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Evaluation of responses embryogenic *Cycas revoluta* thumb., from callus culture obtained *in vitro*

Summary

Processes cell differentiation and dedifferentiation are included in the development of biotechnology protocols to promote somatic embryogenesis as an alternative to the *in vitro* propagation of plants, somatic embryos may be an excellent strategy for both propagation and conservation of fossil species such as cycads. They were evaluated *in vitro* with different strategies, morphogenic responses associated with obtaining somatic embryos of *C. revolute*. calli of megagametophytes, subsequently subcultured in four combinations of basic salts of Murashige and Skoog (1962) MS, with the addition of benzyladenine (BA), and 2, dichlorophenoxyacetic (2,4-D) were used, and kinetin (K), and picloram. In the results, It was possible to characterize potentially embryogenic callus, evaluating the levels of both cellular differentiation, necrosis, texture and color; and increases mass or weight considered to start differentiation or proembryogenic or globular type. Calli were subcultured in a medium containing MS salts, incorporating abscisic acid (ABA) in 0, 0.38, 1.13, 3.78 and 5.67 uM doses influenced both the production and maturation of somatic embryos. Embryonic structures, presented a pink coloration characteristic strongly associated towards maturity. The effect of combinations of BA, Kin, 2,4-D, GA3 and ANA influenced the development and germination of mature somatic embryos. And the combination of 1.36 mM 2,4-D+4.44 uM BA promoted the appearance of calluses with a compact texture, characteristic related to their embryogenic potential. The purpose of this research in *Cycas sp* was to contribute to the study of the *in vitro* morphogenic responses of this group of plants. And somatic embryogenesis, will allow the obtaining and multiplication as well as its preservation of *Cycas sp*. Gender that is evolutionary very important.

Keywords: *C. revoluta*, cycad, somatic embryogenesis, growth regulators

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Introduction

Cycads are a group of ancestral dioecious, which appeared in the pérmico and evolved from the progymnosperms free spores, preceding the Gingkoales and Gnetales, succeeded in developing mechanisms of adaptation and survival, so have been called "living fossils". Biologically are very interesting because they represent an important stage in the evolution of flowering plants and are considered baseline in the evolutionary tree seed plants.[1] Their beauty and rarity, has generated a great demand, so we have become perfect target of ends and people dedicated to the black market collectors of exotic species, causing today all species of the order Cycadales are included in Appendix I or II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2017). *C. revolute* It is the oldest living cycads and is included in Appendix II of CITES (2017). The species symbolizing economic importance of order,[2] ornamental and besides, they have been attributed to nutritional and medicinal properties with clear antibacterial and deleterious effects against colon cancer and epidermoid.[3–5] However, due to slow growth, dioicismo, development, and contradictions in the seed, the spread is directed to the use of buds or buds in the basal part of the parent plants limiting genetic variability significantly. Thus, It has emerged a clear need and unpostponable the use of biotechnological tools that enable effective propagation of the species to meet demand, and contribute to the conservation and avoid extinction. The ES is the *in vitro* development of embryos from cell culture are not the product of a gametic fusion.[6] In the last 70 years are few reports *in vitro* cultivation in these plants. Somatic embryogenesis in cycads is relatively low and the best responses were obtained from ZE.[7] The morphogenic potential tissue in cycads has been recognized for many years and that the use of haploid explants or those that do not require fertilization, can significantly optimize genetic resources, since there are species such as *Encephalartos woodii* Sander., of which only exemplary and have male sexual propagation is impossible. *C. revolute* investigations have sought regeneration from ZE;[8,9] leaf midrib and epicotyls;[10] endosperm[11] and megagametophytes; however, the use of megagametophytes as a source of explant organogenics responses generated (buds and roots), according Ashmore[12] are limited for conservation of genetic resources use due to genetic instability inherent to these plants. EPE development from embryogenic cultures in cycads, It remains inefficient since cultures cannot be synchronized and therefore certain developmental events are difficult to identify. Necrosing problems are frequent and slow responses due to the nature of this type of plant; Litz et al.,[7] have indicated that several species of embryogenic cultures have grown cycad vigorously and are highly morphogenic after 11 years of induction. Growth regulators can promote morphogenesis even if the salt concentration is not adequate. However, when it is desired to develop ES, use of regulators depends on the stage of the process (induction, proliferation, maturation or germination). While in cycad it has suggested the use of 2,4-D induction, Embryogenic cultures should be subcultured in the same formulation of basal medium but lacking growth regulators. Litz et al.,[7] they founded that these embryogenic cultures are not receptive to ABA because of its evolutionary condition in the plant kingdom. However, it is essential to remember that many factors other than growth regulators, influencing embryogenic responses, such as the species and type of explant. Therefore, the objective of this study was to establish the conditions *in vitro* that can promote the main morphogenic responses associated with obtaining somatic embryos of

Cycas revolute Thunb., Which allow to establish a protocol alternative propagation and preservation and contribute to study of these species.

