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Cycads *in vitro*

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Abstract

The Cycadales are a group of botanical and evolutionary importance; however, many species face the threat of extinction due to poaching and habitat destruction. The current investigation reviews previous work on *in vitro* production of cycads, which holds great potential for mass production and germplasm conservation of these unique plants. Megagametophytes and zygotic embryos have been used as explants in most studies, while seedling tissue and new leaf tissue of mature trees have also been used. Callus, coralloid roots and somatic embryos have been formed *in vitro* but direct organogenesis appears to be the most promising method for mass production and germplasm preservation of the cycads, as recent studies have reported the acclimatization of numerous plantlets less than 200 days after initial culture of zygotic embryos.

Cycads have been referred to by Gilbert (1984) as "living fossils" because they are an ancient group of plants, the Cycadales being the oldest of the living members of the gymnosperms (Ikeno 1896) and are therefore an important group in the quest to unravel the mysteries of plant evolution.

Cycads are made up of three families: Cycadaceae, Stangeriaceae and Zamiaceae (Chavez et al. 1998). In the Cycadales, Hill and Stevenson (1999) listed 273 tropical and subtropical species in 11 genera although Chavez et al. (1998) claim 132 species in 10 living families. *Cycas revoluta* Thunb., commonly known as "Japanese sago palm", is probably the most researched member of the Cycadaceae family and is the oldest of the living cycads (Jones 1993). Native to southern China and Japan (Stevenson 1990), it has several characteristic botanical features: it is evergreen, has palm-like, pinnately divided, glossy green leaves

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with narrow leaflets that have a sunken midrib and rolled down margins (Gilman 1999). In addition to its ornamental value, *C. revoluta* has some medicinal properties such as the traditional treatment of cancer (Kowalska et al. 1995) and nutritional value, including eaten raw, cooked, or in a fermented state within "miso" soup (Brenner et al. 2003), but with toxic potential (Chang 2004), possibly due to the presence of cycasin (Tadera et al. 1995). In its ornamental appeal, reputed medicinal properties, nutritional value and toxicity, *C. revoluta* epitomizes the economic importance of cycads.

Cycads are also unique in their possession of motile antherozoids and are the only gymnosperms that have a symbiotic relationship with nitrogen-fixing cyanobacteria (Vessey et al. 2005), which inhabit specialized branching structures of cycads known as apogeotropic or coralloid roots (Webb et al. 1984, Lindblad and Bergman 1990, Korzhenevskaya et al. 1999, Vessey et al. 2005). Cycads of today, however, represent a small fraction of a plant group that once dominated the earth's flora millions of years ago (Bhatnagar and Moitra 1996), and many species now face the threat of extinction in the wild.

While cycad numbers appear to have declined naturally over the millennia, the principal reasons for their rapid decline in recent years include the removal of plants from the wild by collectors, traders and landscapers, and the destruction of habitat for agricultural, domestic and commercial development (Osborne 1995; Donaldson et al. 2003; Vovides et al. 2003). Cycad numbers have declined to such an extent that all species are now included in either Appendix I or II of the Convention in International Trade in Endangered Species of Flora and Fauna (CITES 2014), and are protected by law in various countries. However, despite legislative protection for a number of years, there has not been a substantial increase in cycad numbers in the wild mainly due to reproductive failure, as a very few seeds germinate in nature and few seedlings are observed (Forsyth and van Staden 1983, Giddy 1990).

Cycads are dioecious with males bearing cones and females bearing groups of megasporophylls (Ikeno 1896). It is, therefore essential that male and female plants of the same species cone at the same time for fertilized seed to be produced in nature, which may present a problem in populations with a few individuals. Although a few studies have been conducted, cycad seeds also appear to be recalcitrant (Dehgan and Schutzman 1989, Forsyth and van Staden 1983, Woodenberg et al. 2010), i.e. they are 'wet' and desiccation-sensitive, and may succumb to dehydration in the field during the protracted development of the embryo after seed-shed (Woodenberg et al. 2014). While rapid germination (2-6 weeks) of *Zamia floridana* (*sensu lato*) seeds is possible when the sarcotesta is removed and seeds are soaked in H_2SO_4 for 1 hr, rinsed, then soaked in 1 g/l GA_3

for 48 hrs (Dehgan and Johnson 1948), *C. revoluta* seeds lose viability rapidly and have low morphogenic potential or recalcitrance *in vitro* (Benson 2000). The recalcitrant nature of cycad seeds means that they are not suitable for *ex situ* long term storage and/or conservation and therefore methods – other than seed propagation – are urgently needed to conserve these plants.

Furthermore, cycads are generally slow growers and it may take up to 15 years for a seedling to become sexually mature (Norstog and Nicholls 1997, Tang 1990, Vogel et al. 1995). Hence, cycad numbers in the field increase naturally at a relatively slow rate and there is a pressing need to develop effective biotechnological protocols for the mass production of cycads to satisfy the demand by landscapers and collectors, and to prevent the extinction of these ancient plants.

In this regard, the current review aims to analyze the *in vitro* studies that have been conducted on members of the Cycadales, since biotechnology – specifically tissue culture – is a viable way to conserve the germplasm of this ancient plant group (Litz et al. 2004, 2005).

