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BRIEF COMMUNICATION

Somatic embryogenesis and plant regeneration from leaf, root and stem-derived callus cultures of *Areca catechu*

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Abstract

Plant regeneration through somatic embryogenesis of *Areca catechu* L. was established using leaf, root and stem segments as explants. Embryogenic callus was induced and maintained on medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) or 3,6-dichloro-2-methoxybenzoic acid (dicamba) at concentrations 2, 4, 6 and 8 mg dm^{-3} in darkness. Somatic embryos were found on primary callus in the presence of 2 and 4 mg dm^{-3} dicamba and during subculture on 2 - 8 mg dm^{-3} 2,4-D or 2 - 4 mg dm^{-3} dicamba-containing media. Plantlet conversion from embryos was successfully achieved on growth regulator-free medium. The plants grew well when transplanted to containers in shaded greenhouse.

Additional key words: betel nut, dicamba, 2,4-dichlorophenoxyacetic acid.

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Areca catechu L. (*Arecaceae*), local name betel nut, is one of the most economically important crops in Taiwan. Because of the limited success in tissue culture of this species, generally, the propagation practice is through the seed. Ganapathi *et al.* (1997) reported a method for *in vitro* culture of zygotic embryos of this species. However, to our knowledge, the progeny from sexual propagation are not uniform. Recently, protocols for *in vitro* plant regeneration of betel nut through somatic embryogenesis from zygotic embryo-derived callus (Wang *et al.* 2002) and shoot formation from shoot tip-derived callus (Wang *et al.* 2003) have been described. In this communication, we further established a method for inducing plant regeneration through embryo formation using vegetative tissues of betel nut.

Mature fruits of *Areca catechu* L. were collected from a local farm in Taipei, Taiwan, Republic of China. Zygotic embryos of these fruits were taken, immersed in 70 % alcohol for 1 min, followed by surface sterilization by agitation for 10 min in a solution of with 2 % sodium hypoclorite and 0.05 % *Tween* (1:1, v/v). These zygotic embryos were cultured on Murashige and Skoog (1962; MS) basal medium contained full-strength macro- and micro-elements of MS salts supplemented with [mg dm^{-3}]: myo-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), peptone (1000), NaH_2PO_4 (170), sucrose (30000), and *Gelrite* (2200). Segments (5 mm in length) of leaves, roots and stems were taken from 2-month-old seedlings and were used as explants. Plant growth regulators, 2,4-dichlorophenoxy-acetic acid (2,4-D; 2, 4, 6, 8 mg dm^{-3}) and 3,6-dichloro-2-methoxybenzoic acid (dicamba; 2, 4, 6, 8 mg dm^{-3}), were added into MS media prior to autoclaving for 15 min at 121 °C. The pH of the media was adjusted to 5.7 with 1 M KOH or HCl. Explants were incubated in 20 × 150 mm culture tubes in darkness for callus induction and subsequent proliferation. Embryo induction and plantlet development were performed under a 16-h photoperiod at irradiance of 28 - 36 μmol $m^{-2}s^{-1}$ (daylight fluorescent tubes *FL-30D/29*, 40 W, *China Electric Co.*, Taipei,

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; dicamba - 3,6-dichloro-2-methoxybenzoic acid; MS - Murashige and Skoog (1962) medium; BA - N^6-benzyladenine; TDZ - 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (thidiazuron).

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Taiwan, Republic of China) and temperature at 26 ± 1 °C. The subculture period was two months. Regenerated plantlets were transferred to sandy soil in containers for acclimatization in a shaded greenhouse (30 % irradiance). In the first experiment, 15 explants were tested for each treatment, and the frequency of callusing explants was determined after 60 d of culture. In the second experiment, 6 explants were used in each treatment, and the proliferation rate of callus (the final fresh mass divided by the initial fresh mass) was determined after 60 d of culture. All cultures were examined and photographed under a stereozoom microscope (*SZH*, *Olympus*, Tokyo, Japan). Tissues for histological observations were fixed in FAA (95 % ethyl alcohol + glacial acetic acid + formaldehyde + water, 10:1:2:7), dehydrated in a tertiary-butyl-alcohol series, embedded in paraffin wax, sectioned at 10 µm thickness and stained with 0.5 % safranin-O and 0.1 % fast green (Jensen 1962).

