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IN VITRO SEED GERMINATION OF *CYCAS REVOLUTA* THUNB.

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Abstract

Germination of *Cycas revoluta* zygotic embryo and calli production from endosperm of seeds were succeeded in Litz medium which contains major salts of B5 (Gamborg et al. 1968) and minor and organic salts of MS media (Murashige and Scoog 1962). The induction medium covers organic compounds such as glutamine, arginine and asparagine in a variety of combinations including 2.4-D and Kn as well. The best medium for *Cycas revoluta* zygotic embryos is that with 10 µM 2.4-D and 4.5 µM Kn in which both zygotic embryos germinated healthily and green calli grew from endosperms of megagametophytes.

Introduction

Having been surviving from early periods of mezozoic age until present time, cycads inhabit regions including east Africa, Madacascar, subtropical and tropical Americas, Indian subcontinent, South China, South of Japanese islands and Australia.

Majority of cycads undergoes danger of extinction, even some have already become extinct. The surviving ones are represented by 9 genera and 132 species whose orders have been restructured. Although cycads are of importance in botany, they provide natural sources of biochemicals with antimicrobial and pesticidal character. Slowly growing plants, cycads take 3-10 years to mature (Dyer 1965). They can be reproduced using both short-lived seeds and vegetative shoots. *In vitro* techniques have enabled to protect many species in danger of extiction. Micropropagation protocols established based on organogenesis and somatic embryogenesis, the latter of which was successful in using leaf calli of mature plant and zygotic embryo explants of some species of *Zamia* (Chavez *et al.* 1992c) and *Ceratozamia* (Chavez *et al.* 1992a and Litz *et al.* 1995). Shoot organogenesis was also successful in propagation of *Zamia* and *Cycas* species (Chavez *et al.* 1992c) and *Cycas revoluta* (Rinaldi and Leva 1990, 1995).

Light and nitrogen formulations were factors to stimulate the production of callus cultures. Adventive stem regeneration of *Zamia* zygotic embryo explants were stimulated in constant darkness (Chavez *et al.* 1992c) including major salts of B5 (Gamborg *et al.* 1968) and minor and organic salts of MS media (Murashige and Scoog 1962). Adventive stem regeneration from zygotic embryo explants of *Cycas revoluta* was obtained after they had been subjected to a 12-hour darkness in SH basal medium (Schenk and Hildebrandt 1972) then underwent light (Renaldi and Leva 1990). Stem organogenesis was caused by seedling extracts of *Cycas revoluta* (Rinaldi and Leva 1990, 1995) after their exposure to light in SH basal medium but not in darkness. Shoot differentiation was previously obtained from zygotic embyros and seedling explants of *Cycas revoluta* in the media with BA and 2.4-D (Rinaldi and Leva 1990) but culture conditions were not optimized. Different nitrogen formulations (total and relative

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quantaties of inorganic nitrogen) and effects of 16 hour exposure to light in nitrogen formulation on adventive shoot formation from zygotic embryo and shoot explants (Rinaldi 1999) were performed.

One of rare species, *Cycas revoluta* (sago palm) which have managed to survive from the tertiary were germinated from its zygotic embryo in vitro and calli obtained from endosperms of its seeds.

Materials and Methods

Cycas revoluta seeds and preperation of germination medium: *Cycas revoluta* seeds were obtained from pollinated plants in the botanical garden of Rixos Hotel, Antalya (Fig. 1a, b). They were exposed to a surface sterilization in a solution of 5% hypochloride (37.5 ml), agitated for 15 minutes and passed through sterilised water in vitro for three times to eliminate hypochloride after removing their sarcotesta and sclerotesta (Fig. 1c). Zygotic embryo explants were excised from them so that their endosperms should be 1-1.5 cm. Cultivated zygotic embryos ranged from 5 to 15 mm in length (Fig. 1d). Only embryo-processed plantings managed to germinate whereas one failed to germinate seeds planted by disconnecting embryonic association of endosperm with funiculus. Seeds were surface-sterilized first then planted in their media with the result that inflated embryo were excised from endosperm tissue following a two-week absorption of water (Fig. 1d). Because endosperm is rather rigid, direct embryo planting could not be performed. Seeds whose embryos were to be excised had to be left in their own media for one-week exposure to hydration. Softening of endosperms enabled embryos to be easily removed from endosperms.