The first *in vitro* studies on cycads (Gymnospermae, Cycadales) was reported by La Rue (1948, 1954) for *Zamia floridana* (= *pumila*) (Zamiaceae) and Norstog (1965) for *Zamia integrifolia*. Callus induction from zygotic embryos (ZEs) and/or megagametophytes of *Cycas circinalis* and *Zamia integrifolius* was reported by Norstog and Rhamstine (1967), of *Zamia pumila* by Webb et al. (1983), and of *Dioon edule* by Chavez and Litz (1999). De Luca et al. (1979) conducted studies on the *in vitro* culture of *Ceratozamia mexicana*, *C. revoluta* and *Encephalartos umbeluziensis* megagametophytes and identified structures that were classified as pseudobulbils or globular embryos but that were later corrected in *C. revoluta* to coralloid root nodules (De Luca and Sabato 1980, De Luca et al. 1980) and by Webb and Osborne (1988) as adventitious shoots in *Ceratozamia mexicana*. Chavez et al. (1992a, 1995) then reported somatic embryogenesis from the leaves of *C. mexicana* while somatic embryogenesis and shoot formation were reported in *C. mexicana* and *C. hildae* when ZEs and megagametophytes were used (Chavez et al. 1992b). In those studies, somatic embryos developed within 2 - 4 months and reached the cotyledonary stage after 6 - 8 months (18 months for leaves) in culture. Chavez et al. (1995) criticized how earlier claims of somatic embryogenesis were not substantiated by sufficient histological proof, and also indicate how somatic embryogenesis in *C. mexicana* differed substantially (dicotyledonous in nature when from leaves) from the *in ovulo* development of ZEs as defined by Chamberlain (1912) (monocotyledonous in nature), although Dorety (1908) claimed a dicotyledonous-like development of excised *Ceratozamia* ZEs.

Following a suitable explant disinfection process (Table 1), in most studies, it was common to induce callus from ZEs or megagametophytes using kinetin or 2,4-D, BA and NAA (La Rue 1950, 1954, Webb et al. 1983, Pan et al. 2013), and more recently one case of the use of picloram for callus induction (Ling et al. 2008) (Table 1). Webb et al. (1983) reported that NAA was required for callus initiation, but BA was not always necessary. High frequency of callus initiation occurred with 1.0 mg/l NAA combined with 0.01 or 1.0 mg/l BA. When the concentration of NAA was high relative to that of BA, friable callus formed and as the relative BA concentration was increased, a more compact callus formed. Compact-nodular callus developed at equal concentrations of NAA and BA over a wide range of absolute concentrations. Friable callus formed roots only while compact-nodular callus formed roots, shoots and ZE-like structures. Dhirnan et al. (1998) reported callus formation from the leaves of *C. revoluta*.

The sterilization procedure employed by several authors is quite similar (Table 1). However, Chaplot and Jasrai (2000) used bulbils as explants and a complicated sterilization procedure. Bulbils were treated for 90 min with an aqueous solution containing 0.1% bavistin, 0.1% citric acid, 1% activated charcoal on a gyratory shaker (100 rpm), and surface sterilized with 50% ethanol for 1 min, followed by 0.1% $HgCl_2$ for 3 min. The outer 9 or 10 bulb-scales were discarded and the bulbils were re-treated with 0.05% $HgCl_2$ for 1 min, followed by repeated rinses with sterile distilled water five times. The inner (8 - 10) bulb-scales were used as explants. After incubation for 20 - 25 days, shoot primordia differentiated in 60% of the cultures of only the inner bulb-scales on half-strength MS with 2.21 μM BA. One or two shoot buds differentiated directly on either surface of the bulb-scale without any observable callus formation. Three weeks after culture the explants bearing shoot buds were transferred to fresh medium. At 4.43 μM BA there was slight callus formation. However, the callus failed to differentiate further, even on fresh medium. The choice of gelling agent (Moon et al. 2004) or light intensity (Vargas-Luna et al. 2004) can affect the qualitative and quantitative outcome of somatic embryogenesis. Some parallels have been drawn between somatic embryogenesis of coniferous gymnosperms and cycads, the former having been reviewed elsewhere (Teixeira da Silva and Malabadi 2012).

Luo et al. (2011) reported the formation of callus from root tips or the stem of *in vitro* seedlings of *Cycas guizhouensis* on MS containing 50 mg/l Kn and 20 mg/l 2,4-D or MS with 50 mg/l Kn and 20 mg/l BA. There are no other reports about callus proliferation or plant regeneration for this plant.

Table 1 Micropropagation of members of the Cycadaceae.

Genus, species and/or cultivar	Explant used	Sterilization procedure	Culture medium, PGRs and additives	Culture conditions	Remarks, experimental outcome, acclimatization and variation	Reference
Zamia integrifolia (= *pumila*)	Megagameto-phytes	Ovules were sterilized in 10% Clorox for 10-30 min and bisected longitudinally. ZEs were excised and cultured separately from the halves of the megagametophytes.	White's medium + different concentrations of Kn, adenine, 2,4-D, IAA, glutamine, asparagine and alanine; pH 4.5	25°C; darkness	The organs that regenerated resembled their normal diploid counterparts. Highest percentage of apogamy was 59 after 5 months of culture on medium + 5 ppm IAA. After several subcultures, embryoids formed both on diploid excised ZEs and on haploid megagametophytes.	Norstog 1965
Ceratozamia mexicana, *Cycas revoluta* and *Encephalartos umbeluziensis*	Megagameto-phytes	Seeds were soaked in NaOCl (conc. NR) for 5 min, then in 20% diluted NaOCl for 20 min. Rinses NR.	White's medium + 2,4-D and Kn at various concentrations, asparagine, alanine, glutamine, adenine SO_4	25°C; light (50 $\mu Em^{-2}s^{-1}$)	For *Ceratozamia mexicana*, SEs occurred without prior callus formation on medium devoid of growth regulators; for *Cycas*, coralloid roots developed after callus formation, while *Encephalartos* formed callus with no coralloid root development in medium with a relatively high concentration of 2,4-D.	De Luca et al. 1979
Cycas revoluta	Megagameto-phytes	Seeds coats removed and sterilized, washed and bisected longitudinally. Chemicals used for sterilization are NR.	White's medium + various concentrations of 2,4-D and Kn; 2% sucrose and 0.9% Difco Bacto-Agar	25°C; continuous light (500 lux)	No regeneration on White's medium without growth hormones; spherical outgrowths observed after 3 months on media with growth hormones; spherical outgrowths subsequently developed into coralloid roots; sections of the coralloid roots appeared similar to those of *Cycas in situ*; however, they were free of blue-green algae.	De Luca and Sabato 1980
Zamia latifoliolata	Half ZEs (transverse and longitudinal)	NR	MS; 100 mg/l inositol, 0.4 mg/l thiamine; 30 g/l sucrose; 0.8% agar; pH 5.7. 0.1 or 1.0 mg/l NAA, with or without 0.01-10.0 mg/l BA (CIM)	NR.	BA-containing CIM resulted in nodular callus that could form plantlets in PGR-free CIM. Glutamine inhibited callus formation.	Webb and Rivera 1981
Zamia pumila	ZEs	Following removal of the outer, fleshy integument, the stony layer of each ripe seed was soaked in 10% aqueous dilution of commercial bleach (5.25% NaOCl) for 30 min. ZEs cut into transverse or longitudinal halves.	MS + NAA/BA, or combinations of both; pH 5.7	27°C; darkness	NAA was required for callus initiation, but BA was not always required. High frequency of callus initiation with 1.0 mg/l NAA + 0.01 or 1.0 mg/l BA. At a high NAA: BA ratio, friable callus formed. Higher relative BA concentration formed more compact callus	Webb et al. 1983

