UK) were used for data acquisition and data analysis.

3.2.5. Hormone quantification by HPLC-ESI-MS/MS

The procedure for quantification of ABA and ABA catabolites, cytokinins, auxins, and gibberellins in this tissue was performed using a modified procedure described in Lulsdorf et al. (2013).

Briefly, the analyses utilize the Multiple Reaction Monitoring (MRM) function of the MassLynx v4.1 (Waters Inc) control software. The resulting chromatographic traces were quantified off-line by the QuanLynx v4.1 software (Waters Inc) wherein each trace is integrated and the resulting ratio of signals (non-deuterated/internal standard) is compared with a previously constructed calibration curve to yield the amount of analyte present (ng per sample). Calibration curves were generated from the MRM signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard. The QC samples, internal standard blanks and solvent blanks were also prepared and analyzed along each batch of tissue samples.

3.3. Results of quantification

3.3.1. Gibberillins (GAs)

None of the GAs monitored were present in either the embryo or megagametophytes samples.

3.3.2. Abscisic acid (ABA) and ABA metabolites

Results show the presence of ABA and most of its catabolites in both the embryos and megagametophytes. Results indicate that levels of biologically active ABA alone and overall ABA and ABA catabolites were higher in the embryo tissue compared to those

in the megagametophyte tissue (Figure 3.2). It appears that the conjugation (which resulted in ABAGE, as presented in Figure 3.1) was the predominant catabolism pathway, followed by 7'- and 8'-hydroxylation (resulting in 7'OH ABA, and in PA which was further reduced to DPA, respectively). Trans-ABA was formed as a product of isomerization of natural ABA under UV light. The presence of high levels of catabolites suggests that higher levels of biologically active ABA must have been produced and further catabolized.

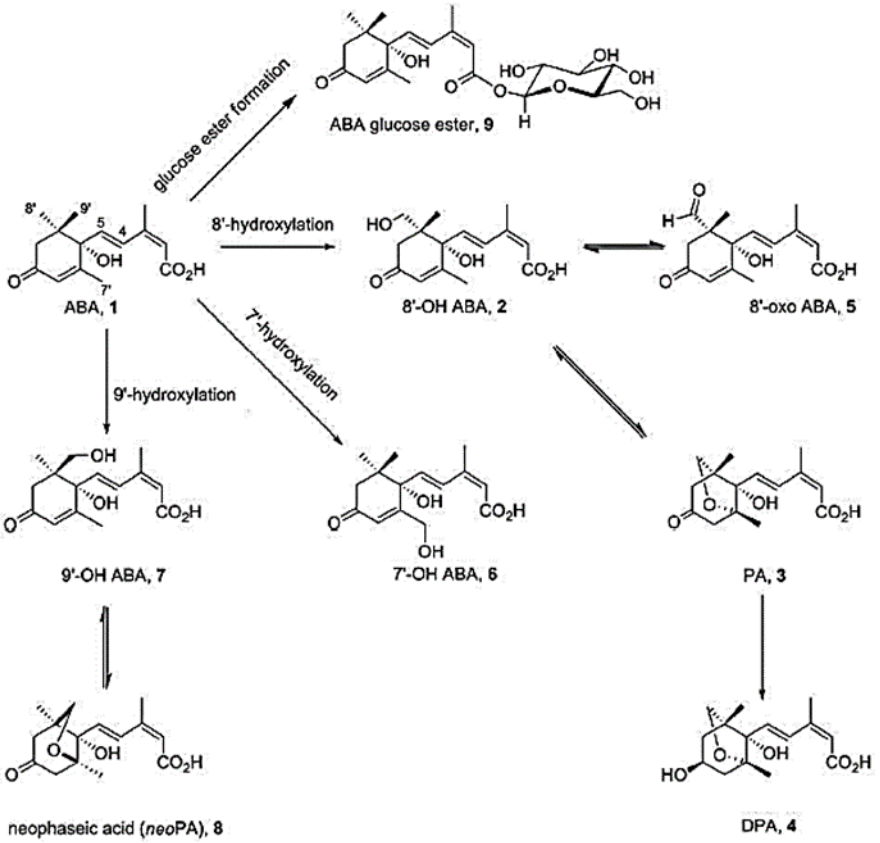
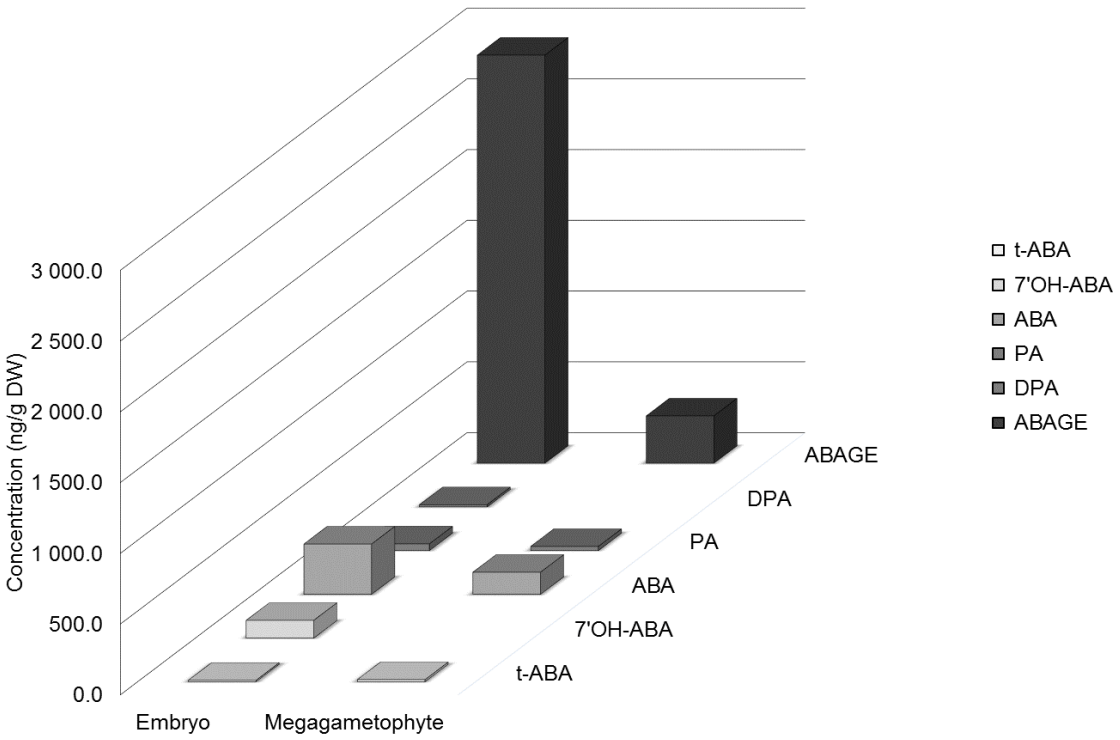