Materials and methods

The research was conducted at the Laboratory of Plant Tissue Culture Department of Plant Science at the Autonomous University Chapingo, Mexico. calli three months old were used obtained from *in vitro* culture of C. revolute megagametophytes from a culture medium with macroelements and salts of B5 medium[13] and microcells MS medium (1962) supplemented with arginine, asparagine and glutamine (100, 100 and 400mg L-1, respectively), 6% sucrose and 6 g L-1 gellam-gum. Calli were obtained under the effect of two combinations of auxin-cytokine (Table 1). The process for promoting somatic embryogenesis was evaluated in several experimental stages and each strategies., Considered as the induction, maturation and germination of embryos obtained.

Table 1 Types and auxin-cytokine concentrations applied to megagametophytes of *Cycas revolute* for callus induction

Treatment	Growth regulators (uM)				
	2,4-D	picloram	Kin	BA	TDZ
one	9.05	-	13.93	-	-
two	-	27.75	-	2.13	2.22

Promotion stage callus. Mass increases, cell differentiation, and obtaining globular structures or proembriogenics. Embryogenic calli were subcultured to promote both their differentiation and embryonic responses using different strategies based, the medium with the inorganic salts of Murashige & Skoog[14] MS (1962), by changing the presence or absence of auxiliaries and regulators increase.

a. **Strategy 1:** MS salts (1962) with the source of nitrate diluted to 50%, supplemented with myo-inositol, thiamine, pyridoxine, folic acid, biotin, mannitol, L-cysteine, PVP (100, 0.40, 0.5, 0.5, 0.5, 170 , 60 and 70 mg L-1, respectively) and 4% sucrose.
b. **Strategies 2:** MS (1962) 100% salts, supplemented with thiamine, myo-inositol, glutamine, arginine, asparagine (0.40, 100, 400, 100 and 100 mg L-1, respectively) and 6% sucrose.
c. **Strategy 3:** MS (1962) diluted to 50% salts, supplemented with myo-inositol, thiamine, pyridoxine, folic acid, biotin, mannitol, PVP (100, 0.40,0.50, 0.50, 0.50, 170 and 70mg L-1) and sucrose 4%.
d. **Strategy 4:** MS (1962) salts, reducing the source of nitrate 50%, supplemented with myo-inositol, thiamine, pyridoxine, folic acid, biotin, mannitol, PVP (100, 0.40,0.50, 0.50, 0.50, 170 and 70mg L -1) and 4% sucrose.
e. The media were adjusted to pH 5.7±0.1, the effect of three different combinations of growth regulators and a control (Table 2) was tested.

Table 2 Types and concentrations of growth regulators applied to amorphous callus *Cycas revolute* in proliferation step

Strategies	Treatment	Growth regulators employees (uM)			
		BA	2,4-D	Kin	picloram
one	0	-	-	-	-
	one	1.33	0.45	-	-
	two	4.44	1.36	-	-
	3	13.32	4.52	-	-
two	0	-	-	-	-
	one	-	0.45	2.32	-
	two	-	2.26	4.65	-
	3	-	6.79	13.94	-
3 and 4	0	-	-	-	-
	one	-	-	0.46	4.14
	two	-	-	2.32	4.14
	3			4.65	4.14

Variables evaluated

To identify the physical characteristics of callus associated with their embryogenic potential was established a model to determine levels of cellular organization or differentiation, until the formation of globular structures or proembriogenics type, having as variables.

1. Levels cell differentiation (DC), with: 0, no differentiation (callus); 1, minimal differentiation; 2, cell aggregates (globular or pro embryonal appearance); and 3, the presence of globular or embryonic structures (EPE).
2. Coloring callus, based on the scale of Pantone. The colors used were:

(1) White
(2) P155 (cream-clear)
(3) 466 (pink)
(4) 141 (yellowish cream)
(5)374 (cream-green)
(6) 399 (green)
(7) 164 (coffee)
(8) Gray / Black

3. Consistency: friable or compact.
4. Levels of blackening necrosing (oxidation) (NO), with: 0, 25% less necrosing or oxidation; 1, 25 to 75%; and 3, greater than 75% of the developed callus.
5. And the number of distinct globular structures or proembrionarias (EC).

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Increase in mass (weight) of the callus (IB)

Both the number of embryonic structures and levels of cell differentiation DC, and the levels of blackening or oxidation and color callus were evaluated every ten days using a stereomicroscope Carl Zeiss Stemi DV4 with a lens (8x/21) for 80dds. IB biomass increases in corns, was taking his weight using an analytical balance every month for three months. In each experimental distribution strategy remain four treatments a Table 2 and with 10 repetitions per treatment. Each experimental unit was a Gerber type flask with 20 ml of culture medium.

Obtaining step and embryo maturation

Callus cell differentiation levels DC were transferred to 3/2 MS medium (1962) by diluting 75 and 25% of nitrate source (N03) and ammonium (NH4), respectively. The medium with a pH of 5.7±0.1 was added glycerol (30 g L-1), glutamine, arginine, asparagine, L-cysteine, PVP (400, 100, 100, 60 and 70mg L-1) and sucrose one%. Incorporating Ac. Abscisic (ABA), in concentrations of 0, 0.38, 1.13, 3.78 and 5.67µM. The experiment was set under a completely randomized design with 10 replications per treatment. Each experimental unit was a Gerber type flask with 20 ml of culture medium.