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					NAA: BA = 1 formed compact-nodular callus over a wide range of absolute concentrations. Friable callus formed roots only but compact-nodular callus formed roots, shoots and embryo-like structures.	
Cycas revoluta Thunb.; *Encephalartos altensteinii*; *Zamia furfuracea*	Megagameto-phytes	Seed surface sterilized in 10% commercial bleach for 30 min, then blotted dry. Megagametophytes excised under sterile conditions and plated directly *in vitro*.	Germination of excised megagametophytes: 1.0% agar; pH 5.7, 27°C, darkness, 7 days. Germinated seedlings grown in 1.5% agar slants with White's minerals + 2% sucrose, 27°C, darkness, 21 days.	Developed seedlings then transferred to light (16-h PP, 5 klux).	Light was generally inhibitory to seedling development and root growth.	Webb et al. 1984, and references therein
Stangeria eriopus	Root of seedlings	Seeds germinated in plastic bags containing loamy soil. Primary roots excised and disinfected in 70% ethanol for 4 min, 20% Jik (commercial 3.5% NaOCl solution) + 1% Teepol for 30 min, then 1% $HgCl_2$ + 1% Teepol for 30 min. After thorough rinse and soak in SDW for 2 h, roots treated again with 20% Jik and rinsed 3X with SDW.	SH + 4.5 µM 2,4-D + 4.5 µM Kin; pH 5.8	25 ± 2°C in darkness for 30 d, then in light (~100 µmol m^{-2} s^{-1}) at 25 ± 0.2°C	After about 25 days in darkness, compact white callus formed on upper surface of all explants. On subculture and transfer to the light environment, each explant developed a small green meristematic zone within 14 days followed by emergence and expansion of a typical circinate leaf.	Osborne and Van Staden 1987
Cycas revoluta	ZEs (5-15 mm; 20/culture dish) from seeds stored for 6 months and derived from open-pollinated trees. In Rinaldi and Leva (1995), 2-6 mm long sections of epicotyl and cotyledonary leaves of 30-	The sclerotesta was surface sterilized for 60 min with 20% NaOCl, pH 6.2 (Sweet and Bolton 1979). After removing the sclerotesta under sterile conditions, megagametophytes were sterilized again for 10 min with 10% NaOCl and rinsed twice with SDW. ZEs were then excised.	SH or MS; 0.5 mg/l BA + 1.0 mg/l 2,4-D; 0.5 µM NAA (RIM); pH 5.7	12 weeks in darkness then transferred to light (16-h PP; 40 µm m^{-2} s^{-1}); 23 ± 1°C	Young, immature ZEs could not produce callus, only the oldest ZEs. SH or MS, both containing ammonium, promoted shoot formation, but not KK medium, which contained nitrate. Darkness promoted callus induction while light promoted shoot formation. On SH, 7.3 shoots/ZE formed after 8 weeks. 20% of cotyledonary leaves of 4-week-old plants yielded more shoots on MS than on SH or KK medium and epicotyls; 20% of epicotyls on SH yielded more adventitious shoots than cotyledonary leaves on MS or KK media. 40% of cotyledonary leaves formed roots in the presence of 0.5 µM NAA. No acclimatization studies were performed.	Rinaldi and Leva 1995, Rinaldi 1999