Figure 3.1: Known ABA catabolism pathways in plant tissue (Abrams et al., 2003).



2229

2230 Figure 3.2: ABA and ABA catabolic content in *Encephalartos altensteinii* embryos and
2231 megagametophytes

2232 ### 3.3.3. Cytokinins

2233 Results show that the overall cytokinin content in the embryo tissue was higher than
2234 that in the megagametophyte tissue (Figure 3.3). Interestingly, among the bioactive
2235 free base cytokinins (Z, dhZ and iP) only Z (cis-isomer) was found, and this in the
2236 megagametophyte tissue. However, their biosynthetic precursors ZR (both cis- and
2237 trans-isomers), dhZR and iPR were detected, as well as the Z catabolism product ZOG
2238 (both cis- and trans-isomers) in both the embryos and megagametophytes.

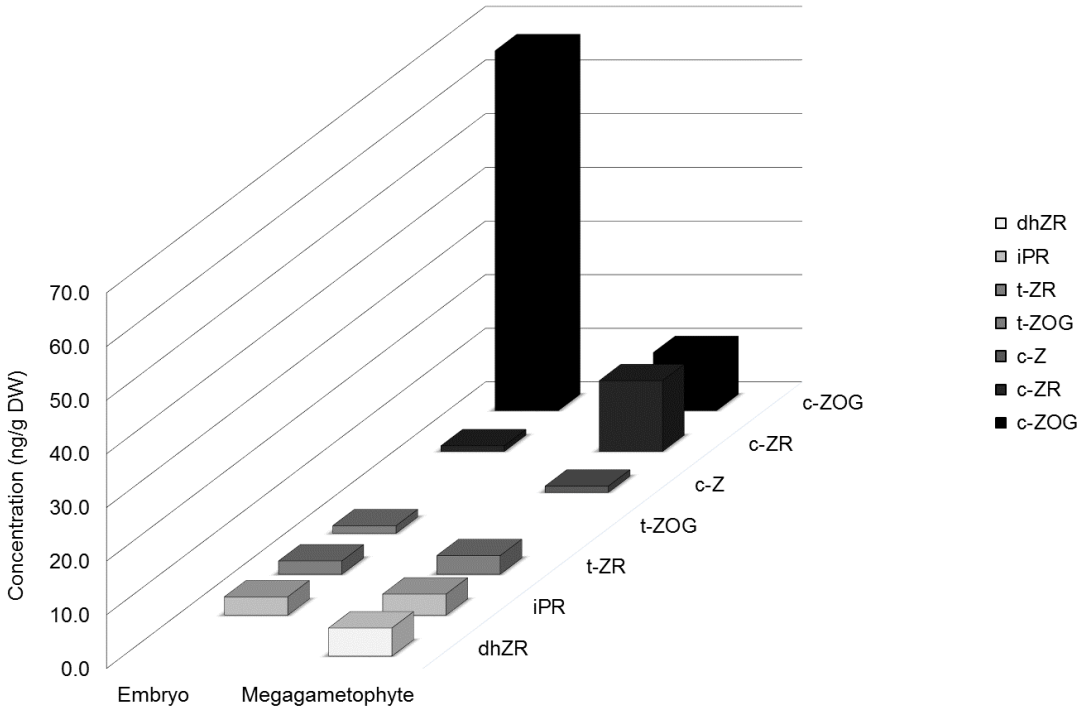
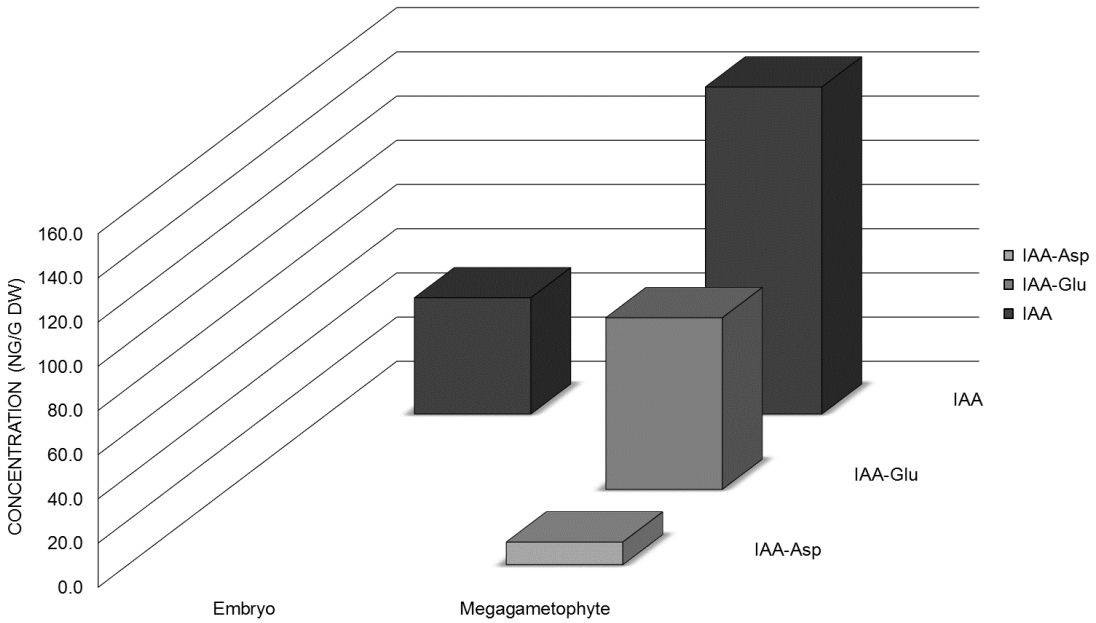


