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Tissue Culture Note

Adventitious embryo formation derived from zygotic embryos in *Cycas revoluta*

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Abstract Numerous adventitious embryos of *Cycas revoluta* were successfully induced and grown on the mature zygotic embryos in the well-ripe, naked seeds used on 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^{-1} BAP and 0.00 to 0.20 mg l^{-1} 2,4-D in combination at pH 5.9 42 to 84 days after the beginning of the culture. Maximum proliferation of 88.7±40.5 adventitious embryos per zygotic embryo was obtained on SH basal medium supplemented with 0.20 mg l^{-1} BAP and 0.02 mg l^{-1} 2,4-D. Root formation was seen on all of the adventitious embryos by continuous culture on the same medium. Each rooted plantlet was isolated individually and then, grew big enough to juvenile stage that had one to three leaves with pinnate leaflets, thick trunk and primary tap root on SH basal medium without any growth regulator 196 days after the beginning of the culture and was ready for acclimatization.

Key words: Adventitious embryo, *Cycas revoluta*, embryo, Schenk and Hildebrandt medium.

Among the living members of the Gymnosperms the Cycadales is the most primitive (Ikeno 1896) and consists of two sub-orders, three families, nine genera and 185 species distributed in the tropics to subtropics in the world (Jones 1993). It has the specific characters such as Permian plants arisen 230 million years or more ago, sexual habit of dioecism, sperm production in pollen tube, pinnate leaflets to form leaf, symbiotic relations with cyanobacteria which are capable of fixing nitrogen from the atmosphere in coralloid roots, and high toxicity of plant bodies and seeds (Ikeno 1896; Stevenson 1990; Jones 1993). *Cycas revoluta* is taxonomically considered as the most primitive species among the living cycads and is placed in the Cycadaceae, the Cycadineae, the Cycadales (Stevenson 1990; Jones 1993). The species is found in Miyazaki and Kagoshima Prefectures in Kyushu District down to the Ryukyu Islands, Okinawa Prefecture in Japan (Jones 1993).

Since the majority of the cycads is endangered in the world as the category of IUCN-SSC and the Appendix I or II of CITES (Prance and Elias 1977), it needs conservation and propagation with aids of rescue and re-location plantations for *in situ* and *ex situ* conservation, asexual reproductions, hand pollination and sexual reproduction, micropropagation by tissue culture (De Luca et al. 1979; Stevenson 1990; Norstog and Rhamstine 1967; Osborne 1990; Rinaldi 1999; El-Shiaty 2005; Li et al. 2007). Regarding the first success case of tissue culture of cycads by Norstog and Rhamstine (1967), young embryos of *Cycas circinalis* and *Zamia interrifoius* were cultured with MS medium (Murashige and Skoog 1962) modified with amino acid and adenine and supplemented with 2,4-D and kinetin and induced pseudobulbous tissues and then, transferred to MS medium with no growth substance to synthesize adventitious embryogenesis. Osborne and van Staden (1987) and Osborne (1990) tried various explants from some species of *Encephalartos* and *Stangeria eriopus* for tissue culture. They successfully induced their calli from main roots of 2/3 of the species studied as the explants on SH medium (Schenk and Hildebrandt 1972) supplemented with auxin and cytokinin in combination in the dark within 3–4 weeks. Their shoots were differentiated in the same medium under light conditions.

The mature seeds of *C. revoluta* used were collected in Ohgimi Village and Miyagi-Jima, Okinawa Island, Okinawa Prefecture, Japan. After their sarcotesta and sclerotesta were removed, the naked seeds were rinsed in tap water for 30 minutes and sterilized in 70% ethanol

Abbreviations: BAP, 6-benzylaminopurin; CITES, Convention on International Trade in Endangered Species of Wild Fauna and Flora; IUCN, International Union for Conservation of Nature and Natural Resources; SSC, Species Survival Commission; 2,4-D, 2,4-dichlorophenoxyacetic acid.

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for 1 minute and in 30% purelox for 5 minutes. Then, the mature embryo used were cut together with the megagametophytes into a block smaller than 20 mm×20 mm×20 mm size and was rinsed in the distilled, sterilized water. They were planted on SH basal medium.