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	and 45-day-old seedlings were tested.					
Ceratozamia hildae Landry and Wilson, *C. mexicana* var. *robusta* (Miq.) Dyer strobili	Bisected megagameto-phyte and ZEs	After removing the sarcotesta, the sclerotesta surrounding the megagametophyte was surface-disinfested for 30 min with 1.05% NaOCl (20% Clorox) (v/v) containing 2-3 drops of Tween 20. The sclerotesta was removed under sterile conditions, and the megagametophyte, together with the enclosed ZE was disinfested for 10 min with 0.52% NaOCl (10% Clorox) (v/v), and rinsed twice with SDW. The megagametophyte was bisected longitudinally, and the ZE in each immature seed was removed intact. Both halves of the bisected megagametophyte and the ZE from each seed were used as explants.	B5 macronutrients + MS micronutrients. Following the Norstog and Rhamstine (1967) protocol, 400 mg/l glutamine, 100 mg/l ascorbic acid, 100 mg/l casein hydrolystate, 100 mg/l arginine, 100 mg/l asparagine were added. For *C. hildae*, 4.7 µM Kin + 2.3 µM 2,4-D (SIM); 4.7 µM Kin + 0.5 µM 2,4-D or 4.5 µM 2,4-D alone (SEIM). For *C. mexicana*, 4.5 or 9.0 µM 2,4-D alone (SIM); 4.5 µM 2,4-D alone (SEIM). 60 g/l sucrose; 6.5 g/l Difco bacto-agar; pH 5.7	25°C; darkness	In *C. hildae*, 60% of megagametophyte explants formed shoots and 50% of ZEs formed somatic embryos. In *C. mexicana*, 100% of megagameto-phyte explants formed shoots and 60% of ZEs formed somatic embryos. No histological proof of somatic embryogenesis was provided. No acclimatization was performed. Histological proof of somatic embryogenesis in Chavez et al. 1995. No acclimatization studies were performed.	Chavez et al. 1992a, 1992c, 1995, Litz et al. 1995a
Zamia fisheri, *Z. furfuracea*, *Z. pumila*	Bisected megagameto-phyte and ZEs	As above.	As above.	25°C; darkness	Organogenesis and somatic embryogenesis occurred from the megagametophyte and zygotic embryo explants of *Zamia pumila* and *Z. furfuracea* tissue cultures, but only from the megagametophyte of *Z. fischeri*. Nucellar callus of *Z. pumila* produced globular structures that failed to develop further. Plantlets were recovered from somatic embryos of *Z. pumila*.	Chavez et al. 1992b
Cycas revoluta	Seedling epicotyls and hypocotyls (1 cm long).	Kernels were aseptically germinated on cotton layers moistened with sterile water at 28°C in the dark for 1-2 months then completely sterilized with 70% ethanol for 1	½ MS; 10 mg/l 2,4-D; 30 g/l sucrose; 0.1% AC (CIM). CIM – 2,4-D + 10 mM NAA + 1	30°C; darkness	MS, LS and White's (1943) media could not induce callus in the presence of 3% sucrose and 2,4-D and/or IAA. No callus could differentiate into shoots. Cycasin detected in mother tissues	Tadera et al. 1995

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		min and 10% $Ca(OCl)_2$ for 20 min, and then 3 rinses in SDW.	mM Kin (callus proliferation). 1% agar; pH NR		(highest in seedling epicotyls) and in callus cultures. No acclimatization studies were performed	
Encephalartos cycadifolius	ZEs	Seeds were washed with tap water, surface-sterilized in 70% ethanol for 4 min, rinsed and treated with 1% sodium hypochlorite for 30 min before rinsing three times in sterile water.	Modified B5 medium containing 1 mg l^{-1} 2,4-D and 1 mg l^{-1} Kin established callus, SEs formed on media containing various combinations of 2,4-D and Kin ; matured on medium containing 1 mg l^{-1} ABA.	26°C; darkness ; transfered to 16h light/8h darkness (13.6 mol m^{-2} s^{-1}) for SE development	Rapid callus growth was obtained on the induction medium; SEs developed in the same sequence as reported in *Ceratozamia* (Chavez et al. 1992a) and *Zamia* (Chavez et al. 1992b) species; after transfer to medium containing ABA for maturation, embryos were rooted in medium that was free of growth regulators.	Jager and van Staden 1996a
Encephalartos dyerianus and *E. natalensis*	ZEs	Sclerotesta-enclosed seeds were washed in tap water before surface sterilization in 70% aqueous ethanol for 4 min, rinsed twice with sterile water before immersion in 1% aqueous sodium hypochlorite for 30 min, then rinsed three times with sterile water.	Modified B5 macronutrients, MS micronutrients and amino acids; 4.5 µM 2,4-D and 4.6 µM Kin ; 0.8% agar; pH 5.7 (induction medium) ; various combinations of 2,4-D and Kin used in initiation medium ; transferred to medium supplemented with 3.8 µM ABA.	26°C ; darkness for induction medium ; transfered to 16h light/8h darkness (13.6 mol m^{-2} s^{-1}) for somatic embryo development	In *E. dyerianus*, SEs were formed on media with either no growth hormones or growth hormones in low concentrations; in *E. natalensis*, SEs formed on media with same concentration of 2,4-D and Kin as induction medium; SEs of both species started-off as pro-embryos that grew suspensors and ultimately became dicotyledonary; maturation of SEs with radicle development was not achieved.	Jager and van Staden 1996b
Ceratozamia euryphyllidia Vazquez Torres, Sabato and Stevenson	Young leaf flushes (1-3 cm long) of mature trees.	Leaf pinnae removed from the rachis and washed in RTP for 5 min. Surface sterilized with 30% (v/v) commercial bleach for 20 min. Leaf pinnae washed 3X with SDW and cut into square explants (~ 0.3–0.5 cm^2).	Almost the same as Chavez et al. 1992a-c. 2 g/l Gellan gum.	Darkness for somatic embryo induction then transfer to light for plantlet conversion: 16-h PP; first 25-30 then 140 µm m^{-2} s^{-1}; 25°C	Approximately 3 years from germination to plant formation. Somatic embryogenesis not quantified nor were significant differences between treatments assessed: only dissecting microscopy images and proof of somatic embryogenesis provided, but claim the same successful PGR combinations as Chavez and Litz (1999). No acclimatization studies were performed.	Chavez et al. 1998