Figure 3.3: Cytokinin content in *Encephalartos altensteinii* embryos and megagametophytes

3.3.4. Auxins

Results indicate that the overall auxin content in mega gametophyte tissue was significantly higher than that in the embryo tissue (Figure 3.4). Only the biologically active IAA was present in the embryo, whereas its conjugates with glutamic acid IAA-Glu and aspartic acid IAA-Asp were present in the mega gametophyte tissue.



2247

2248 Figure 3.4: Auxin content in *Encephalartos altensteinii* embryos and

2249 megagametophytes.

3.4. Discussion

In this study *E. altensteinii* seeds were assessed for phytohormones and the predominant trends found were Abscisic acid (ABA) and its metabolites followed by cytokinins and auxins. Phytohormones are provided by the mother plant (Frey et al., 2004) and the seed coat plays a role in the regulation of the development and maturation of embryos (McCarty, 1995). The presence of these phytohormones in the megagametophyte supports the hypothesis that megagametophytes are a nutrient source for the embryos and thus may regulate germination (see also King & Gifford, 1997).

3.4.1. Abscisic acid

The findings of this study show the presence of both ABA and its catabolites in both the embryos and megagametophytes. While ABA is associated with various functions during seed development (including induction of reserve accumulation, desiccation tolerance and dormancy), germination and response to harsh conditions, the catabolic pathways as well as the active hormone levels determine the actual role it plays (Jacobsen et al., 2002). The presence of ABA in high levels is often associated with high abiotic stress (i.e. hydraulic stress) during seed development (Nambara & Marion-Poll, 2003). However, in *E. altensteinii* seeds, the presence of ABA is unlikely to be related to hydraulic stress. High levels of ABA and its catabolites in the embryos could be an indication that ABA was inhibiting germination of the seeds while promoting embryo maturation (Gazzarrini et al., 2004). Given that there was a range of two months in maturity of the embryos sampled, it is therefore possible that there is a slow breakdown of ABA, and a slow release from “dormancy”. If the megagametophytes

are a source of nutrition, higher ABA levels earlier (prior to the assessment) could have been present.

There are multiple routes in which ABA catabolism may occur to further regulate ABA concentrations in plant tissues, either by oxidation or conjugation (see Figure 3.1) (Cutler & Krochko, 1999). The most common pathway is that of oxidation which is triggered by hydroxylation at C-8' to give rise to PA and DPA (Kushiro et al., 2004). Although PA has been shown to exhibit some ABA biological activity, DPA has been described as a fully inactive state of ABA (Walton & Yi, 1995). Therefore, the low levels of both PA and DPA suggests the ABA was still predominately active in *E. altensteinii* seeds. Although oxidation is the most common pathway, the conjugation of ABA to form ABAGE was the predominant pathway for ABA inactivation in this study. Moreover ABAGE was predominately higher in the embryo. This conjugation forming ABAGE is biologically inactive (Cutler & Krochko, 1999) and further suggests a possible slow breakdown of active ABA in maturing embryos..

3.4.2. Gibberillins

Several studies have shown the interplay between ABA and GA in facilitating seed dormancy and germination (Debeaujon & Koornneef, 2000; Karssen & Laçka, 1986; White et al., 2000). While ABA is associated with seed dormancy, GAs are known for promoting seed germination following dormancy period (Bewley, 1997). The absence of GAs was observed in both the embryos and megagametophytes. While this may suggest that there was no real germination preparation taking place, it is also surprising that they were found in neither the embryos nor megagametophytes. In cycad seeds, GAs are thought to occur in the megagametophytes and absorbed by the embryo and depletes from the megagametophytes as the seed matures (Bewley

& Black, 1978; Dehgan & Schutzman, 1989). These 8-12 month old *E. altensteinii* seeds seem to be in either a metabolic stasis or dormant while awaiting germination. This is supported by high levels of ABA and the absence of GAs. However, this could also indicate the stage that is known to be mature cycad seeds are not really mature seeds. And the absence of GAs in these seeds may be an indication that these embryos are probably still developing.