As SH medium was primarily employed at pH 5.9 and was supplemented with 20% coconut milk, 3.0% sucrose and 0.6% agar for the basal medium and prepared various media by combinations of BAP and 2,4-D at the concentrations of 0.00, 0.02, 0.20, 2.00 and 10.00 mg l^{-1}, respectively. Five examinations were repeated per medium.

Grown cultures were fixed in 1 : 3 mixture of glacial acetic acid and absolute ethanol, hand sectioned and put and smeared with the mixture of zeratin, phenol and gycerol on glass slides and then, put one or two drops of formalin and dried. Dried slides were dipped in the series of 3 : 1 mixture, 1 : 1 mixture and 1 : 3 mixture of xylen and ethanol, respectively and were, then, soaked the alcohol series from 10.5, 20, 30, 50, 60 and 80%, and double stained with 2% safranine aqueous solution and 1% light green in 30% ethanol and sealed with eukitt. The slides were observed with dissecting microscope.

If adventitious embryos together with culture tissues were less than 7 mm×7 mm×7 mm size, they were fixed in 10% formalin and were, then, dehydrated with 2-methoxymethanol, 2-methoxyethanol, absolute ethanol, n-propanol and n-butanol before they were saturated with the monomer 189 : 1 : 10 mixture of glycol methacrylate, 2,2′-azobis isobutyronitrile and polyethylene glycole. They were placed in zeratin capsule filled with the monomer mixture solution and later polymerized and were embedded with glycol methacrylate by Feder and O'Brien's method (1968). Serial longitudinal sections of adventitious embryos, 10 μm in thickness, were made by cutting on a rotary microtome. The sections were mounted on glass slides with a drop of 30% ethanol and then, air dried. The slides were stained with 0.05% toluidine blue aqueous solution and sealed with eukitt.

Although white calli were formed on the surface of the embryos planted on SH basal medium supplemented with 2.00 and 10.00 mg l^{-1} 2,4-D, they did not show any progress of differentiation. However, these white calli were temporary products and died together with the explants after 112 days culture.

The mature zygotic embryos produced numerous adventitious embryos if they were cut into the blocks of approximately 15 mm×15 mm×15 mm used as the explants on SH basal medium supplemented with 0.00, 0.02, 0.20 mg l^{-1} BAP and 0.00, 0.20, 2.00, 10.00 mg l^{-1} 2,4-D in combination. The explants got firstly swollen and secondly reached double the first size after 42 days culture, and then, they started to produce adventitious embryos, increased gradually their populations and