Germination and calli induction: Zygotic embryo explants were cultivated in two different induction media of Litz *et al.* (2005) and SH (Schenk *et al.* 1972), nitrogen contents of which are presented in Table 1.

Litz induction media are composed of contents of micro nutrients and organic compounds [glutamine (400 mg/l), arginine (100 mg/l), asparagine (100 mg/l)] of MS and macro nutrients of B5 media. In addition, four different experimental designs were created by adding carbon source of 60 g/l sucrose, 4g gellan gum, 9.05, 10, 9.05 and 8,5 µM 2.4-D and 9.3, 4.5, 11 and 10 µM kinetin. 2 g agar added-media whose pH was modified to pH 5.8 were autoclaved under 103.4 kPa pressure at 121ºC for 15 minutes. Media were incubated in illumination of 40 µm $m^{-2}s^{-1}$ and 16/8 photoperiod at 25ºC.

With the addition of 30 g/lt sucrose, agar (5 g/l), 9 µM BA and 0.04 µM 2.4-D in SH medium, the explants were cultivated. Semi-strengthened macro elements were added to hormoneless medium of 10 g sucrose into which explants were then transferred. The medium whose pH was changed to 5.7 was autoclaved under 103.4 kPa pressure at 121ºC for 15 minutes. The medium was incubated in illumination of 40 µm $m^{-2}s^{-1}$ and photoperiod of 16/8 and all explants cultivated every six mounths with the experiment being repeated under photoperiod of 16/8 h five times.

Statistical analyses: Percentages of regenerated explants were compared using x^2 test ($P \leq 0.05$). Data of number of shoots regenerated from each embryo was calculated by variance analysis (ANOVA), with the result that test ($p \leq 0.05$) showing the least important difference was the mean of comparisons (Steel and Torrey 1980).

Results and Discussion

Initiation and growth of morphogenetic tissues from *Cycas revoluta* embryos was of radicular appearance especially in a three-week exposure of explants to the medium (Fig. 1d). Brown callus formation was observed in the Litz medium with the addition of 9.05 µM 2.4-D and 9.30 µM Kinetin (Litz *et al.* 2005). However, calli were seen to loose their vitality 1-2 weeks after their second subculture (Fig. 1e). The additon of 10 µM 2.4-D and 4.5 µM Kn to the Litz medium caused a 80% healthily callus formation from endosperms and 80% seedlings from embryos (Fig. 1 f, g; Fig. 2 a, b). It was observed that the Litz medium supplied with 9.05 µM 2.4-D and 11 µM Kn produced a 40% callus (Fig. 1 h) and 40% seedlings (Fig. 2 c). Although the calli were green in the 3rd week of cultivation, they became brown to dark and died in 4th and 5th weeks. The medium did not create seedlings from embryos but endosperms produced calli by 10% (Fig. 1 i) which died later (Table 2). Comparisons between media 4 and 2 in callus formation and those between 2 and 3 in seedling formation were found to be statistically significant (Table 3). Germination was not observed in seeds planted in SH medium only with superficial crackings on them during the 12-month study (Fig. 2 d).