3.4.3. Auxins

Indole-3-acetic acid (IAA) was detected in both tissues, but the conjugates were only detected in the megagametophytes. Furthermore, the highest concentrations of IAA were detected in the megagametophytes compared to that of the embryos. IAA is a naturally occurring biologically active auxin that is essential for various functions in plants such as flowering and fertilization. More importantly, IAA is required in high amounts for embryo development (Zhao, 2010) and has also been assumed to play a role in specifying the identities of gametic and nongametic cells in *Arabidopsis* plants (Pagnussat et al., 2009). Thus low levels of IAA may be observed in embryos of mature seeds (Bialek & Cohen, 1989; Cohen & Bandurski, 1982). Other studies on gymnosperms with recalcitrant seeds found an opposite trend in mature seeds of *Araucaria angustifolia* (Pieruzzi et al., 2011) and *Pinus sylvestris* (Ljung et al., 2001). However, considering that this study assessed only one developmental stage of so-called “mature” seeds, it cannot be predicted what the levels of IAA were in the earlier or later stages. In gymnosperms, the levels of IAA and ABA in megagametophytes were observed to decline as seed germination was taking place, which suggests that the megagametophyte transfers the phytohormones to the embryo (Ljung et al., 2001; Pieruzzi et al., 2011). Although the biological roles of IAA-Asp and IAA-Glu are

unclear, they have been described in several studies as a starting point to removing excess auxins (Ludwig-Müller, 2011; Woodward & Bartel, 2005). Thus their presence could mean that IAA levels in the megagametophyte are in the declining phase. The reason for the absence of these two conjugates in the embryo is unclear. The storage form of IAA (IAA-Leu), IAA-Ala and IBA were not detected in either embryos or megagametophytes, suggesting that further synthesis of IAA in these tissues was ongoing at the time of sampling. This does need to be confirmed using measurement of levels of enzymes involved in IAA synthesis. This can also be confirmed by analysing phytohormones at different developmental stages of the seeds.

3.4.4. Cytokinins

The trans-isomer of Zeatin riboside (*t*-ZR) was detected in both the embryos and the megagametophytes. The bioactive cytokinin, trans-Zeatin (*t*-Z), was not detected in either the embryos or megagametophytes; however, its catabolic product trans-Zeatin O-glucoside (*t*-ZOG) was detected in low concentrations in the embryo. This study also detected dhZR in the megagametophytes only and IPR in both the embryos and the megagametophytes, while their bioactive cytokinin forms dhZ and IP were not detected. Amounts of biologically active cytokinins (IP and Z) were shown to decline in the mature seeds of the mangrove *Avicenia marina* and remained low during germination (Farrant et al., 1993). Since both these seed types are desiccation sensitive, this similarity in trend is interesting. In the current study, *c*-Z was the only biologically active cytokinin detected. Furthermore, the levels *c*-Z were detected in very low concentration only in the embryos, again fitting a general observation of active cytokinin levels in mature stages of desiccation-sensitive seeds (Dodd & van Staden, 1982; Farrant et al., 1993). This study cannot confirm that the absence of IP and *t*-Z,

and the low levels of *c*-Z could be a result of the decline in cytokinin after the development of the embryos as observed by Farrant et al. (1993). Thus, future studies could work on phytohormone profiling *E. altensteinii* seeds following the different stages of development to assess the changes in these phytohormones.

Unlike t-Z, very little is known about the role of c-Z in embryo development and its influence in the response to environmental stress. However they do have a role during seed dormancy (Schäfer et al., 2015). The limited knowledge on c-Z is mainly because c-Z was ignored in cytokinin studies for decades. In this study c-Z, its precursors and its conjugate, were the most dominant cytokinins compared to t-Z, IP and dhZ. This pattern has been observed in other gymnosperms (*Pinus sylvestris*) and angiosperms (*Urtica dioica*) (Gajdošová et al., 2011). However, the dominance of *c*-Z in the current study was attributed to the high levels of the catabolic product (*c*-ZOG) in the embryos, which is the storage form of c-Z (Mok et al., 1992).

What is known about c-Z, is that its precursor c-ZR under abiotic stress, maintains minimal cytokinin activity, while t-ZR the precursor of *t*-Z is known to exhibit high activity that promotes cell division (Schäfer et al., 2015). In this study, two contrasting detections were observed in the embryos and the megagametophytes. There were high levels of t-ZR compared to those of c-ZR in the embryos, while the opposite was observed in the megagametophytes. A possible explanation for this finding could be that t-ZR is higher than c-ZR in the embryos to promote germination through cell division and that this activity is no longer essential in the megagametophytes.

3.5. Conclusion

The overall aim of this study was to assess the endogenous phytohormones that are present in 8-12 month old *E. altensteinii* seeds. This information could help in developing a successful germination and *in vitro* regeneration protocol for *E. altensteinii* and perhaps an indication of the exogenous hormones that may be applied to improve germination and *in vitro* regeneration. *Encephalartos* seeds are thought to be mature and ready for germination between four to seven months after shedding from the cones depending on the different species (Giddy, 1974; Woodenberg et al., 2007). However, this study suggests this to not be true for *E. altensteinii* seeds. The high levels of ABA compared to auxins and cytokinins suggest that these seeds are either in a dormancy state or show very slow developmental processes where the ABA is simply preventing precocious germination. Furthermore, there were lower levels of IAA in the embryos compared to the megagametophytes. It is then possible that the transfer of IAA to the embryos had not been completed and thus the embryos were probably not yet matured. IAA and ABA are also known to interact with each other; this is especially observed when seed maturity begins and excess ABA needs to be diminished (Pieruzzi et al., 2011). IAA is said to then activate a transcription factor (FUS3) that regulates the synthesis of ABA (Gutierrez et al., 2007). This regulation was observed for *Ocotea odorifera* which also has recalcitrant seeds, showing a decrease in ABA levels and an increase in IAA levels as the seeds germinates (Pieruzzi et al., 2011). Thus, should the *E. altensteinii* seeds be mature it would be expected that the levels of IAA would be higher than those of ABA. Moreover, the absence of GAs in both tissues suggest that no real germination was taking place. These findings suggest that what was thought to be mature *E. altensteinii* seeds may in fact still be immature seeds.

