Morphogenetic routes of long-term embryogenic callus culture of *Areca catechu*

H.C. WANG*, J.T. CHEN** and W.C. CHANG*

*Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan 115, Republic of China**
*Institute of Biotechnology, National University of Kaohsiung, Kaohsiung, Taiwan 811, Republic of China***

Abstract

Early morphogenetic events and repetitive embryogenesis from callus culture of betel nut palm (*Areca catechu* L.) were studied using scanning electron microscopy. On Murashige and Skoog (MS) medium supplemented with 2 mg dm^{-3} dicamba, callus culture has capacity to form plantlets *via* somatic embryogenesis and to form secondary embryos for about 4 years. However, various abnormal embryos without differentiation of the leaf sheath and shoot apical meristem were observed, which showed bell-shaped and then cup-shaped or mushroom-shaped structures. These abnormal embryos contained distinctive structures, including a disk-shape interior region, surfaces with grooves and a stalk-like posterior region. During subculture, these abnormal embryos enlarged, became deformed and gradually lose their shape and then converted into nodular, compact embryogenic callus. It was also found that secondary embryos originated from interior surfaces or posterior regions of abnormal embryos, and gave rise to the next cycle of normal and abnormal embryos.

Additional key words: abnormal embryo, betel nut palm, dicamba, secondary embryogenesis, somatic embryogenesis.

Introduction

A major advantage of developing a somatic embryogenic system is the potential for cultures to undergo secondary embryogenesis, and to repetitively produce somatic embryos from developing somatic embryos or embryogenic callus (Baker and Wetzstein 1995). Embryogenic cultures of some plants can be subcultured for a prolonged period on medium containing plant growth regulators (PGRs) and still retain their full embryogenic potential. However, the occurrence of somaclonal variation increases with prolonged culture (Von Arnold *et al.* 2005).

In betel nut palm, the propagation practice is mostly through seeds. Previously we reported regeneration systems *via* shoot formation and somatic embryogenesis from callus cultures using shoot tips, zygotic embryos and vegetative tissues of *in vitro* germinated plants (Wang *et al.* 2002, 2003, 2006). However, complexity of regeneration pathways and some uncertain structures of long-term maintained callus may reduce the potential use of this system. Hence, this study aims to clarify morphogenetic routes of long-term subcultured callus of betel nut, and demonstrates the origin of secondary embryos as a basis for the further physiological studies.

Materials and methods

Mature fruits of betel nut palm *Areca catechu* L. (*Arecaceae*) 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 hypochlorite and 0.05 % *Tween* (1:1, v/v). These zygotic embryos were cultured for obtaining donor plants on Murashige and Skoog (1962; MS) basal medium 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). Callus induction followed the protocol established previously (Wang *et al.* 2006), and callus maintenance was performed on MS medium supplemented with 2 mg dm^{-3} 3,6-dichloro-2-methoxybenzoic acid (dicamba) with a

Received 26 May 2008, *accepted* 15 December 2008.
Abbreviations: dicamba - 3,6-dichloro-2-methoxybenzoic acid; MS - Murashige and Skoog (1962) medium.
** Corresponding author; fax: (+886) 7 591 9404, e-mail: jentsung@nuk.edu.tw

two-month interval of subculture. Dicamba was added into the 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 and at 26 ± 1 °C for callus induction and subsequent proliferation. Samples for scanning electron microscopy were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 4 h at 4 °C, dehydrated in ethanol (Dawns 1971) using critical point dryer (*HCP-2*, *Hitachi*, Tokyo, Japan), and coated with gold in an ion coater (*IB-2*, *Giko Engineering Co.*, Tokyo, Japan). A scanning electron microscope (*DSM-950*, *Carl Zeiss*, Jena, Germany) was used for examination and photography of the specimen.