Variables evaluated

As variable was the formation of mature somatic embryos obtained every ten days, counted with a stereomicroscope Stemi DV4 Carl Zeiss. Calluses They were incubated with 16 hour photoperiod light (26°C) and 8 hours darkness (24°C).

Stage, germination and development of somatic embryos

Mature somatic embryos were subcultured on MS basal medium (1962) with a pH of 5.7±0.1, supplemented with myo-inositol, thiamine, pyridoxine, folic acid, biotin, Polivinylpilorridone (PVP), mannitol (100, 0.40, 0.50, 0.50, 0.50mg L-1), 4% sucrose and 7mg L-1 agar. Evaluating the percentage of embryos with responses associated with maturity and germination, evaluating the set of auxins, cytokinins and gibberellins (Table 3) effect. Calli were incubated under the same conditions of light and temperature in step above.

Table 3 Types and concentrations of growth regulators used in the germination of somatic embryos *of Cycas revolute*

Treatment	Growth regulators (µM)				
	2,4-D	BA	Kin	ANA	AG3
0	-	-	-	-	-
one	-	-	4.65	1.61	1.44
two	-	-	13.93	5.37	2.88
3	1.33	0.45	-	-	-
4	4.44	1.35	-	-	-
5	13.32	4.53	-	-	-

Statistic analysis

To determine the effect of growth regulators in vitro responses embryogenic callus cultivated on an analysis of variance for variables CE and IB, the proliferation phase is performed; and number of mature embryos with a completely randomized design with Tukey test with α=0.05 and comparison of means using SAS (Statistical Analysis System, 2002).

The statistical model used was:

$$Yij = \mu + Tij + eij$$

where: Yij, Variable Response; µ, average general; Tij, Effect of growth regulators; ij, experimental error.

For variables: DC levels, blackening, necrosing (oxidation), color and consistency, the proliferation stage, pictures with the Chi-squared Pearson (X2) were performed.

Results and discussion

The calli were subcultured in basic medium with the inorganic salts of MS (1962), for each of the steps aimed at obtaining somatic embryos, and according to the results obtained, the concentration of salts and mainly the type and concentration growth regulators employed influenced decisively on this particularly that regulators can promote embryogenic responses, even when the concentration of salts is not adequate.

Quantification of globular structures or proembrionarias and (IB)

Strategy 1: Nitrate reduction 50% MS medium (1962), did not influence the formation of structures proembrionarias EPE, before 80 dap; however, the combination of regulators BA+2,4-D promoted enhanced differentiation of embryonic structures. Noting that the top level of cytokinin BA, limits the growth of calluses in their increasing mass, but promotes cell differentiation processes, to obtain proembrionarias structures (IB), as shown by the results with α=0.05 , that there were significant differences in the number of EPE arising under different doses employed BA+2,4-D (Table 4); However, the variability obtained is due to the effect of treatments, as described Fieire (2003), which explains that embryonic responses are grouped characteristics callus cultured.he combination of 4.44 uM +1.36 (BA+2,4-D), had a higher response in obtaining embryonic structures (average 2.30), at 80dds. (Figure 1). Thus, the behavior was callus EPE concerning the number of the most obvious development period.

Strategy 2: The basic MS medium (1962) over the combination of kinetin+2,4-D promoted more EPE compared to other strategies co callus culture in the differentiation stage. Statistically significant

differences (α=0.05) IB when the concentrations of kin+2,4-D increased; however, compared to the results obtained in strategy 1, treatment stimulated greater IB, also generated a higher number of EPE (Table 5); recognizing the effect of promoting embryogenesis due to the presence of 2,4-D coinciding with Cangahuala et al.,15 and Konieczny et al.,16 that evaluated this effect, regarded as one of the factors that will determine the ES promotion rates. Comparison of means showed greater variability treatment, indicating precisely one ESAF. The number of EPE was a significant increase according to the concentration of growth regulators used, evident from dds 40 (Figure 2).

Table 4 Effect of four levels BA +2,4-D on the amount of callus and number of structures proembriogenics *Cycas revolute* to 80 dap

Concentration BA + 2,4-D (M)	Biomass increase (mg)	Number of different structures
0 + 0	88.44 toO▪	0.00 b▪
1.33 + 0.45	86.82 to	0.00 b
4.44 + 1.36	72.26 to	2.30 to
13.32 + 4.52	61.79 to	0.8 ab

▪Valores with the same letter within columns are equal according to the Tukey test at P≤0.05.

Table 5 Effect of four levels kin + 2,4-D on the amount of callus and number of structures proembrionarias *Cycas revolute* to 80 dap

Concentration KIN + 2,4-D (µM)	Biomass increase (mg)	Number of different structures
0 + 0	44.00 b▪	0.00 toO▪
2.32 + 0.45	73.74 b	2.10
4.65 + 2.26	ab 143.53	2.70 to
13.94 + 6.79	236.96 to	10.00

▪Values with the same letter in column are equal according to the Tukey test at P≤0.05.

Strategy 3: MS (1962) medium diluted 50%, the average development allowed EPE 1.30 to 80 dds in the absence of growth regulators. Meanwhile combining 2.32+4.14 uM (kin+picloram) stimulated average 5.5 differentiated structures (of which 25.45% were poorly differentiated structures development. The increased cell differentiation under the effect of 0.46+4.14 mM (kin+picloram), was reflected in a numerically greater IB, the high concentration auxin versus cytokinin source (Table 6). Production structures differentiated under the effect of 2.32+4.14 uM (+picloram kin) was higher after 40 dds (Figure 3).