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CHAPTER FOUR: Synthesis

The aim of this study was to develop an efficient and successful *in vitro* regeneration protocol for threatened desiccation-sensitive *Encephalartos* cycad seeds (*E. altensteinii* and *E. manikensis*). The two species chosen for this study were based on the availability of seeds at Kirstenbosch National Botanical Garden (KNBG). Following the *in vitro* study, a phytohormone profiling analysis on *E. altensteinii* seeds was conducted, to test which phytohormones were prevalent in seeds that were the same age as those used for the *in vitro* regeneration study (8 -12 month old seeds). Phytohormone profiles were only assessed on *E. altensteinii* seed (embryos and megagametophytes), as these were the only seeds available at the time these experiments were conducted. The lack or presence of phytohormones in these seeds could possibly have an impact on the regeneration of *E. altensteinii* embryos in *in vitro* tissue culture, and germination as a whole. As such, this study may possibly be a key in understanding the complexity of *in vitro* cycad embryo regeneration and germination. Moreover, this may offer practical solutions for improved *in vitro* regeneration and germination protocols.

General discussion

Successful *in vitro* regeneration of plants is a complex phenomenon and is influenced by a wide range of factors including but not limited to light, sucrose concentrations and application of PGRs in plant growth media (variables tested in Chapter 2). The current study illustrated that while both *E. altensteinii* and *E. manikensis* were subject to the same treatments, they however did not respond in a similar manner with regards to the tested variables. *E. altensteinii* responded with increased callus formation while *E. manikensis* showed increased embryo necrosis and contamination with increased

sucrose concentrations. Furthermore, these two species responded differently to PGRs; where *E. altensteinii* rooting was significantly affected by the presence of Kinetin. The hormone profiling experiment (Chapter 3) showed low levels of active cytokinin and high levels of auxins (IAA) in the embryos, the application of Kinetin in the media may have induced the germination process in these immature seeds. While *E. manikensis* rooting was significantly affected by the presence of PGRs, rooting was higher where there were no PGRs. The lower rates of rooting for *E. manikensis* in the presence of PGRs was due to high necrosis and contamination with increased sucrose. These findings suggest that, although the two species are related, belonging to the same genus (*Encephalartos*), it cannot be assumed that a regeneration protocol that works for one species will have the same effect on other species. For improved results, a successful *in vitro* regeneration protocol for cycads needs to be species specific. As such moving forward during subculture, moderate sucrose levels (15 g/L) were used to reduce the rate of necrosis and contamination for both species and the PGR concentrations were increased from 0.5 mg/L to 1.0 mg/L on *E. manikensis*, which improved regeneration.

This study further illustrated that *in vitro* regeneration of *E. altensteinii* and *E. manikensis* was possible in a relatively short period of time (12 week culture period), although this was achieved at low rates as a result of embryo necrosis and contamination. While plant growth media is a source of nutrients for explants, it is also a good source for microbes which compete with explants for nutrients, resulting in increased culture mortality, variable growth, tissue necrosis, reduced shoot proliferation, and reduced rooting (Odutayo et al., 2007b; Kane, 2003). In attempts to combat the necrosis and contamination, activated charcoal was incorporated in the culture media regime and stringent surface sterilization procedures to produce aseptic

cultures were employed in this study, however, these problems were not completely eliminated. The problem of necrosis and contamination in tissue culture is well documented in other plant species, however none of the reviewed literature in this thesis on *in vitro* regeneration of cycads had dealt with these issues. Embryo necrosis and contamination were evident in the early stages of culture and thus resulted in the loss of explants, contributing to the low numbers of successfully regenerated plants. This is reported to be a common problem amongst woody plant species (Reed et al., 1998).

Successful *in vitro* regeneration of plants depends on recovery of well acclimatized and hardened plantlets (Thomas et al., 2010). Previous studies on *C. revoluta*, conducted by Motohashi et al. (2008) and Naderi et al. (2015) resulted in low numbers of successfully regenerated plantlets, furthermore the frequency of acclimatized and hardened *C. revoluta* plantlets was also low. Likewise, in this study successful acclimatization of *E. manikensis* was achieved at a low rate of 3.5%. In contrast, *E. altensteinii* was also able to regenerate *in vitro*, however, acclimatization was not achieved, as the regenerated plantlets dried out and died within two weeks of primary hardening. The low acclimatization rates may be due to a number of biotic and abiotic factors during transfer from *in vitro* to *ex vitro*. *In vitro*-derived plantlets have been found to have reduced photosynthetic activity, lower cuticular wax, poorly functioning stomata, and underdeveloped root system, (Sivaprasad & Sulochana, 2004) thus making them susceptible to death and low survival rates *ex vitro*, as seen in this study. High mortality following transfer to *ex vitro* may further be caused by the decline in high relative humidity that the explants received *in vitro* as well as the absence of microbial elicitors that trigger metabolic pathways (Thomas et al., 2010). The absence of these microbial elicitors render *in vitro*-derived plantlets susceptible to

pathogen attack and environmental stress once transferred to *ex vitro* conditions (Thomas et al., 2010). Therefore establishing ways to overcome these problems could result in improved growth of *in vitro*-derived plantlets upon transfer to *ex vitro* growth conditions.