Results and discussion

Normal development of somatic embryos from callus culture: During the first year under *in vitro* conditions most of callus-derived embryos underwent normal embryogenesis (Wang *et al*. 2002, 2006). The embryogenic cells were generally originated from outer cell layers of callus which gave rise to form globular proembryos (Wang *et al.* 2006). The globular proembryo was hemispherical in shape and subsequently shifted to bilateral symmetry due to unilateral expansion of one cotyledon (Fig. 1*A*). The swelling of dome region on one side of the embryo indicated the initiation of the shoot apical meristem (SAM) enclosed by the cotyledon (Fig. 1*B*). The embryo kept growing in both the axial and the lateral directions and the SAM was entirely covered by the first leaf primordium (Fig. 1*C*). A mature embryo contained the first plumular leaf with trichomes on the surface and the SAM inside (Fig. 1*D*). In *Cocos* (and probably in all other palms) the zygotic embryogenesis shows a number of primitive characters, such as differentiation of the embryo from one cell of the pluricellular proembryo, origin of the single cotyledon from a position lateral to the terminal stem tip, and a tendency to cleavage polyembryony (Mainz and Kerala 1979). In the present study, the development and the structure of somatic embryos of betel nut palm were mostly similar to those in zygotic embryogenesis of coconut, except for the polyembryonic tendency. Besides, it has been reported in coconut tissue culture that somatic embryos contained the haustorial tissue which was located at the distal end (Verdeil *et al.* 1994, Chan *et al.* 1998, Perera *et al.* 2007). However, similar structure was not observed in betel nut palm. At maturity, the cotyledons gradually diminished and were replaced by growing plumular leaves.

Abnormal development of somatic embryos observed in long-term callus culture: After several years of maintenance on dicamba-containing MS medium, a range of abnormal embryos as well as normally developing embryos were obtained from callus. Scanning electron microscopy revealed that these abnormal embryos had repeatable but unusual development when compared with normal embryos. At globular stage, there was no obvious difference in size and shape between normal and abnormal embryos. However, after attaining bilateral symmetry, abnormal embryos differentiated a ring of cotyledon structure emerging to encircle the apex rather than unilateral expansion of the cotyledon (Fig. 2*A*). The cotyledon structure subsequently enlarged without normal developing of SAM and the entire embryo became a bell-shaped structure (Fig. 2*B*). As a consequence, abnormal embryos failed to form normal leaf primordia. According to occurrence of various stages of embryos on one piece of callus, it was suggested that abnormal embryos emerged asynchronously (Fig. 2*C*). When differentiation progressed, the interior part of abnormal embryos showed a shallow disk-shaped structure with a flat central region instead of dome shape of normal apex (Fig. 2*D*). These abnormal embryos usually have smaller size than mature normal embryos with first plumular leaf (*cf*. Figs. 1*D*, 2*D*).

Another abnormality was found on the edge of interior embryo, which developed nodules with shallow or deep grooves (Fig. 3*A*). This type of abnormal embryos showed a cup-shape structure (Fig. 3*A*,*B*). In some cases,

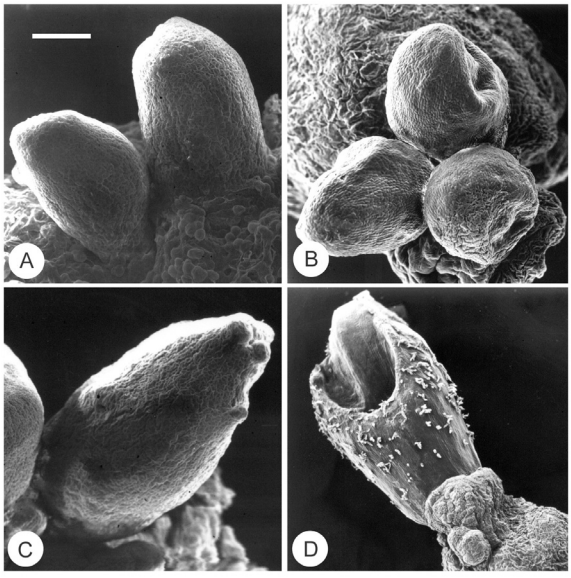

Fig. 1. Scanning electron microscopy of normal somatic embryogenesis from root-derived callus culture of *Areca catechu* L. (*bar* in upper left refers to all panels). *A* - somatic embryos attaining bilateral symmetry *(bar* = 0.1 mm). *B* - somatic embryos differentiate a cotyledon and the shoot apical meritems (*bar* = 0.2 mm). *C* - developing somatic embryo with the plumular leaf primordia inside (*bar* = 0.2 mm). *D* - mature embryos contains first extending plumular leaf with trichomes on the surface (*bar* = 0.4 mm).