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Dioon edule Lindley	ZEs (~2.5 cm long) and megagameto-phytes of 1.5 year-old cones	Immature seeds, removed from the cone, were washed thoroughly in RTW, then surface sterilized for 20 min in 0.25% (w/v) NaOCl + 2-3 drops Tween 20/100 ml. Seeds rinsed 3X in SDW and dissected under sterile conditions.	Almost the same as Chavez et al. 1992a-c. 8 g/l Bacto agar.	Darkness for callus induction, subcultured every 4 weeks. Transfer to light when shoots formed: 16-h PP; 60 µm $m^{-2} s^{-1}$; 25°C.	Callus induction possible from both explants from a range of Kin × 2,4-D combinations. For megagametophytes, 2.26 µM 2,4-D resulted in most shoots (indirect) (9/explant) while for ZEs, shoots (indirect) formed from callus in the presence of 9.29-13.94 µM Kin + 0.45-9.05 µM 2,4-D (max. 2/explant). No acclimatization studies were performed.	Chavez and Litz 1999
Cycas revoluta	Bulbil inner bulb-scales	Bulbils treated for 90 min with aqueous 0.1% bavistin, 0.1% citric acid, 1% AC on gyratory shaker (100 rpm). Surface sterilized with 50% ethanol for 1 min, then 0.1% $HgCl_2$ for 3 min. Outer 9-10 bulb-scales discarded and bulbils re-treated with 0.05% $HgCl_2$ for 1 min. 5X rinses with SDW. Inner 8-10 bulb-scales used as explants.	½ MS + 2.21 or 4.43 µM BA	16-h PP; 30 µE $m^{-2} s^{-1}$, 25°C.	After 20-25 days incubation, shoot primordia differentiated in 60% of the cultures of only inner bulb-scales on ½MS + 2.21 µM BA. 1-2 shoot buds differentiated directly on either surface of bulb-scales without any callus formation. Explants formed shoot buds after 3 weeks. Buds subcultured on fresh medium. At 4.43 µM BA, some callus formed but failed to develop further, even on fresh medium.	Chaplot and Jasrai 2000
Cycas circinalis; *Zamia integrifolia*	Megagameto-phytes (2 cm long, 0.5-0.7 cm wide)	0.1-0.2% $HgCl_2$ for 2 min. Rinses NR.	MS; 5-10 µM 2,4-D or 2-10 µM NAA with or without 1-5 µM Kin (CIM); pH 5.8.	NR.	Only callus was induced, becoming more nodular when Kin was added. No shoot or plantlet formation was reported. No acclimatization studies were performed.	Dhiman et al. 2000
Cycas revoluta	Seed, cotyledon, squama (plant scales) and shoot tips of *in vitro* seedlings	0.1% $HgCl_2$ for 10-20 min. 3-5X rinses in SDW.	ER, MS, and MT + different concentrations of BA, IBA and *C. revoluta* episperm juice; pH 5.8.	Seed: 25°C and 30 °C, in darkness then into natural light after germination; cotyledons: 30°C; squama and shoot tips: 25-28°C in natural light.	Seed germination *in vitro* 6 months earlier (only 1-3 months) than conventional sowing. 76.7% of seeds formed multiple shoots on ER+ 2.0 mg/l BA + 0.4 mg/l IBA + 2 m/l *C, revoluta* episperm juice. 53.3% and 33.3% of cotyledons and squama formed shoot buds. Survival > 90% when plantlets transplanted from April to July.	Lin et al. 2007
Cycas revoluta	ZE. Endosperm (most likely the megagameto-phyte)	Seeds were washed under running tap water for 20 min then dipped in 30% Clorox® containing 1 to 2 drops of Tween-20 (time period NR). Surface sterilized seeds were rinsed with SDW for 5-10 min then immersed in 70% ethanol for 5 min, and two	MS; 20 µM picloram (CIM); 30 g/l sucrose; 0.8% Vietnam agar; 1 g/l PVP; pH 5.7	16-h PP, PPFD NR; 23 ± 1°C	Callus formed after 17.8 days in CIM, but not in the presence of 2,4-D. No acclimatization studies were performed.	Ling et al. 2008

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		rinses with SDW for 5 min each rinse. ZE endosperm was cut to 1 cm^2 explants.				
Cycas revoluta	Mature ZEs	Sarcotesta and sclerotesta removed from mature *C. revoluta* seeds. Naked seeds rinsed in RTP for 30 min, dipped in 70% ethanol for 1 min and 30% purelox for 5 min. Mature ZEs were cut, together with megagametophytes, into blocks smaller than 20 mm^3, rinsed in SDW then cultured.	SH; 0.2 mg/l BA + 0.02 mg/l 2,4-D; 30 g/l sucrose; 20% CM; 0.6% agar; pH 5.9	Light, PP and temperature conditions NR	Multiple adventitious shoots formed directly in 42-84 days after culture initiation: maximum of 88.7±40.5 adventitious shoots/ZE. Rooted plantlets ready for acclimatization formed 196 days after the start of culture.	Motohashi et al. 2008
Cycas guizhouensis	Root, stem, squama (plant scales)	NR	MS + different concentration 2,4-D, Kin, BA, IBA, and NAA; pH NR	12-h PP, PPFD NR; 25 ± 1°C	Callus formed from root or stem on MS + 50 mg/l Kin + 20 mg/l 2,4-D or MS + 50 mg/l Kin + 20 mg/l BA.	Luo et al. 2011
Cycas revoluta	Leaf and midrib	The explants were subjected to rigorous surface sterilization by treating with 70% ethanol, followed by hypochlorite. Sterilants were removed by repeated washing with autoclaved distilled water.	MS and B5 media + different concentrations of 2,4-D, Kin, BA, IBA, and NAA; pH 5.8	NR	Only swelling was observed at 50 mg/l 2,4-D in MS medium from leaves.	Pan et al. 2013
Cycas revoluta	Mature ZEs 2-cm long	Mature *C. revoluta* seeds surface sterilized for 30 min with 30% sodium hypochlorite. After removing the sclerotesta under sterile conditions, megagametophytes were sterilized by soaking in 70% ethanol for 2 min and then in 50% NaOCl containing 2-3 drops of Tween-20 for 25 min, then rinsed twice with SDW for 5-10 min each rinse. After surface sterilization, megagametophytes were longitudinally bisected and the ZE was excised from each seed. ZEs were placed on sterile tissue culture media in Petri dishes (15 cm deep, 10 cm in diameter), at 3 ZEs/Petri dish.	½ MS (micro- and macronutrients); 0.5 mg/l BA (SIM). 0.54 µM NAA (RIM; based on Rinaldi et al., 1999). 30 g/l sucrose; 0.6% (w/v) agar; pH 5.9	16-h PP, 4 µm $m^{-2} s^{-1}$, 26 ± 1°C	3.67 and 29.67 direct and indirect (from callus) shoots formed/ZE, respectively. Plantlets acclimatized after 185 days. Rooted plantlets acclimatized in perlite at high RH (covered) under *in vitro* light and temperature conditions, then left uncovered in the greenhouse under ambient conditions.	Naderi R, Mohaiseni K, Teixeira da Silva JA, Omidi M, Naderi B 2014 (unpublished data)