Leaf, root and stem explants were cultured on MS medium supplemented with 2, 4, 6 and 8 mg dm^{-3} 2,4-D or dicamba to induce callus formation. All the explants became necrotic and no callus was formed on basal

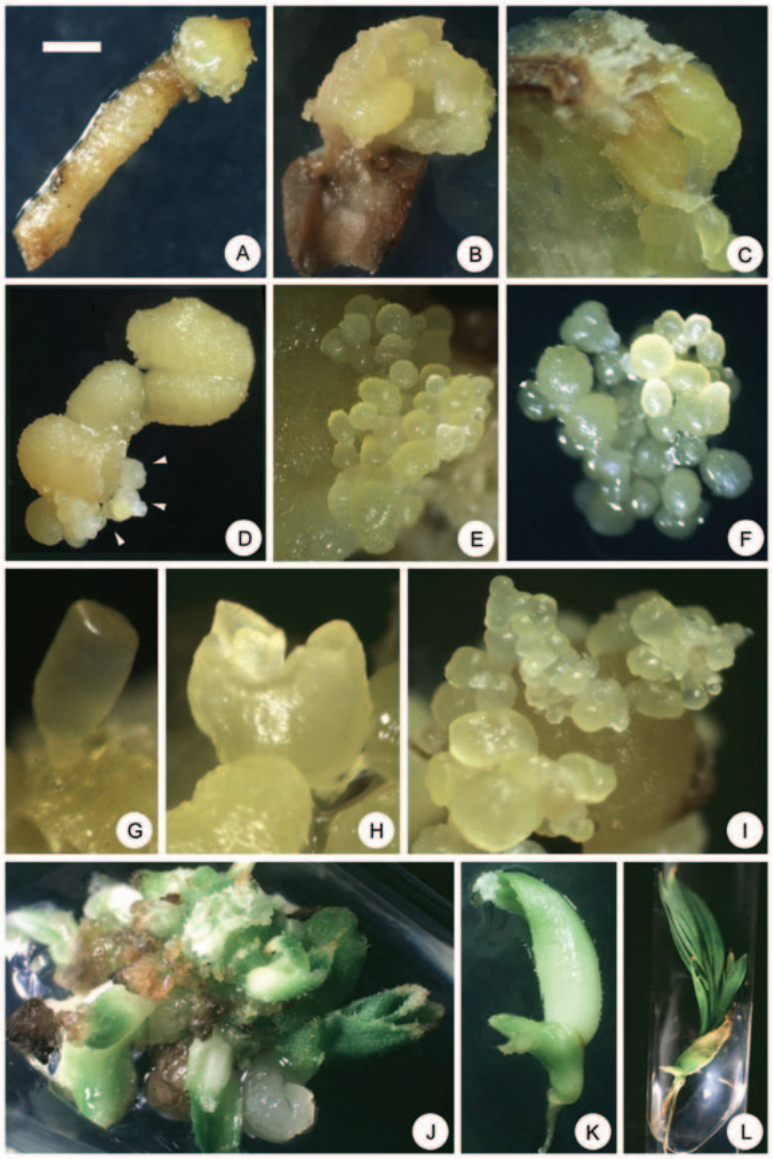

Fig. 1. Callus induction, somatic embryogenesis and plant regeneration of *Areca catechu* L. (*bar* in upper left refers to all panels).: *A* - callus formed from the root explant (*bar* = 0.2 cm); *B* - callus formed from the stem explant (*bar* = 700 µm); *C* - callus formed from wound regions of the leaf explant (*bar* = 200 µm); *D* - yellow-white nodular calluses formed during subculture (*bar* = 0.3 cm); *E* - somatic embryos formed from nodular callus (*bar* 300 = µm); *F* - callus-derived embryos enlarged and in globular shape (*bar* = 400 µm); *G* - callus-derived embryo developed (*bar* = 500 µm); *H* - a mature embryo (*bar* = 800 µm); *I* - secondary embryos formed from the surface of embryos (*bar* = 200 µm); *J* - embryos formed shoots (*bar* = 0.3 cm); *K* - a plantlet that converted from the callus-derived embryo (*bar* =0.6 cm); *L* - a regenerated plantlet that ready to be transplanted (*bar* = 1 cm).