Strategy 4: The basic MS medium (1962) diluting nitrate 50% without the presence of growth regulators, allowed development of average 1.10 EPE. Moreover employed combinations of kin+picloram developed statistically significant responses (α=0.05) in IB and number of EPE dds 80 (Table 7). Figure 4 shows the production of EPE upward under the effect of the doses used kin+picloram from 40 dds.

Physical characteristics of calluses

The combination of BA+2,4-D in the MS (1962) with the source of nitrate diluted to 50%, stimulated the development of callus with a level of DC (2) having 50%; nodular characteristics and little response to the formation EPE. However, statistically significant differences with α=0.05 between the necrosing and the texture type and coloring according to the concentrations of growth regulators used, including the control (Table 8) were presented. Accepting cytokinins high doses promotes accumulation of phenolic compounds by Abohatem. So 13.32µM BA presented 3 necrosing levels. The effect of kin+2,4-D in the MS medium (1962), promoted the development of calluses a DC level 2; However, unlike strategy 1, it expressed a clear friable texture in 72.5% of the calli and necrosing of 47.5%. I exist significant differences with α=0.05 in the color corns between treatments, including the control (Table 8). MS basal medium (1962) with reducing salts 50%, and the effect of kin+picloram, calli promoted structures with differing levels of 45% and compact texture at 70%; however, statistically significant differences, with α=0.05 in the necrosing between treatments, including the control (Table 8). Incorporating joint kin+picloram to MS medium (1962), 50% reducing nitrate source, compact calli produced by 72.5%. Statistically significantly different at α=0.05 level of DC (Figure 5).

Table 6 Effect of four levels kin + picloram (50% salts) on the amount of callus and average number of proembrionarias structures *Cycas revolute* to 80 dap

Concentration of kin + picloram (uM)	Biomass increase (mg)	Number of different structures
0 + 0	109.9 a▪	1.30 toO▪
0.46 + 4.14	580.9 to	1.70 to
2.32 + 4.14	390.8 to	5.50 to
4.65 + 4.14	321.6 to	3.50 to

▪Valores with the same letter within columns are igualdes according to the Tukey test with a P≤0.05.

Table 7 Effect of kin + picloram levels (source of nitrate 50%) on the amount and number of callus proembrionarias structures Cycas revolute to 80 dap

Concentration of kin + picloram (uM)	Biomass increase (mg)	Proembrionarias number of structures
0 + 0	136.2 b▪	1.10 b
0.46 + 4.14	803.8 ab	5.50 to
2.32 + 4.14	925.6 to	ab 2.80
4.65 + 4.14	659.3 ab	1.90 b

▪Values with the same letter in column are equal according to the Tukey test with a P≤0.05.

Obtaining somatic embryo maturation

The combination 4.44µM 1.36µM BA+2,4-D and diluting MS inorganic salts (1962) 50%, potentially promoted obtaining embryogenic callus, to 170 dds, with differing levels of DC (2), color (2) and compact texture (Figure 6), initially calluses were cultured on MS medium (1962), without the presence of growth regulators, and light. The presence of regulators in the doses mentioned, promoted embryonic structures from 10 dds and pink. Differentiation was progressive from 40 days (Table 9); and maturity from 80 dds.

According to their histology was observed on meristematic activity of cells in apical zones. 6, the development of pre-cotyledon and cotyledon structures presented in some somatic embryos (Figure 8). Embryogenic developments C. hildae obtained from the culture of leaves of young leaves.EPE 80, 60, and 100 dds, generated in the means of proliferation stage were subcultured to maturity; however, responses were not satisfactory due to the presence of auxin in some treatments inhibiting subsequent embryonic development as reported Vinas and Jimenez.16 Also, tripe with DC (2) level less than 100 days old, were subcultured in the same medium and showed no embryonic responses, indicating that the time of the callus culture is essential in obtaining somatic embryos, as reported by Ruiz.18

Table 8 Significance obtained by Pearson X2 test in the evaluation of statistically significant contrast between treatments mode in proliferative stage callus *Cycas revolute* to 80 dap

		variables			
MS mode (1962)	statisticians	**Levels of cell differentiation**	**Necrosing levels**	**Coloration**	**Consistency**
one	P-value	0.13	0.01*	0.00*	0.01*
	X2	2.51	2.26	0.02	0.09
two	P-value	0.71	0.17	0.01*	1.00
	X2	10.80	2.86	0.84	-
3	P-value	0.18	0.03*	0.20	0.95
	X2	2.90	1.25	5.38	8.00
4	P-value	0.03*	0.04*	0.01*	0.48
	X2	1.27	3.13	4.81	2.27

*, P≤0.05 statistically significant differences between treatments.

The use of ABA in culture medium showed that the higher the dose, the lower the number of developed somatic embryos (Table 9); meanwhile, von Arnold et al.,19 mentioned that ABA can promote the synthesis of storage reserves in embryos during maturation; however, Litz et al.,7 reported that somatic embryos in cycads are not receptive to ABA because of its evolutionary condition in the plant kingdom. The observation of the gradual development of somatic embryos obtained from C. revolute, allowed to show their morphological development is similar to that expresses a ZE, since the development of pro-embryos (being those which showed internal progress) erythrocyte and Torpedo type until the pre and cotyledon stage (Figure 7).