After 12 weeks of incubation period, about 23.7% embryos (99 out of 417) of *E. altensteinii* had successfully produced roots and shoots (Chapter 2: 15.6% roots only, 2.4% shoots only, 2.2% roots and shoots and 3.6% shoots and callus). The high level of failed regeneration (76.3%) was due to necrosis and contamination, as well as 7.2% of healthy embryos that did not regenerate. Upon conducting phytohormone profiling analysis, it became apparent that these 8-12 months old seeds may be immature (Chapter 3). This was evident by the high levels of ABA which is known for its germination inhibition function and probable indication that the embryos were still developing (Gazzarrini et al., 2004). This was further supported by the absence of GAs, which are known to be essential for breaking seed dormancy and inducing germination (Bentsinka & Koornneef, 2008; Finkelstein et al., 2008). These findings may explain why there is a low level of regeneration during the *in vitro* experiment (Chapter 2). The application of exogenous PGRs may have activated certain enzymes that regulate germination (Dehgan & Schutzman, 1989) which led to embryo regeneration, although this was successful only in few embryos (23.7%).

Embryo regeneration for *E. manikensis* after 12 weeks of incubation period was about 15.7% (41 out of 261). This consisted of 4.6% roots only, 0.4% shoots only, 9.2% roots and shoots and 1.5% shoots and callus). Although regeneration for this species was low, the findings in chapter 2 suggest that *E. altensteinii* and *E. manikensis* may not follow the same *in vitro* regeneration protocol for successful regeneration. Therefore it cannot be assumed that phytohormones contents in *E.*

manikensis are the same as that of *E. altensteinii*. Furthermore, rooting in *E. manikensis* was significantly higher in PGR free media (in the first six weeks of culture), which may indicate that endogenous phytohormones could have played a role in their regeneration. However, increasing PGR concentration during subculture resulted in even greater embryo regeneration.

Tissue culture techniques are of great interest for collecting, multiplying and storing plant germplasm and are quite useful for conserving plant biodiversity, including genetic resources of recalcitrant seeds and threatened plant species (Engelmann, 1991; Bunn et al., 2007; Engelmann, 2011). As such i*n vitro* techniques have an important role to play within *ex situ* conservation strategies, especially for threatened plant species like cycads, particularly where it is important to conserve specific genotypes or where normal propagules, such as recalcitrant seeds may not be suitable for long-term storage (Cruz-Cruz et al., 2013). These tissue culture techniques involve the use of conventional micropropagation systems, slow growth techniques and cryopreservation (Blakesley et al., 1996). The *in vitro* regeneration of *E. altenstinii* and *E. manikensis*, as well as the phytohormone analysis of *E. altenstinii* embryos and megagametophytes conducted in this thesis, therefore, serve as an alternative pathway towards the conservation of these threatened desiccation-sensitive plants. As part of a longer conservation plan the successfully regenerated plantlets – their embryos and germplasm, provided they survive over a long time period could potentially be used for future *in vitro* regeneration/germplasm conservation studies, as these plants could potentially be contamination free. Although this would not ensure completely virus-free plants tissue, shoot tip or meristem culture of vegetatively propagated plants have been used to eliminate viruses in many plant species (Ashmore, 1997; Faccioli & Marani, 1998). This is a result of having uneven

distribution of viruses in the youngest tissues i.e. shoot apex as virus concentration usually decreases more towards the apical meristem of the stem, where the cells are constantly and rapidly dividing (Wang & Valkonen, 2009). Since not all cells in the actively dividing tissue would be, it is possible to dissect out a non-infected region and manipulate this explant *in vitro* to produce virus-free plants (Grout, 1990; Kane, 2005). Thus, this could potentially result in an increased number of regenerated plants where contamination and perhaps necrosis could no longer be factor that results in a decreased number of successfully regenerated plants. Furthermore *in vitro*-derived plants could also be reintroduced to the wild as part of a conservation strategy.

Biodiversity hotspots, like South Africa are at risk of losing their precious biodiversity, and *in vitro* tissue culture methods have been shown to be effective in rescuing and conserving threatened plants (Pence, 1999; Berjak, 2011), such as cycads. While standard *in vitro* propagation protocols are readily accessible, threatened species have shown to have unusual growth requirements and, thus, may need modified procedures for *in vitro* culture (Cruz-Cruz et al., 2013) and this thesis has clearly indicated the need for *in vitro* protocols to be species specific. This current study has therefore provided important, new and additional scientific knowledge in cycad species conservation particularly for the highly threatened *Encephalartos* genus. This further strongly emphasises the need to have systematically integrated conservation strategies, and the current *ex situ* conservation concepts should be modified accordingly to accommodate technological advances such as *in vitro* tissue culture.

In conclusion, it should be emphasized that *in vitro* tissue culture techniques used in this study are not aimed at replacing conventional *ex situ* conservation methods. They should be used as additional tools for optimizing the cycad germplasm.

The information presented in this thesis will also be useful in monitoring the ability of recalcitrant seeds to germinate after storage. This would further add knowledge to the much needed somatic embryo research an in conservation programmes for *Encephalartos* cycads.

Future studies

Based on the findings of the *in vitro* regeneration experiments and the phytohormone profiling experiments, recommendations for future studies on *E. altensteinii* and *E. manikensis* are as follows:

1) To investigate the influence of AC on the PGRs. Should these be as experienced in this study (negating the application of PGRs) the charcoal must be eliminated (applicable to both *E. altensteinii* and *E. manikensis*).
2) To combat contamination and obtain contamination free cultures, in addition to sterilization protocols, antibiotics might minimize or eliminate contamination. However, this must be specific to each species. Furthermore, the microbes (bacteria or fungi) present in *E. altensteinii* and *E. manikensis* need to be identified and thus determine the effects of various antibiotics on these microbes without adversely affecting the health of *in vitro* grown plant material.
3) To initiate embryo culture in darkness in the first six weeks of culture to promote embryo rooting. Although this resulted in over 50% embryo rooting in media supplemented with 0.5 mg/L Kinetin in this study, this may be increased by supplementing the media with auxins (not tested in this study) which are associated with root initiation.
4) After the first 6 weeks during subculture, introduce a 16h photoperiod to promote shooting. According to this study, shoot regeneration was best induced by 0.5 mg/L Kinetin + 0.5 mg/L 6-BAP.
5) While sucrose is necessary, this study showed that this must be in moderate amounts of sucrose i.e. 15 g/L, as excess amounts seemed to promote embryo necrosis and contamination.