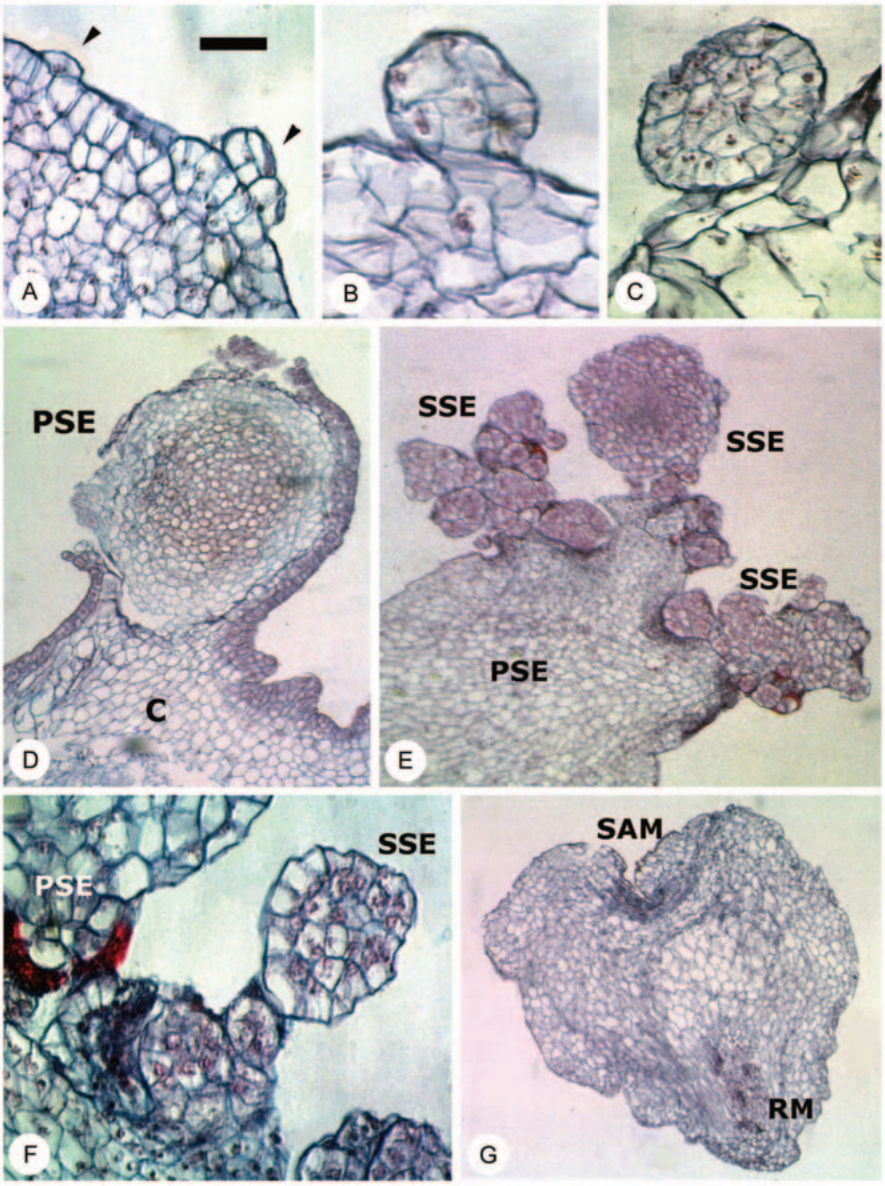

Fig. 2. Histological observation on somatic embryogenesis from callus cultures of *Areca catechu* L. (*bar* in upper left refers to all panels).: *A* - embryogenic cells (*arrows*) formed from outer cell layers of callus (*bar* = 250 µm); *B* - a somatic proembryo (*bar* = 100 µm); *C* - a somatic embryo in globular stage (*bar* = 100 µm); *D* - an enlarged primary somatic embryo (PSE) formed from callus (C) (*bar* = 450 µm); *E* - clusters of secondary somatic embryos (SSE) formed from primary somatic embryos (PSE) (*bar* = 450 µm); *F* - a secondary somatic embryos (SSE) in globular stage (*bar* = 250 µm); *G* - a mature somatic embryo shows shoot apical meristem (SAM) and root meristem (RM) (*bar* = 450 µm).