Table 9 Maturation of somatic embryos of *Cycas revolute* expressed in the maturation medium under five concentrations of ABA

ABA dose (µM)	**Days after subculture (dds)**			
	10	**twenty**	**30**	**40**
0	1.70	2.60	3.00	3.00
0.38	1.00	1.30	1.90	2.20
1.13	1.00	1.20	1.70	1.90
3.78	0.60	0.90	1.20	1.40
5.67	0.60	0.80	0.90	0.90

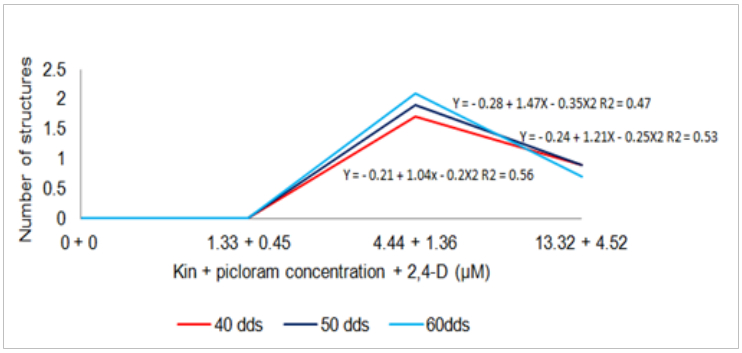

Figure 1 Schematic comparison of the development of EPE (40-60 dds) of *Cycas revolute*, under the effect of BA +2,4-D.

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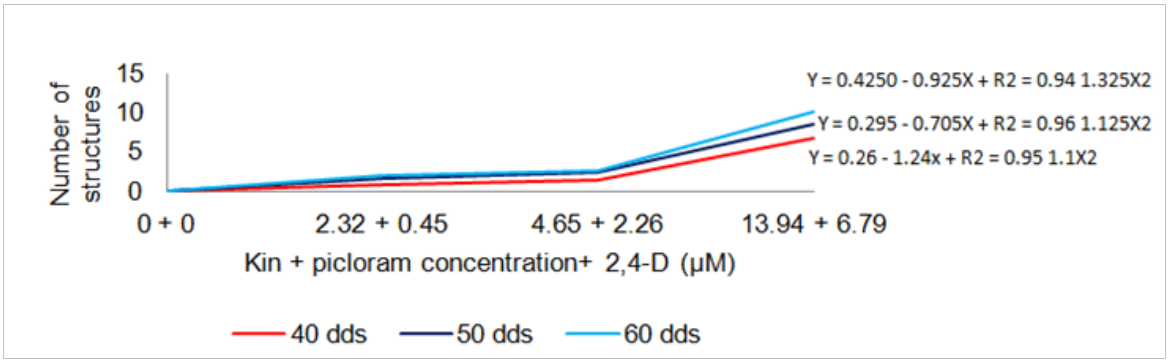

Figure 2 Schematic comparison of the development of EPE (40-60 dds) of *Cycas revolute*, under the effect of kin+2,4-D.

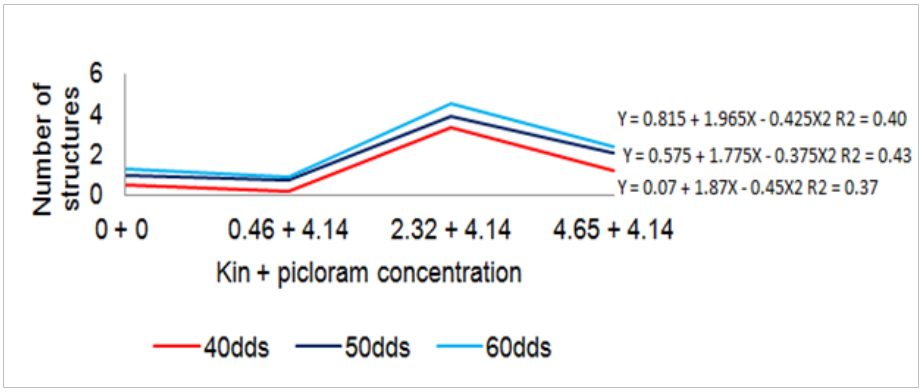

Figure 3 Schematic comparison of the development of EPE (40-60 dds) of Cycas revolute, under the effect of kin+picloram (inorganic salts 50%).