2,4-D, AC, activated charcoal; BA, N^6-benzyladenine (BA is used throughout even though BAP (6-benzylamino purine) may have been used in the original, according to Teixeira da Silva, 2012; B5, Gamborg et al. (1968) medium; CIM, callus induction medium; CM, coconut milk; ER medium (Eriksson 1995); $HgCl_2$, mercuric chloride; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; Kin, kinetin; KK, Klimaszewska and Keller (1985) medium; LS, Linsmeier and Skoog (1965) medium; MS, MT, Murashige and Tucker (1969) medium ; NAA, α-naphthaleneacetic acid; NaOCl, sodium hypochlorite; NR, not reported; PP, photoperiod; PPFD, photosynthetic photon flux density; PVP, polyvinylpyrrolidone; RIM, root induction medium; rpm, revolutions per minute; RTP, running tap water; SDW, sterile distilled water; SEIM, somatic embryo induction medium; SH, Schenk and Hildebrandt (1972) basal medium; SIM, shoot induction medium; White's medium (White 1943); ZE, zygotic embryo.

Lin et al. (2007) reported plantlet regeneration *in vitro*, particularly the formation of multiple shoots derived from seed, cotyledon, squama (plant scales) and shoot tips of *C. revoluta*. *C. revoluta* seed cultured *in vitro* could germinate six months earlier than when sown under normal conditions (*ex vitro*) at 25°C. When seeds were inoculated on ER media (Eriksson 1995) containing 2.0 mg/l BA, 0.4 mg/l IBA, and 2 ml/l of juice of *C. revoluta* episperm (testa), multiple shoots formed on 76.7% of explants. Shoot buds differentiated from 53.3% of cotyledons and from 33.3% of squama. Survival rate exceeded 90% when plantlets were transplanted from April to July on a substrate of sand and garden soil, or a 1 : 1 (v/v) mixture of perlite and coco coir.

An effective *in vitro* protocol serves several purposes for cycads: (1) to conserve rare or endangered germplasm; (2) to establish protocols that will allow for the clonal production of standardized material, suitable for gardening; (3) with standardized protocols, aspects such as gametogenesis can be examined without the dependence on environmental cues and tissues subjected to genetic transformation can have a better chance of recovery of transgenic callus, plantlets or somatic embryos; (4) the ability to obtain regenerable transformants would allow for the introduction of novel traits such as disease or pest resistance; (5) the ability to induce somatic embryos would allow for the mass production of plantlets through bioreactor culture; (6) *in vitro* production of algae-free coralloid roots may be important for studies on cycad-cyanobacteria symbiosis – especially studies looking at the path of entry of cyanobacteria; and (7) to produce suitably-sized explants for the cryopreservation of these endangered plants.

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Conflicts of interest

The authors declare no conflicts of interest.

References

Benson EE (2000) *In vitro* plant recalcitrance *in vitro* plant recalcitrance: An introduction. In Vitro Cell. Dev. Biol. - Plant **36:** 141-148.

Bhatnagar SP and **Moitra A** (1996) Gymnosperms. New Age International Limited, New Delhi.

Brenner ED, Stevenson DW and **Twigg RW** (2003) Cycads: evolutionary innovations and the role of plant-derived neurotoxins. Trends Plant Sci. **8:** 446-452.

Chamberlain CJ (1910) Morphology of *Ceratozamia*. Bot. Gaz. **53:** 1-18.

Chang SS (2004) Acute cycas seed poisoning in Taiwan. J. Toxicol. Clin. Toxicol. **42:** 1119-1126.

Chaplot BB and **Jasrai YT** (2000) Differentiation of shoot buds in bulb-scale cultures of *Cycas revoluta* Thunb. Phytomorphology **50:** 37-40.

Chávez VM and **Litz RE** (1999) Organogenesis from megagametophyte and zygotic embryo explants of the gymnosperm *Dioon edule* Lindley (Zamiaceae, Cycadales). Plant Cell Tiss. Org. Cult. **58:** 219-222.

Chávez VM, Litz RE and **Marquez J** (1995) Histology of somatic embryogenesis of the cycad *Ceratozamia mexicana* var. Robusta (Miq.) Dyer. Plant Sci. **108:** 191-200.

Chávez VM, Litz RE, Monroy M, Moon PA and **Vovides AM** (1998) Regeneration of *Ceratozamia euryphyllidia* (Cycadales, Gymnospermae) plants from embryogenic leaf cultures derived from mature-phase trees. Plant Cell Rep. **17:** 612-616.

Chávez VM, Litz RE and **Norstog K** (1992a) *In vitro* morphogenesis of *Ceratozamia hildae* and *C. mexicana* from megagametophytes and zygotic embryos. Plant Cell Tiss. Org. Cult. **30:** 93-98.