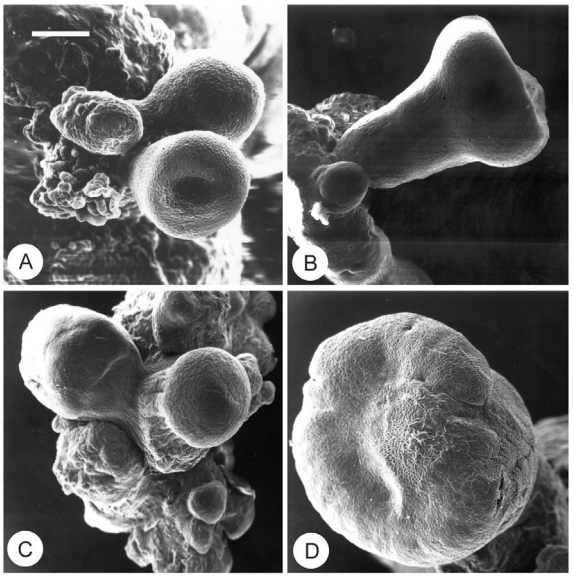

Fig. 2. Scanning electron microscopy of representative abnormal embryos obtained from long-term maintained callus culture of *Areca catechu* (*bar* in upper left refers to all panels). *A* - the abnormal embryo attaining bilateral symmetry but differentiated a ring of cotyledon structure encircling the apex (*bar* = 0.2 mm). *B* - a side view of an abnormal embryo developed into a bell-shaped structure (*bar* = 0.25 mm). *C* - abnormal embryos failed to develop the shoot apical meristems (*bar* = 0.5 mm). *D* - an abnormal embryo developed a flat interior region rather than a cotyledon and the shoot apical meristem (*bar* = 0.2 mm).

expanding cotyledon structure caused curling borders (Fig. 3*B*). It was found that grooves developed on both sides of cotyledon (Fig. 3*A*,*B*). These abnormal embryos usually have larger size when compared with normal embryos (Fig. 3*A*,*B*). Occasionally, some more complicated and larger structures (about 1 - 1.5 cm in diameter) than above-mentioned were obtained from callus (Fig. 3*C*,*D*). At maturity, these embryos differentiated nodular cotyledon structures with grooves and numerous trichomes on the circumambiency (Fig. 3*C*) or with flexuous grooves and trichomes on the front surface (Fig. 3*D*). In some cases, abnormal embryos with less grooves were found which showed uneven cotyledon structures (Fig. 4*A*) or developed highly extended cotyledon structures with stalks of posterior parts to make them a mushroom-shape (Fig. 4*B*). Perera *et al.* (2007) reported that some abnormal shoots were obtained from ovary-derived callus. In addition, morphological somaclonal variants were obtained in tissue culture of oil palm (Jaligot *et al.* 2000) and bottle palm (Sarasan *et al.* 2002). However, there is no detailed documentation on the abnormality of somatic embryogenesis, the age of callus or the morphogenetic route of abnormal embryos.

The origin of secondary embryos: Secondary embryos are formed from other somatic embryos in culture. In date palm or oil palm tissue culture, it has been observed that secondary embryogenesis occurs from callus-derived embryos (Rajesh *et al.* 2003, Konan *et al.* 2006). However, little is known about the morphorgenetic route and detailed structures of secondary embryogenesis. In this present study, we clarified the origins of secondary embryos in order to establish a model system for further morphological studies and improvement of vegetative propagation of betel nut palm.

Secondary globular proembryos and multicellular proembryogenic masses of different size and age formed on the posterior region of the abnormal embryo (Fig. 4*A*). It was found that secondary embryogenesis in this case is an asynchronous process. Along with the growing of the primary embryo, more and more secondary embryos appeared (Fig. 4*B*). Other secondary embryos took places on the interior surface of cotyledon structures of abnormal embryos, including the mushroom-shaped structure (Fig. 4*B*), the non-grooved structure (Fig. 4*C*) and the grooved structure (Fig. 4*D*). It was found that repetitive embryogenesis was asynchronous process.