medium devoid of plant growth regulators. In leaf explants, initially, the callus showed yellow, soft, glutinous structures, and then pale-yellow, compact, granular callus developed (Fig. 1*A*, Table 1). After 60 d in culture, 2 - 8 mg dm^{-3} 2,4-D and 2 - 8 mg dm^{-3} dicamba induced 13 - 26 % and 53 - 100 % of explants to form callus, respectively. At 4 mg dm^{-3} dicamba, small amount of somatic embryos spontaneously formed from the callus. In root explants, either on 2,4-D- or dicamba-containing media, only yellowish callus was obtained (Fig. 1*B*, Table 1). The percentages of callus-forming root explants were 66 - 100 % and 86 - 100 % at 2 - 8 mg dm^{-3} 2,4-D and dicamba, respectively. At 2 and 4 mg dm^{-3} dicamba, somatic embryos formed from callus during the first period of culture. In stem explants, yellowish callus was obtained only at 4 mg dm^{-3} dicamba. Other treatments showed necrotic of explants or developed brown callus. In palm tissue culture, very high levels of 2,4-D (50 - 100 mg dm^{-3}) were usually used to induce callus proliferation (Gabr and Tisserat 1985, Bhaskaran and Smith 1992, Dias *et al.* 1994, Guerra and Handro 1998, Fernando and Gamage 2000). However, working with *Areca catechu*, low concentrations of 2,4-D were effective in callus induction from vegetative tissues and

subsequent callus proliferation (Table 1). Moreover, in our previous paper, somatic embryos formed from zygotic embryo-derived callus after 2 - 3 passages of subculture (at an interval of 8 wks) on 2 - 8 mg dm^{-3} dicamba-containing medium (Wang *et al*. 2002). However, in this report, it was found that leaf and root-derived callus could form embryos on 2 - 4 mg dm^{-3} dicamba-containing medium within 60 d without subculture.

Table 1. Effects of 2,4-D and dicamba on callus formation and proliferation from leaf, root and stem explants of *Areca catechu* L. Data were scored after 60 d of culture (6 replicates were used in each treatment).

Growth regulators	[mg dm^{-3}]	Callus formation [%] leaf	root	stem	Proliferation rate
Control		0	0	0	1.45
2,4-D	2	80	100	13	3.98
	4	100	100	66	3.45
	6	93	93	6	3.36
	8	53	86	0	2.68
Dicamba	2	20	66	53	3.93
	4	26	100	6	3.30
	6	13	86	13	3.23
	8	20	93	0	3.15

With a regular subculture every 8 weeks on 2,4-D or dicamba-containing media, the primary calli proliferated into palm-yellow, compact and nodular structures (Fig. l*C*,*D*). Except for the control treatment and 6 - 8 mg dm^{-3} dicamba, the nodular calluses formed somatic embryos spontaneously during subculture (Fig. l*E*, Table 1). At 2 - 8 mg dm^{-3} 2,4-D, the range of callus proliferation rate was between 2.68 to 3.98. At 2 - 8 mg dm^{-3} dicamba, the range was 3.16 - 3.93. The numbers of embryos could be further increased many fold by subdivision of calli.

When somatic embryos derived from calli were transferred onto hormone-free medium and kept under a 16-h photoperiod, embryos continued germinating and further developing shoots and roots (Fig. 1*F*). The embryos could be converted into normal plantlets after 10 weeks of culture. After three passages of subculture (at an interval of one months), the plantlets were transplanted in containers in shaded greenhouse and most grown well.

The embryogenic cells were generally originated from outer cell layers of callus (Fig. 2*A*). As the differentiation progressed, the proembryo (Fig. 2*B*) and then the globular embryo (Fig. 2*C*) started to initiate from the callus. The callus-derived primary somatic embryo (PSE) enlarged and had small densely stained embryonic cells (Fig. 2*D*). Clusters of secondary somatic embryos (SSE) with small densely stained cells were originated from PSEs (Fig. 2*E*,*F*). The embryo kept enlarging and further developing into the mature embryo with shoot apical meristem (SAM) and root meristem (RM) (Fig. 2*C*).

A protocol for *Areca catechu* L. regeneration was established in this paper. Plantlets were obtained through somatic embryogenesis using root, stem and leaf as explants. Further investigations were needed to modify this protocol for mass propagation and gene transformation of this species.

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