Chávez VM, Litz RE and **Norstog K** (1992b) Somatic embryogenesis and organogenesis in *Zamia fisheri*, *Z. furfuracea* and *Z. pumila.* Plant Cell Tiss. Org. Cult. **30:** 99-105.

Chávez VM, Litz RE, Moon PA and **Norstog K** (1992c) Somatic embryogenesis from leaf callus of a mature gymnosperm *Ceratozamia mexicana* var Robusta (Miq.) Dyer (Cycadales). In Vitro Cell. Dev. Biol. – Plant **28:** 59-63.

CITES (Convention in International Trade in Endangered Species of Flora and Fauna) (2014) http://www.cites.org/eng/app/appendices.php. Last accessed: 3 January, 2015

Dehgan B and **Johnson CR** (1983) Improved seed germination of *Zamia floridana* (sensu lato) with H_2SO_4 and GA_3. Sci. Hortic. **19:** 357-361.

Dhirnan M, Moitra S, Singh MN and **Bhatnagar SP** (1998) *In vitro* studies on some non-coniferous gymnosperms. Curr. Sci. **75:** 1113-1115.

De Luca P, Moretti A and **Sabato S** (1979) Regeneration in megagametophytes of cycads. Giorn. Bot. Ital. **113:** 129-143.

De Luca P and **Sabato S** (1980) Regeneration of coralloid roots on cycad megagametophytes. Plant Sci. Lett. **18:** 27-31.

Dhiman M, Moitra S, Singh MN and **Bhatnagar SP** (2000) Organogenesis in female gametophyte and embryo cultures of cycads: *Cycas circinalis* and *Zamia integrifolia*. J. Plant Biochem. Biotechnol. **9:** 111-113.

Donaldson JS, Hill KD and **Stevenson DW** (2003) Cycads of the World: An Overview. In: Donaldson JS (ed) Cycads. IUCN - The World Conservation Union. Charlesworth & Co Ltd., Huddersfield, UK, pp 3-8.

Dorety HA (1908) The embryo of *Ceratozamia*. Bot. Gaz. **45:** 412-416.

Dyer RA (1965) The cycads of Southern Africa. Bothalia **8:** 405-514.

Gamborg OL, Miller RA and **Ojima K** (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50:** 151-158.

Gilbert S (1984) Cycads: Status, Trade, Exploitation and Protection 1977-1982. World Wildlife Fund, Washington, DC, USA.

Gilman EF (1999) *Cycas revoluta*: General information and description. Review paper of Institute of Food and Agriculture Sciences. Environment Horticulture Department, Florida.

Hill KD and **Stevenson DW** (1999) The cycad pages. World list of cycads. http://plantnet.rbgsyd.gov.au/PlantNet/cycad/wlist.html. Last accessed: 3 January, 2015

Ikeno S (1896) Das Spermatozoid von *Cycas revoluta*. Bot. Mag. (Tokyo) **10:** 367-368.

Jager AK and **van Staden J** (1996a) Somatic embryogenesis in *Encephalartos cycadifolius*. Plant Cell Rep. 15: 437-440.

Jager AK and **van Staden J** (1996b) Somatic embryogenesis and organogenesis in *Encephalartos dyerianus* and *E. natalensis*. Plant Cell Tiss. Org. Cult. **45:** 99-102.

Jager AK and **van Staden J** (1999) Somatic embryogenesis in African cycads (*Encephalartos*). In: Mohan Jain S. et al. (eds.) Somatic Embryogenesis in Woody Plants, Volume 4, Kluwer Academic Publishers, pp. 449-460.

Jones DL (1993) Cycads of the World. Reed Book, New York Bot Gardens, Chatswood. pp. 312.

Klimaszewska K and **Keller WA** (1985) High frequency plant regeneration from thin cell layer explants of *Brassica napus.* Plant Cell Tiss. Org. Cult. **4:** 183-197.

Korzhenevskaya TG, Lobakova ES, Dol'nikova GA and **Gusev MV** (1999) Peculiarities of microsymbiont topography in apogeotropic roots of *Cycas revoluta* and *Encephalartos horridus*. Mikrobiologiya **68:** 33-38.

Kowalska MT, Itzhak Y and **Puett D** (1995) Presence of aromatase inhibitors in cycads. J. Ethnopharmocol. **47(3):** 113-116.

La Rue CD (1948) Regeneration in the gametophyte of *Zamia floridana*. Bull. Torrey Bot. Club **75:** 597-603.

La Rue CD (1950) Regeneration in cycads. Am. J. Bot. **37:** 664.

La Rue CD (1954) Studies on growth and regeneration in gametophytes and sporophytes of gymnosperms. Brookhaven Symp. Biol. **6:** 187-208.

Lin QL, Shi RB, Lin YC, Lin XB, Gong JC and **Zou N** (2007) Plantlets with multi-shoots of *Cycas revoluta* Thunb. *in vitro*. Acta Hortic. Sin. **37(6):** 1575-1578.

Lindblad P and **Bergman B** (1990) The cycad–cyanobacterial symbiosis. In: Rai AN (ed) *Handbook of Symbiotic Cyanobacteria*, Boca Raton: CRC Press, pp. 137-159.

Ling PKA, Thing YS, Gansau JA and **Hussein S** (2008) Induction and multiplication of callus from endosperm of *Cycas revoluta*. African J. Biotechnol. **7:** 4279-4284.

Linsmaier EM and **Skoog F** (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. **18:** 100-127.

Litz RE, Moon PA, Benson EM, Stewart J and **Chávez VM** (2004) A biotechnology strategy for medium- and long-term conservation of cycads. Bot. Rev. **70:** 39-46.

Litz RE, Moon PA and **Chávez VM** (1995) Somatic embryogenesis from leaf callus derived from mature trees of the cycad *Ceratozamia hildae* (Gymnospermae). Plant Cell Tiss. Org. Cult. **40:** 25-31.