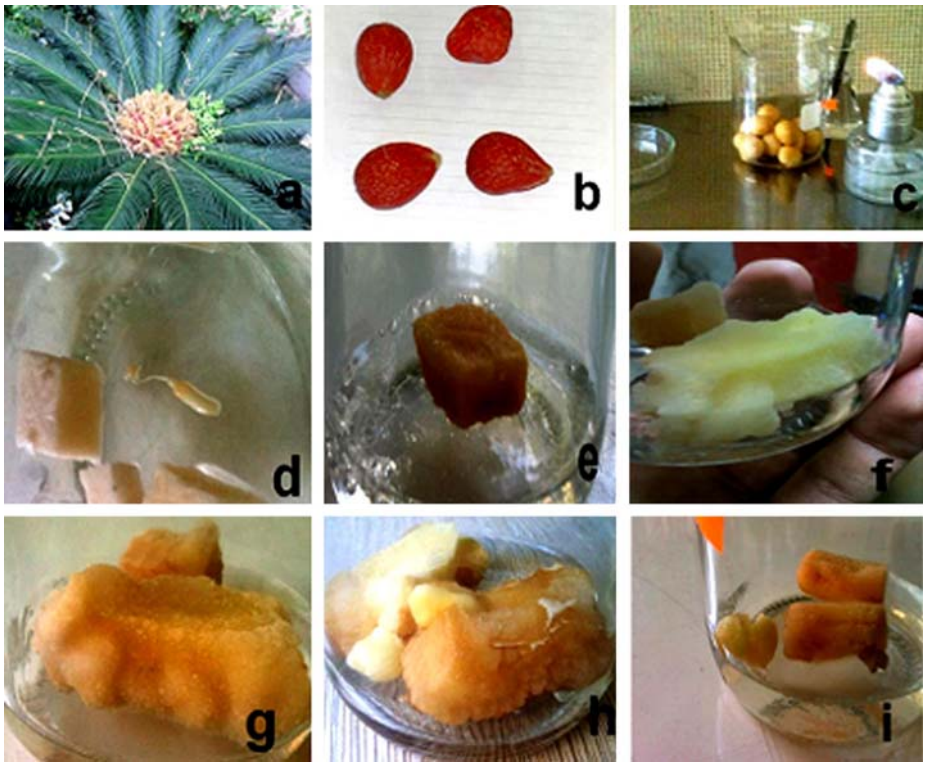

Fig. 1. *Cycas* seeds (a) with red testas (b). Surface-sterilized (c) and embryo extracted with its funiculus (d) from endosperm of 3-week *Cycas revoluta* seeds. Brown callus formation in Litz (2005) medium supplied with 9.05 µM 2.4-D and 9.30 µM Kn (e), green callus formation with 10 µM 2.4-D and 4.5 µM Kn for 5-week cultivation (f); for 9-week cultivation (g), yellowish callus formation with 9.05 µM 2.4-D ve 11 µM Kn for 5-week cultivation (h), and light brown callus formation with 8.5 µM 2.4-D and 10 µM Kn for 5-week cultivation (i).

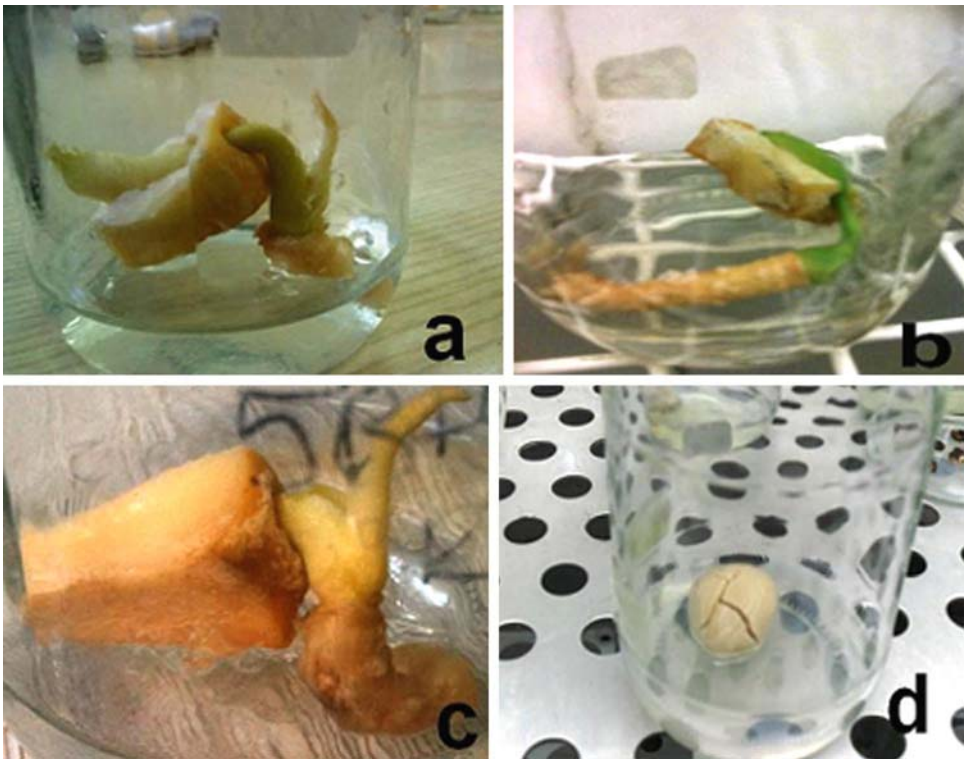

Fig. 2. Seedling formation in Litz (2005) medium supplied with 10 µM 2.4-D and 4.5 µM Kn (a), root (b); 9.05 µM 2.4-D and 11 µM Kn for 10-week cultivation (c). Seeds cracked and non germinated in SH (Schenk et al. 1972) medium (d).

