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PLANT REGENERATION THROUGH SOMATIC EMBRYOGENESIS FROM ZYGOTIC EMBRYO-DERIVED CALLUS OF *ARECA CATECHU* L. (ARECACEAE)

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Summary

An *in vitro* culture procedure was established for somatic embryogenesis and plant regeneration from callus cultures of the important palm 'betel nut' (*Areca catechu* L.). Segments of zygotic embryos of *Areca catechu* L. were cultured on Murashige and Skoog basal medium supplemented with dicamba (9.05, 18.1, 27.15, and 36.2 μ*M*). After 7–8 wk in darkness, wounded regions of explants formed callus with yellow, soft, glutinous structures. Proliferation and maintenance of callus was on the same dicamba-containing medium. With regular subculture every 8 wk, the callus showed pale yellow, compact and nodular structures. During subculture, somatic embryos were formed spontaneously from nodular callus tissues within 2–4 mo. The embryos developed into plantlets after 10 wk of culture on basal medium free of plant growth regulators. After subculturing every month for 3 mo., the plantlets were transferred to containers for acclimatization in the greenhouse. The survival rate was 24%.

Key words: betel nut; palm; somatic embryo.

Introduction

Areca catechu L. (Arecaceae), betel nut, is one of the economically important palms. The fresh fruit of this species is the major part of a natural masticatory in south-eastern Asia, called 'betel quid' (Bhonsle et al., 1992). The general propagation of this species is through seed. However, the seeds are short-lived, and the progeny from seeds are not uniform. Conventional vegetative propagation methods such as cutting, grafting, or layering are not possible for *Areca* species. With an increasing demand for fruit production of *Areca* palms, there is a need to develop approaches for efficient propagation of this species. In our previous paper, a protocol for vegetatively propagating *Areca catechu* L. through *in vitro* shoot bud regeneration was described (Wang et al., 2002). In this report, we established a tissue culture protocol to regenerate this species through somatic embryogenesis in defined conditions.

Materials and Methods

Mature fruits of *Areca catechu* L. were collected from a local farm in Taipei, Taiwan, ROC. Zygotic embryos of these fruits were collected (Fig. 1*a*), immersed in 70% alcohol for 1 min, followed by surface sterilization by agitation for 10 min in a solution of 2% sodium hypochlorite and 0.05% Tween (1:1 v/v). Segments of zygotic embryos were used as explants and were placed on the surface of MS (Murashige and Skoog, 1962) basal medium containing full-strength macro- and micro-elements of MS salts supplemented with (mg l^{-1}): *myo*-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), peptone (1000), NaH_2PO_4 (170), sucrose (30 000), and Gelrite (Sigma Chemical Co., St. Louis, MO) (2200). Plant growth regulators were added prior to autoclaving as optional additives according to the experimental objectives. The pH of the media was adjusted to 5.7 with 1 *N* KOH or HCl prior to autoclaving for 15 min at 121°C. Explants were incubated in 20 × 150 mm culture tubes in darkness for callus induction. Embryo induction and plantlet formation were performed under a 16:8 h photoperiod at 28–36 μmol m^{-2} s^{-1} (daylight fluorescent tubes FL-30D/29, 40 W, China Electric Co., Taipei) and 26 ± 1°C. Six replicates (tubes) each with four explants were used for each treatment. The percentage of explants forming callus was determined for each trial. Cultures were examined and photographed with a stereozoom microscope (SZH, Olympus). Differences between means were scored with Duncan's multiple range test (Duncan, 1955).