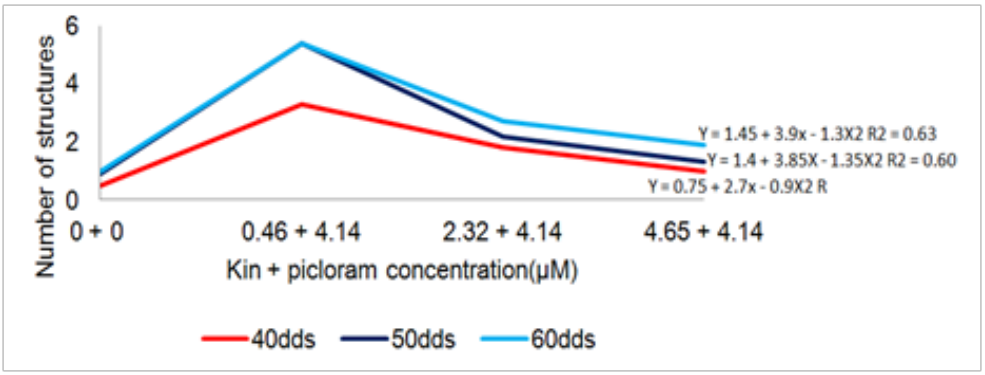

Figure 4 Schematic comparison of the development of EPE (40-60) dds Cycas revoluta, under the effect of kin+picloram (source of nitrate 50%).

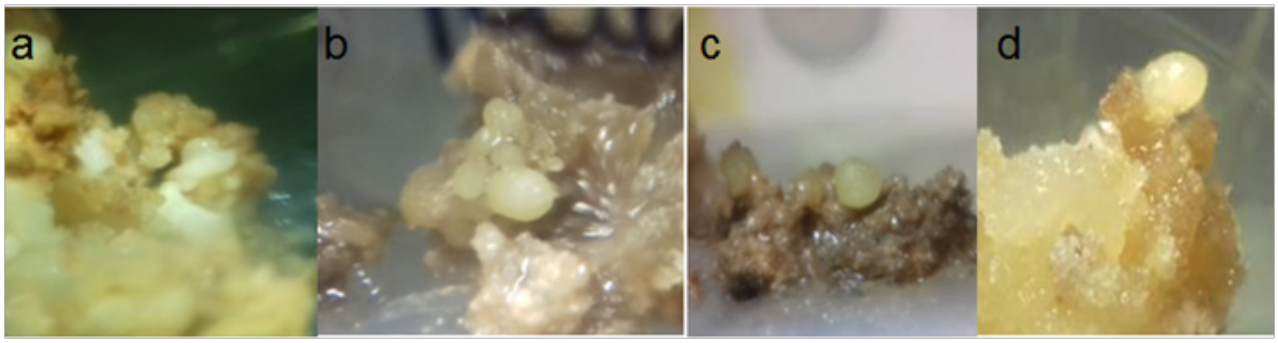

Figure 5 Development of Cycas revolute proembrionarias structures 70 days old at the MS (1962) proliferation step.A) 4.44+1.36 uM (BA+2,4-D; nitrate 50%); B) 13.94+6.79 uM (kin+2,4-D, salts 100%); C) 2.32+4.14 uM (kin+picloram, salts 50%); D) 2.32+4.14 uM (kin+picloram; nitrate 50%).

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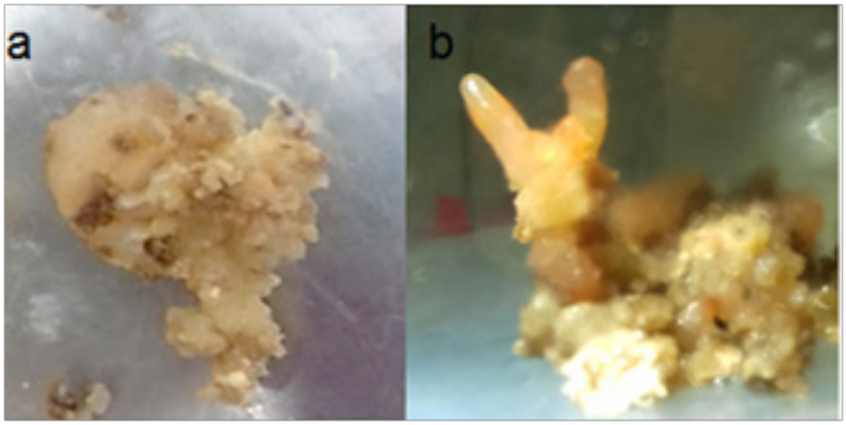

Figure 6 A) Embryogenic calli of Cycas revolute obtained by combining 4.44 uM BA+1.36 .mu.M 2,4-D; B) Polyembryony observed in medium without growth regulators.