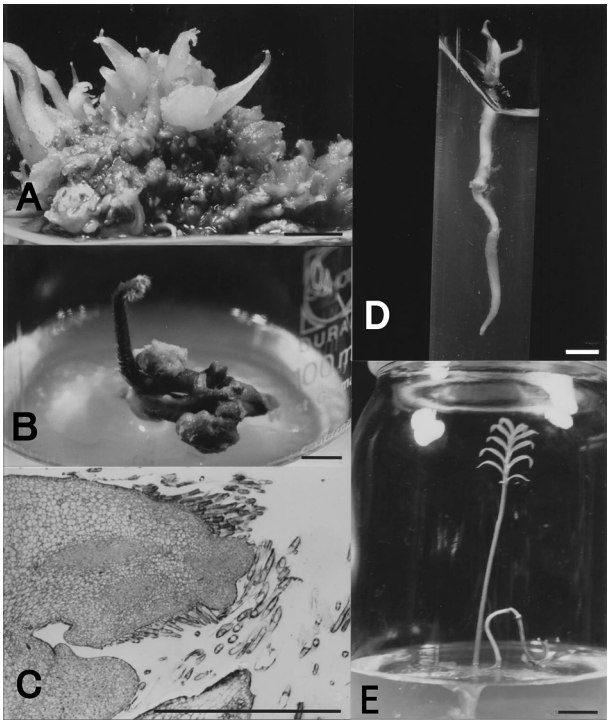

Figure 1. Adventitious embryo formation derived from zygotic embryos and plant regeneration in *Cycas revoluta*. A. Many adventitious embryos formed on a zygotic embryo 112 days after the beginning of the culture on SH basal medium supplemented with 0.20 mg l^{-1} BAP and 0.02 mg l^{-1} 2,4-D. Bar=1 cm. B. An adventitious embryo with young, true leaf contained numerous trichomes, isolated from a mass of adventitious embryos 125 days after the beginning of the culture. Bar=1 cm. C. Longitudinal section of a well-grown adventitious embryo with numerous trichomes. Bar=1 mm. D. A young plantlet individually isolated formed very thick, trunk and primary tap root. Bar=1 cm. E.Well developed plantlet with a juvenile leaf with pinnate leaflets, same as the first leaf of the seedling establishment, 196 days after the beginning of the culture. Bar=1 cm.

covered entirely the cut-surface of the zygotic embryos after 56 days culture. Some of the adventitious embryos began to produce shoots on SH basal medium with 0.2 mg l^{-1} BAP and 0.02 mg l^{-1} 2,4-D after 112 days (Figure 1A).

Table 1 showed the inductions of adventitious embryos and roots on the seed explants of *C. revoluta* cultured on SH basal medium supplemented with BAP and 2,4-D at various concentrations after 84 days culture during the course of investigation. The highest relative frequency of zygotic embryos which produced adventitious embryos and roots (both 71.4%) occurred on SH basal medium supplemented with 0.20 mg l^{-1} BAP (Table 2). Some of the adventitious embryos started to produce true leaves which contained numerous trichomes (Figure 1B, 1C).

Adventitious embryos occurred not only on the cut-surface of the embryo in the seed but also on the plumule or tips or leaf axil of the newly grown shoot from the embryo. Coconut milk supplemented in SH basal medium contributed good formation of roots probably

Table 1. Adventitious embryos induced on embryos of *Cycas revoluta* on SH basal media supplemented with BAP and 2,4-D at various concentrations in different combinations

Growth regulator (mg l^{-1})		No. of	No. of zygotic embryos produced adventitious cultured	No. of zygotic embryos rooted adventitious embryos	No. of adventitious embryos produced on single embryo (mean ± SD)
BAP	2,4-D	embryos			
0.2	0	7	5	5	77.0 ± 54.8
0.2	0.02	12	7	5	88.7 ± 40.5
0.2	0.2	9	3	1	62.0 ± 19.7
2.0	0	7	4	0	70.3 ± 56.1
2.0	0.02	7	3	0	48.0 ± 11.4

Those embryos were cultured for 84 days.

due to its contents of sucrose, amino acid, myo-inositol, phenylurea, indol acetic acid, gibberellic acid, and so on (Harada and Komamine 1979; Komamine et al. 1989). Kinetin at concentrations of 0.01, 0.10 and 1.00 mg l^{-1} added in SH basal medium also performed good formation of roots.

Osborne (1990) reported callus formation on mature, zygotic embryos of *Encephalartos villosus* on SH medium supplemented with 3.0 mg l^{-1} BAP and 0.3 mg l^{-1} 2,4-D and that on leaves of *E. woodii* on SH medium supplemented with 10 mg l^{-1} NAA and 10 mg/l kinetin. Both 2,4-D and NAA were auxin, however, their high concentrations had different effects on differentiation and dedifferentiation. The present study showed high concentration of cytokinin (BAP) in SH basal medium was not effective for embryo culture in *C. revoluta*. The differentiated plantlets were small, yellowish-green in color and looked like weak but they formed leaves within 90 days.

Young plantlets with a few leaves, thick trunk and primary tap root developed from the adventitious embryos were isolated individually and transplanted to SH basal medium supplemented with no growth regulator 125 days after the beginning of the culture. Well developed plantlet with a juvenile leaf with pinnate leaflets 196 days after the beginning of the culture were ready for acclimatization. Since total duration of proliferation and growth of adventitious embryos to individual plantlets on the mature, zygotic embryos of *C. revoluta* were much shorter than that from seed germination to seedling establishment in nature or *in vivo* cultivation for 300 to 360 days it could rescue, propagate and conserve vegetatively the slow growing, endangered cycads.

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