Results and Discussion

Callus induction. In palm tissue culture, very high levels of 2,4-dichlorophenoxyacetic acid (2,4-D; 226.2–452.5 μ*M*) are 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, in our preliminary experiment, explants of *Areca catechu* L. tended to be necrotic even in the presence of low concentrations (4.52–22.62 μ*M*) of 2,4-D. Instead, low dosages (9.05, 18.1, 27.15, and 36.2 μ*M*) of dicamba were found to be effective in callus induction from segments of zygotic embryos of this species (Table 1). In the control, on hormone-free medium, the explants became necrotic and no callus was formed after 2 mo. of culture. By contrast, on dicamba-containing media, the explants formed calluses with yellowish, soft and glutinous structures from the wound regions (Fig. 1*b*). The frequency of callus formation ranged from 62.5 to 91.6% on dicamba-containing media (Table 1). In oil palm, activated charcoal was added to the culture media to prevent browning, but the auxin requirement was raised 50-fold (Teixeira et al., 1993). In *Phoenix canariensis*, the addition of activated charcoal completely inhibited the induction of embryogenic callus, even when the concentration of

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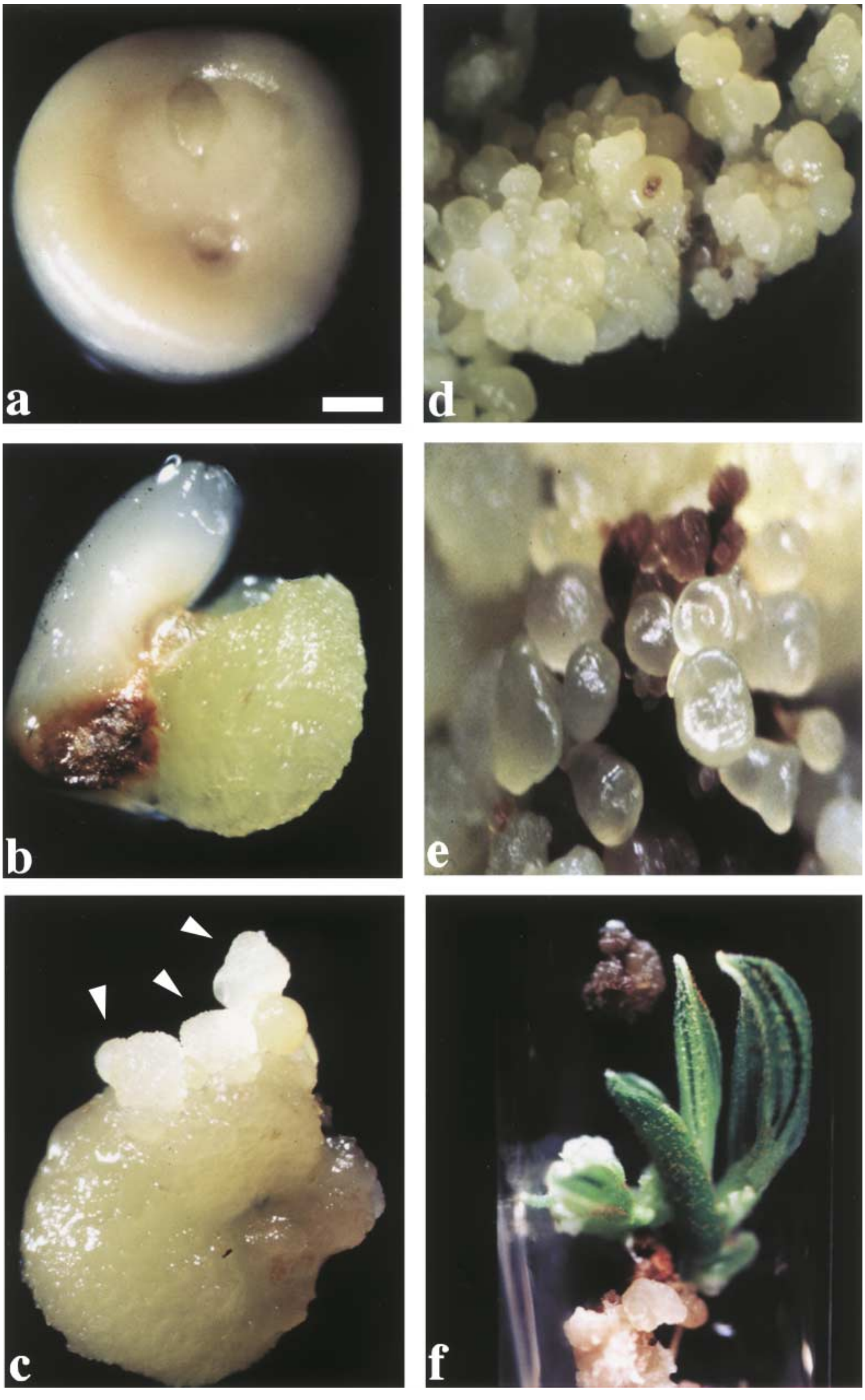