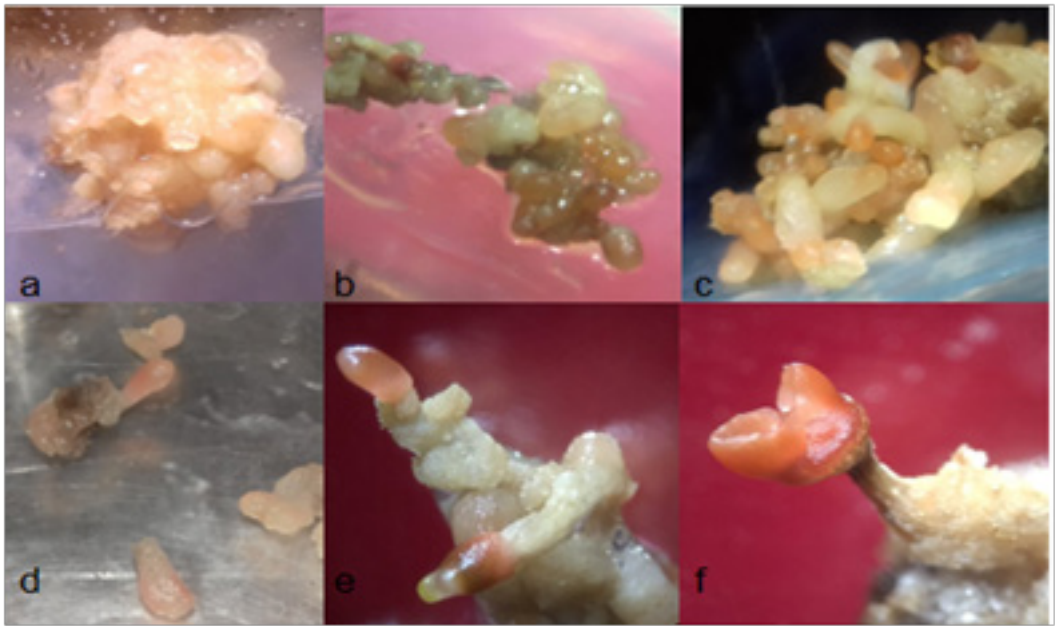

Figure 7 Embryogenic callus responses in Cycas revolute in medium without growth regulators; A) development of rosacea coloration and pro-embryos (10 dds); B) globular embryonic structures immature (30 DAP); C) Callus (70 dds); D) differentiation of somatic embryos with (70 dds) suspensor evident; E) the presence of pre-cotyledon structures (70 dds); F) cotyledon (110 dds).

Germination of somatic embryos

Embryonic structures 129 days old, had responses associated with the development and germination by 90% under the combined effect of 4.44+1.35 uM (BA+2,4-D). Germination is a naturally slow process; and also under in vitro conditions; in Figure 8, embryonal observed at 10, 40 and 220 dds, whereas short term are favorable compared to ZE responses, which develop from 1 to 1.5 years under in situ conditions. The percentage of germination of somatic embryos combining kin, ANA, AG3, BA and 2,4-D at 40 and 210 dds, shown in Figure 9, where obviously the medium without the presence of growth regulators had no effect positive in embryonic development; comparing the combination 4.44+1.35uM (BA+2,4-D).

Embryonic development and responses associated germination were observed in embryos meristematic cell division in apical region type. Which shows an embryonic indispensable maturation in the conversion process plant (germination). Development related to leaf development and the apparent photosynthetic activity could be observed (Figure 8) structures, which coincides with Litz et al.,7 who reported that the expansion of the first sheet always circinada or after germination.

The production of embryonic structures derived from apical meristem area growth mature somatic embryos from 30 dds was evident (Figure 10), averaging 8.7 and 12.1 somatic embryos under the effect of 1.33+0.45 and 4.44+1.35 (μM) BA+2,4-D, respectively. This, evidenced a recurring somatic embryogenesis (ESR), coinciding with who point out that mature somatic embryos are allowed to form new embryos derived from epidermal cells. However, the 2.48% exhibit deformations in morphology (fan shape), and promptly let this kind of derangement may be related to bipolar endogenous auxin transport by Hiraga et al.20

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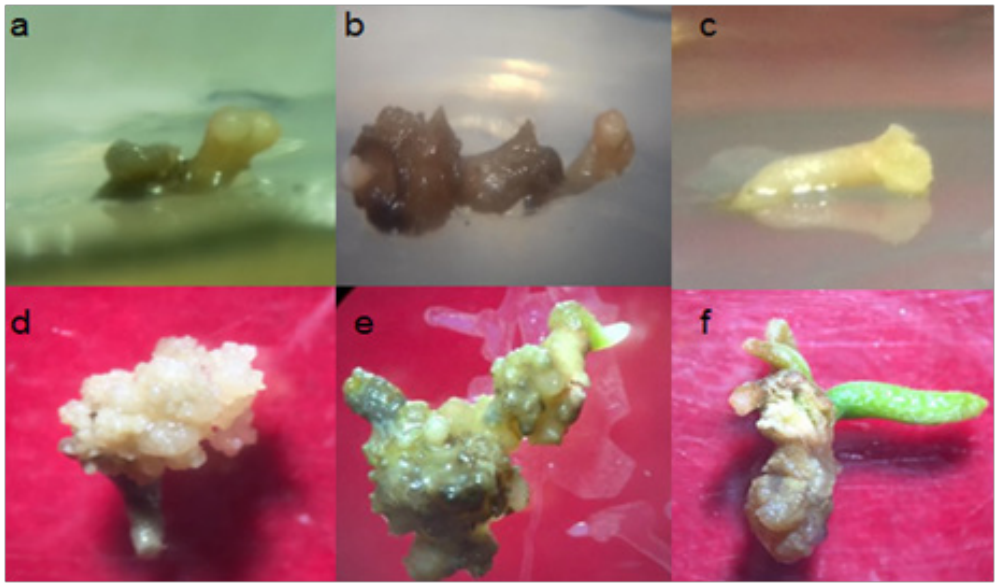

Figure 8 Production of somatic embryos of Cycas revolute with the effect of 4.44+1.35 uM (BA+2,4-D). ab) bipolar meristematic growth evident from the 10 (A) and 40 dds (B); apical meristem growth and radical 10 (C) and 220 dds (D); E) coleoptilar expression of an embryo of 27 dap; F) development of primary leaflets, apparent photosynthetic activity at 70 dds (F).