6) Wounding experiments did not lead to induction of roots, shoots or callus; rather all the wounded plantlets were lost due to necrosis and contamination. Therefore, wounding is not recommended.
7) While both *E. altensteinii* and *E. manikensis* were able to regenerate successful i*n vitro* derived plantlets, most of the plantlets of *E. manikensis* (and all for *E. altensteinii*) died during the transition from *in vitro* to *ex vitro* conditions. Thus it is crucial to assess the optimum conditions for hardening and acclimatization of embryo-derived *in vitro* plantlets.
8) Embryos of *in vitro*-derived *Encephalartos* cycad plants that survive for a long term period may be used for future *in vitro* for cryopreservation or reintroduction in the wild for future conservation purposes.
9) With regards to phytohormone profiling, it may be crucial to conduct comparative studies on several cycad species and genera, and further assess phytohormones at different stages of maturity of seeds (embryos and megagametophytes) as this would give a clear indication as which phytohormones are present in different species and genera, and how these phytohormones increase or declining over time as the seeds develop. Furthermore, this would increase knowledge of the physiological changes that occur during seed growth and development. Studies on seeds further along the developmental pathway used here (i.e. older than 8-12months) should be used to assess progression towards a more germinative state as this would allow better understanding on the communication embryos and megagametophytes.
10) Phytohormone profiling should precede *in vitro* regeneration studies, as this might give an indication as to which exogenous PGRs need to be supplemented in plant growth media in order to achieve the best regeneration for embryo-

derived plantlets. While this was not done in the present study, the following conclusions can be drawn retrospectively; exogenous application of GA in plant growth media to break dormancy and induce germination.

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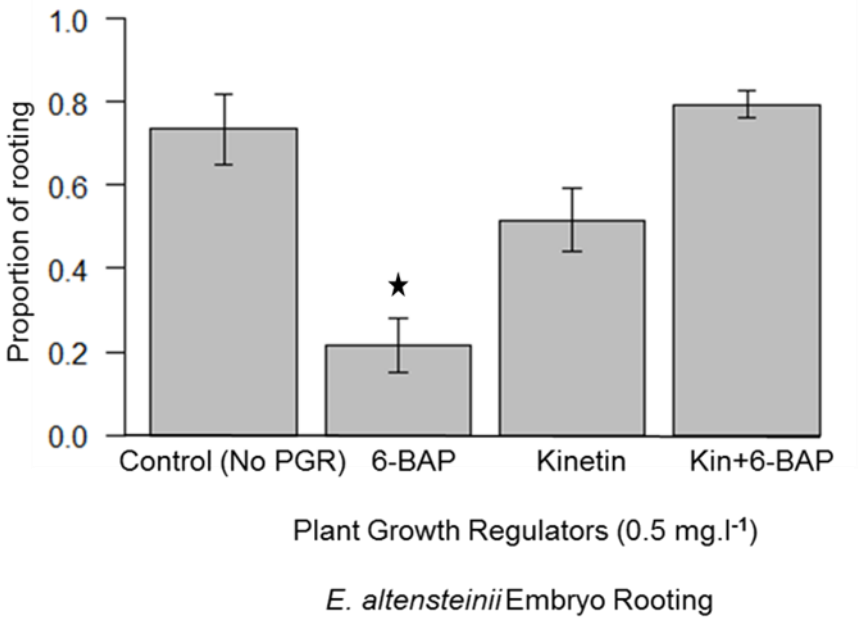
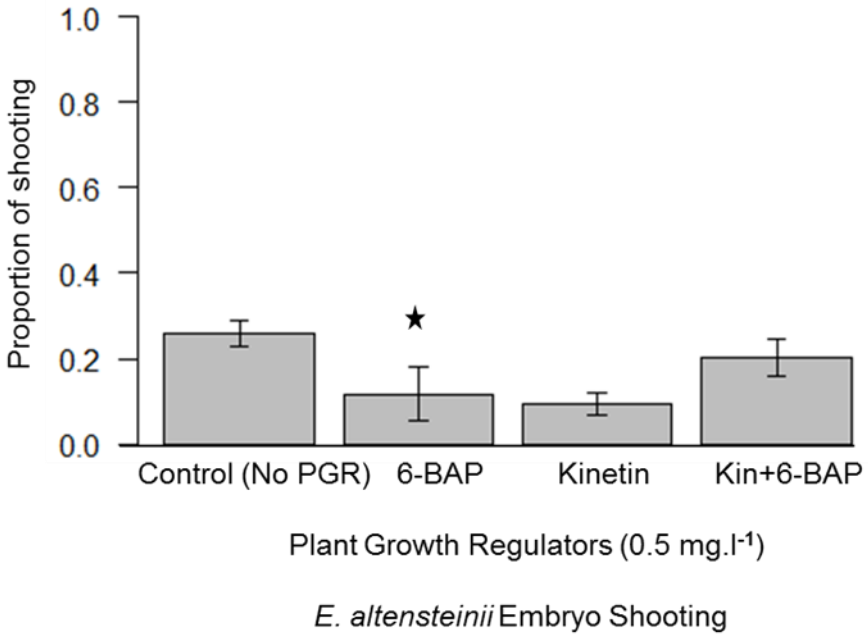
Appendices