Litz RE, Moon PA and **Chávez VM** (2005) Somatic embryogenesis and regeneration of endangered cycad species. Acta Hortic. (ISHS) **692:** 75-79.

Lobakova ES, Dubravina GA and **Zagoskina NV** (2004) Formation of phenolic compounds in apogeotropic roots of cycad plants. Russian J. Plant Physiol. **51(4):** 486-493.

Luo ZQ, Wang LH, Li WJ and **Zhu J** (2011) Study on tissue culture of Guizhou *Cycas guizhouensis* L. J. Anhui Agric. Sci. **39(2):** 859-886.

Moon PA, Litz RE and **Chávez VM** (2004) Effect of gelling agent on growth and development of *Ceratozamia hildae* somatic embryos. Bot. Rev. **70(1):** 47-53.

Motohashi T, Toda M and **Kondo K** (2008) Adventitious embryo formation derived from zygotic embryos in *Cycas revoluta*. Plant Biotech. **25:** 589-591.

Murashige T and **Skoog F** (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. **15:** 473-497.

Murashige T and **Tucker DPH** (1969) Growth factor requirement of citrus tissue culture. In: Proc. 1st Int. Citrus Symp., University of California, Riverside. **3:** 1155-1161.

Norstog K (1965) Induction of apogamy in megagametophytes of *Zamia integrifolia*. Amer. J. Bot. **52:** 993-999.

Norstog K and **Rhamstine E** (1967) Isolation and culture of haploid and diploid cycad tissues. Phytomorphology **17:** 374-381.

Osborne R (1995) An overview of cycad conservation in South Africa. In: Donaldson J (ed) Cycad Conservation in South Africa, Issues, Priorities and Actions. Cycad Society of South Africa, South Africa, pp. 1-7.

Osborne R and **van Staden J** (1987) *In vitro* regeneration of *Stangeria eriopus*. HortSci. **22:** 1326.

Pan S, Neeraj A, Srivastava KS, Kishore P and **Sarethy IP** (2013) Effects of growth regulators on *in vitro* response and multiple shoot induction in some endangered medicinal plants. OA Biotechnol. **31;2(1):** 3.

Rinaldi LMR (1999) Factors affecting shoot regeneration from zygotic embryo and seedling explants of *Cycas revoluta* Thunb. In Vitro Cell. Dev. Biol. - Plant **35:** 25-28.

Rinaldi LMR and **Leva AR** (1995) *In vitro* organogenesis from diploid tissues of *Cycas revoluta* Thunb. Plant Cell Tiss. Org. Cult. **43:** 37-41.

Schenk RU and **Hildebrandt AC** (1972) Medium and techniques for the induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. **50:** 199-204.

Stevenson DW (1990) Morphology and systematics of the Cycadales. Mem. N. Y. Bot. Gardens **57:** 8-55.

Sweet HC and **Bolton WE** (1979) The surface decontamination of seeds to produce axenic seedlings. Amer. J. Bot. **66:** 692-698.

Tadera K, Gin'ya H, Sawada R, Motani Y, Aikawa Y, Nozaki A, Yagi F and **Minami Y** (1995) Cycasin formation in tissue cultures of Japanese cycad. Phytochemistry **38:** 1199-1201.

Teixeira da Silva JA (2012) Is BA (6-benzyladenine) BAP (6-benzylaminopurine)? Asian Austral. J. Plant Sci. Biotech. **6:** 121-124.

Teixeira da Silva JA and **Malabadi RB** (2012) Somatic embryogenesis in conifers. J. For. Res. **23:** 503-515.

Vargas-Luna I, Ortiz-Montiel G, Chávez VM, Litz RE and **Moon PA** (2004) Biochemical characterization of developmental stages of cycad somatic embryos. Bot. Rev. **70:** 54-62.

Vessey JK, Pawlowski K and **Bergman B** (2005) Root-based N_2-fixing symbioses: Legumes, actinorhizal plants, *Parasponia* sp. and cycads. Plant Soil **274:** 51-78.

Vovides AP, Pérez-Farrera MA, Gonzáles-Astorga J, González D, Gregory T, Chemnick J, Iglesias C, Octavio-Aguilar P, Avedaño S and **Bárcenas C** (2003) An outline of our current knowledge on Mexican cycads (Zamiaceae, Cycadales). Curr. Topics Plant Biol. **4:** 159-174.

Webb DT and **Osborne R** (1988) Cycads. In: Bajaj YPS (Ed) Biotechnology in Agriculture and Forestry Trees 2, Springer-Verlag, Berlin. Pp. 591-613.

Webb DT, Nevarez M and **de Jesus S** (1984) Further *in vitro* studies of light-induced root nodulation in the Cycadales. Env. Exp. Bot. **24:** 37-44.

Webb DT and **Rivera MS** (1981) Effects of NAA, BAP and L-glutamine on callus formation and organogenesis of *Zamia latifoliolata* embryo. Env. Exp. Bot. **21**(3-4): 433-434.

Webb DT, Rivera ME, Starszak E and **Matos J** (1983) Callus initiation and organized development from *Zamia pumila* explants. Ann. Bot. **51:** 711-717.

White PM (1943) *A Handbook of Plant Tissue Culture*, J. Cattell Press, Lancaster, PA.

Woodenberg WR, Berjak P and **Pammenter NW** (2010) Development of cycad ovules and seeds. 1. Implication of the ER in primary cellularisation of the megagametophyte in *Encephalartos natalensis* Dyer and Verdoorn. Plant Growth Regul. **62:** 265-278.

Woodenberg WR, Berjak P, Pammenter NW and **Farrant JM** (2014) Development of cycad ovules and seeds. 2. Histological and ultrastructural aspects of ontogeny of the embryo in *Encephalartos natalensis* (Zamiaceae). Protoplasma **251(4):** 797-816.