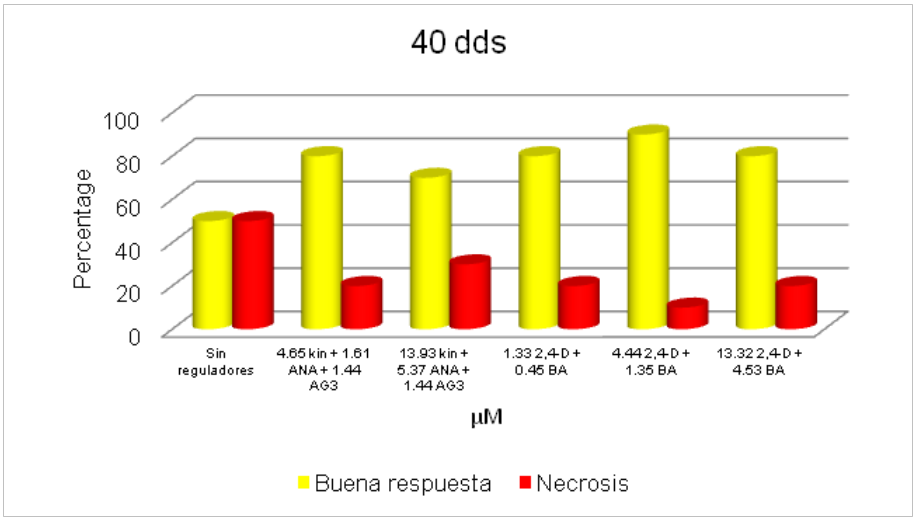

Figure 9 Combination regulators and blackening levels of cultured in vitro responses observed according to their viability tissues to promote somatic embryogene.

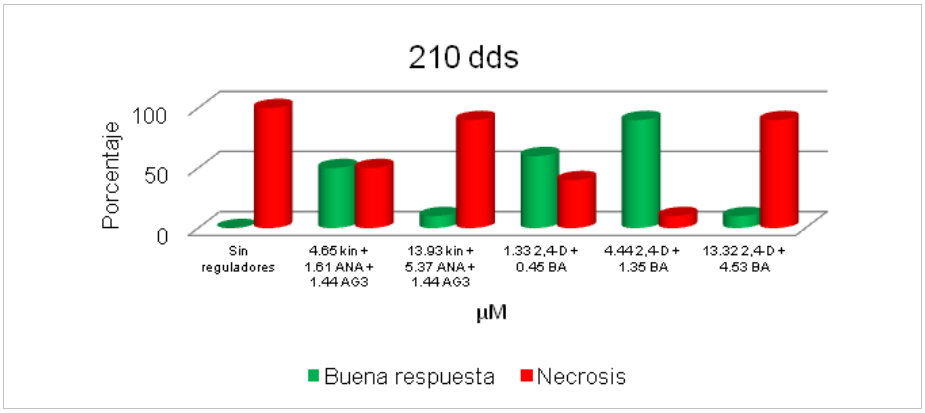

Figure 10 Percentage of responses associated with germination of somatic embryos of Cycas revolute at 40 and 210 dds.

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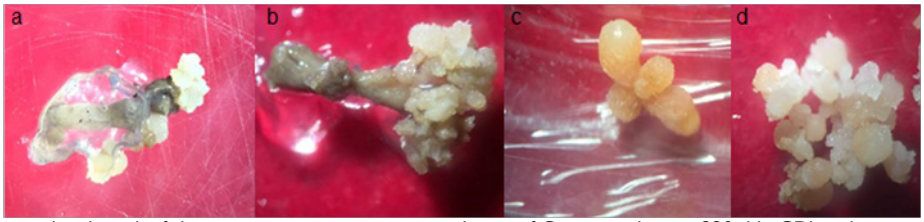

Figure 11 AB) blackening (oxidation) of the root tip apparent somatic embryos of Cycas revolute to 220 dds; CD) embryonic globular structures generated from ESR.

Conclusions

The combination of 4.44+1.35 uM BA+2,4-D in MS basal medium (1962) with 50% reduction in the source of nitrate, stimulate the formation of potentially embryogenic callus at 170 days of cultivation, which characterized by a creamy color, compact consistency and nodular appearance.MS basal medium (1962) with NH4NO3 and KNO3 reduction at 75 and 25%, respectively stimulated the production of somatic embryos from 30 days; however, maturation occurs not before 100 days after subculturing. The combination of 4.44+1.35 uM BA+2,4-D stimulated responses associated germination of somatic embryos.Tissue maturation in each of the stages of somatic embryogénics cycad, is substantial for somatic embryos with good regeneration potential. The application of activated carbon under a previous nutritional stress, recurrent somatic embryogenesis stimulated somatic embryos more than 220 days after subculture. Finally, in Cycas sp. according to the results obtained, we consider that a limitation were the in vitro responses that are very slow, and the assessment of the each stages, as well as various constituents of the growing medium.21–25

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Conflicts of interest

The authors declare there is no conflict of interest.

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