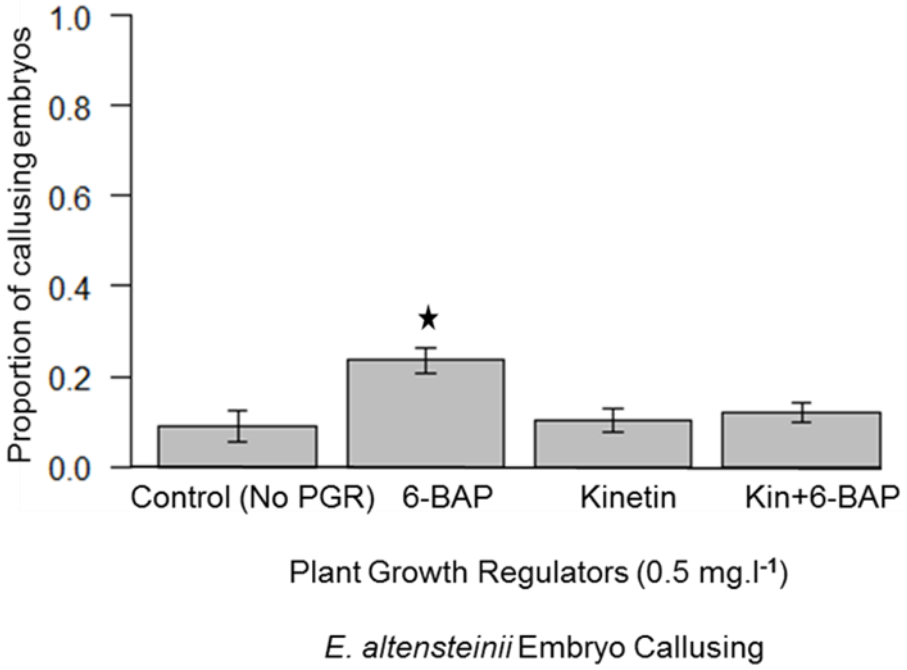
Appendix 1**:** The effect of PGRs on *E. altensteinii* embryo rooting after a 12 week incubation period, obtained from generalised linear models with beta distributions. Embryo rooting was significantly low in 0.5 mg/L 6-BAP (Z = -7.6, P < 0.0001) significance is indicated by ★. Sample size (n = 10) and bars represent Standard Error (±SE).



2840

2841 Appendix 2: The effect of PGRs on *E. altensteinii* embryo shooting after a 12 week
2842 incubation period, obtained from generalised linear models with beta distributions.
2843 Embryo shooting was significantly low in 0.5 mg/L 6-BAP (Z = -2.6, P = 0.009)
2844 significance is indicated by ★. Sample size (n = 10) and bars represent Standard Error
2845 (±SE).

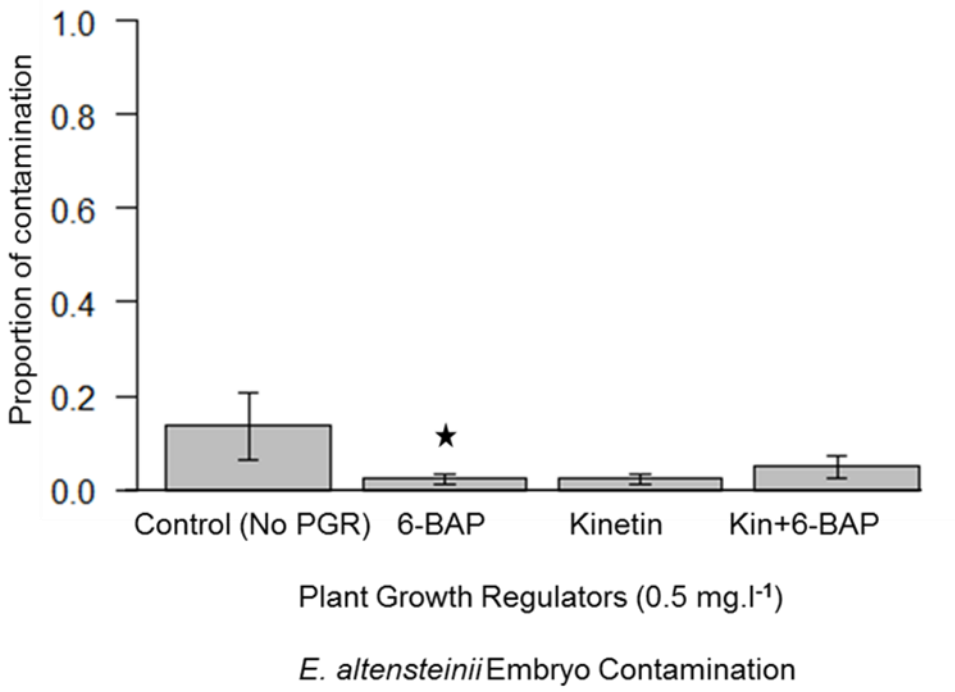
2846



2847

2848 Appendix 3: The effect of PGRs on *E. altensteinii* embryo callusing after a 12 week
2849 incubation period, obtained from generalised linear models with beta distributions.
2850 Embryo callusing was significantly high in 0.5 mg/L 6-BAP (Z = 6.8, P < 0.0001)
2851 significance is indicated by ★. Sample size (n = 10) and bars represent Standard Error
2852 (±SE).

2853



2854

2855

2856 Appendix 4: The effect of PGRs on *E. altensteinii* embryo contamination after a 12
2857 week incubation period, obtained from generalised linear models with beta
2858 distributions. Embryo callusing was significantly low in 0.5 mg/L 6-BAP (Z = -4.4, P <
2859 0.0001) significance is indicated by ★. Sample size (n = 10) and bars represent
2860 Standard Error (±SE).