FIG. 1. Plant regeneration through somatic embryogenesis from segments of zygotic embryos of *Areca catechu* L. *a*, A zygotic embryo (*bar* = 0.1 mm). *b*, Yellowish callus formed from wounded regions of the zygotic embryo (*bar* = 0.1 mm). *c*, Compact nodular masses formed from the primary callus during subculture (*bar* = 0.2 mm). *d*, After two or three subcultures, all of the calluses showed nodular structures (*bar* = 0.2 mm). *e*, Somatic embryos formed from subcultured callus (*bar* = 0.09 mm). *f*, Rooted plantlets developed from somatic embryos (*bar* = 0.3 mm). (The same *bar* is suitable for all figures.)

TABLE 1

EFFECTS OF DICAMBA ON CALLUS FORMATION FROM SHOOT TIP EXPLANTS OF *ARECA CATECHU* L.

Dicamba (μM)	Callusing frequency (%)
0	0 c
9.05	75.0 ab
18.1	91.6 a
27.15	66.6 ab
36.2	62.5 b

The percentage of explants forming callus was scored after 60 d of culture.
Means of six replicates (tubes, each containing four explants) with the same *letters* are not significantly different at $P < 0.05$ (Duncan, 1955).

auxin was raised (Huong et al., 1999). Working with *Areca catechu* L. on dicamba-containing medium, browning was not a problem during callus induction and subculture, even without the addition of activated charcoal.

Embryo formation. In *Genoma gamiova* (Arecaceae), embryogenic cell masses were obtained from zygotic embryos with 452.5 μM 2,4-D, and somatic embryogenesis was achieved after transferring onto medium supplemented with a lower level of 2,4-D (90.5 μM) (Dias et al., 1994). In Canary Island date palm, somatic embryos were obtained from callus when transferred to media with reduced auxins (Huong et al., 1999). In coconut (*Cocos nucifera* L.), somatic embryogenesis is generally induced by gradual reduction of auxin concentration in the culture medium and the incorporation of cytokinins (Fernando and Gamage, 2000). Working with *Areca catechu* L., callus induction and subsequent embryo formation were easily achieved on a simple dicamba-containing medium without any change in the concentration. With regular subculture every 8 wk on dicamba-containing media, almost all the primary calluses formed pale yellow, compact and nodular structures (Fig. 1*c*). These nodular calluses proliferated more with further subculture. After two or three subcultures, all the calluses showed nodular structures (Fig. 1*d*). The nodular calluses of *Areca catechu* L. formed somatic embryos spontaneously during subculture on the same dicamba-containing media (Fig. 1*e*).

Plantlet conversion. Huong et al. (1999) reported that 2 μM abscisic acid (ABA) is required for somatic embryo development in *Phoenix canariensis* (Arecaceae). In bottle palm, although 6-benzyladenine (BA) stimulated 7% of embryos to show plumular development, the further conversion into plantlets failed (Sarasan et al., 2002). In this report, embryos of *Areca catechu* L. developed well and successfully converted into plantlets on medium without any plant growth regulators under a 16:8 h photoperiod. Under this condition, about 50% of embryos germinated and further developed into plantlets after 9–10 wk of culture (Fig. 1*f*). After subculturing every month for 3 mo., the plantlets were transferred to sandy soil in a container for acclimatization in a 30% shaded greenhouse. The survival rate was 24%.

In conclusion, a reliable protocol for *Areca catechu* L. regeneration was established on a simple defined medium. Regenerated plantlets were obtained through somatic embryogenesis by using zygotic embryos as explants. Further investigations are needed to modify this protocol for mass propagation and gene transformation of this species.

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