Table 1. Comparison of inorganic nitrogen sources in two nutrition media.

Inorganic nitrogen	Litz [a] mM	SH[b] mM
KNO_3	24.7	24.7
NH_4SO_4	13.4	----
$NH_4H_2PO_4$	1.3	2.6
Total [N]	38.1	27.3
Total [N]-NO^-_3	24.7 (90%)	24.7 (90%)
Total [N]-NO^+_4	1.3 (5%)	2.6 (10%)

[a]Litz (2005), [b] Schenk and Hildebrandt (1972)

Adventive stem induction is reported to be successful in cultures from zygotic embryos of Cycad species *Ceratozamia hildae* and *Ceratozamia mexicana*, *Zamia* in all but not in *Cycas revoluta* (Chavez *et al.* 1992b, 1992c). However, only zygotic embryos of *Zamia pumila* are inductive in cycads. This study succeeded to occurr adventive stems from zygotic embryos of *Cycas revoluta*. Moreover, research on regeneration from seedling explants is within the scope of further cultivation studies. All experimented explants were shown to be available resources for adventive stems. Experiments which were previously conducted with zygotic embryo

explants demonstrated that artificially-induced photoperiod process and totally darkened cultivation affected embryos. Daily 16 hour photoperiod for 4-8 weeks is reported to show that adventive stem induction was normal but abnormal under totally darkened circumstances (Rinaldi 1999). The experiments were conducted in combination of ammonia with nitrate in MS and SH media. On the other hand, numerous adventive stems of *Cycas revoluta* were successfully induced and grown on the zygotic embryos of the ripe naked seeds used in Schenk and Hildebrandt (SH) medium supplemented with 3.0% sucrose, 20% coconut milk, 0.6% agar and the growth regulators of 0.20 to 2.00 mg/l BAP and 0.00 to 0.20 mg/l 2,4-D in combination at pH 5.9 42 to 84 days after the onset of the culture (Motohashi *et al.* 2008).

Table 2. Ratios of seedling and callus formation in endosperms and embryos from seeds.

Media	Seed number	Endosperm	Callus %	Embryo	Seedling %
1. 9.05 µM 2.4-D ve 9.30 µM Kn	50	50	20	50	0
2.10 µM 2.4-D ve 4.5 µM Kn	50	50	80	50	80
3. 9.05 µM 2.4-D ve 11 µM Kn	50	50	40	50	40
4. 8.5 µM 2.4-D ve 10 µM Kn	50	50	10	50	0

Table 3. Statistically significant x^2 results of our data.

Media	1	2	3	4
Calli numbers	10 ± 0.707^{b}	40 ± 1.224^{acd}	20 ± 0.707^{bd}	5 ± 0.705^{bc}
Seedlings number	-	40 ± 1.227	20 ± 0.702*	-

The difference between "a" and media 1, "b" and media 2, "c" and media 3, "d" and media 4 , "*" and media 2 is statistically important ($P<0.05$)

The present experiments indicated that SH medium failed while the Litz medium with macro and micro elements of B5 and MS media respectively showed occurence of adventive stems and rooted seedlings from zygotic embryos. The induction medium contains organic compounds such as glutamine, arginine and asparagine in a variety of combinations including 2.4-D and Kn as well. Studies conducted with the same medium report that adventive stem regeneration succeeded using calli from zygotic embryos of the cycad *Dioon merolea* about to be exposed to extinction in Mexico (Litz *et al.* 2005). *Dioon merolea* successfully produced adventive stem in the Litz medium in combination of 9.05 µM 2.4-D and 9.30 µM Kn hormones whereas *Cycas revoluta* zygotic embryos did not exhibit any growth. The best medium for *Cycas revoluta* zygotic embryos is that with 10 µM 2.4-D and 4.5 µM Kn in which both zygotic embryos germinated healthily and green calli grew from megagametophytes. As a result, seed propagation is currently the most practical method of large-scale commercial cycad production although an increasing number of plants can be produced with suckers and offsets from stock plants in nursery plantations. Cycads are dioecious, many species of which do not produce seed unaided in cultivation either because their specific insect pollinators are absent or because male and female plants produce cones asynchronously.

Acknowledgement

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