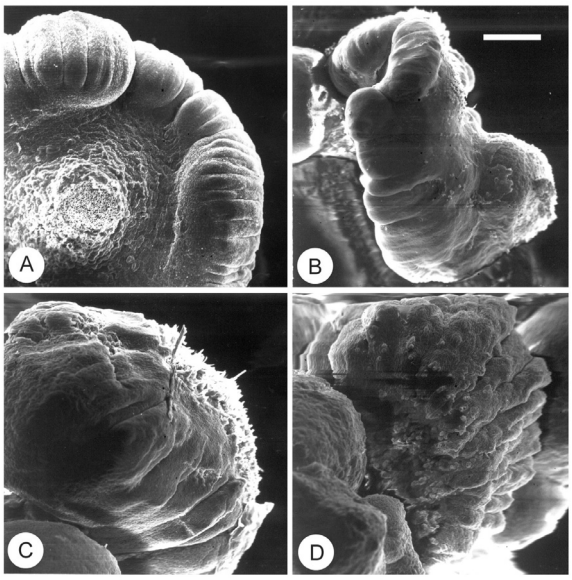

Fig. 3. Scanning electron microscopy of mature abnormal embryos obtained from long-term maintained callus culture of *Areca catechu* (*bar* in upper right refers to all panels). *A* - the cotyledon structure expanded and the edge developed into nodules with grooves in the front side of an abnormal embryo which blocked the attainment of shoot apical meristem (*bar* = 0.3 mm). *B* - a side view of an abnormal embryos developed into cup-shaped structure with grooves on both sides of surfaces (*bar* = 0.5 mm). *C* - abnormal embryos differentiated the nodular cotyledon structure with grooves and trichomes on the edge (*bar* = 0.2 mm). *D* - abnormal embryos differentiated the nodular cotyledon structure with flexuous grooves and trichomes on the surface of front side (*bar* = 0.3 mm).

Overall view on embryo development: During the first year of culture, somatic embryos were found to arise directly from peripheral cell layers of callus nodules. They were separated to each other and loosely contacted with the parent callus (Wang *et al.* 2006). However, more and more multiple-state embryos which fused with each other at their basal parts as well as single-state embryos formed

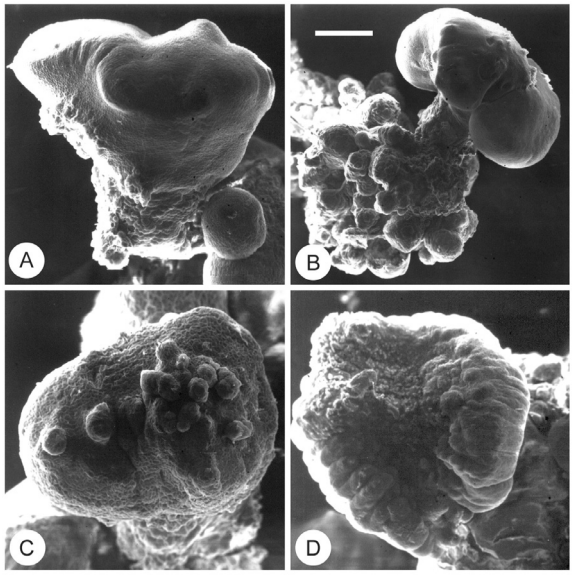

Fig. 4. Scanning electron microscopy of mature abnormal embryos obtained from long-term maintained callus culture of *Areca catechu* (*bar* in upper right refers to all panels). *A* - an expanding abnormal embryo with less grooves on the interior surface but developed a roughen posterior surface with secondary embryos (*bar* = 0.2 mm).*B* - an expanding abnormal embryo became a representative mushroom-shaped structure with secondary embryos (*bar* = 0.4 mm). *C* - secondary embryos formed on the interior surface of an abnormal embryos (*bar* = 0.2 mm). *D* - a top view of an expanding abnormal embryos with a roughen interior surface with grooves (*bar* = 0.35 mm).

closely from the same clump of callus (Fig. 5*A*). In some cases, embryos fused together and/or were tightly connected with the parent callus (Fig. 5*C*,*D*). These multiple embryos mostly showed abnormal structures especially the non-grooved type (Fig. 5*C*). However, it was found that a small number of abnormal embryos with shallow grooves connected with the parent callus tightly (Fig. 5*D*). In our previous paper, secondary embryos formed directly from posterior epidermal cells of normal embryos asynchronously (Wang *et al.* 2006). As a consequence, most of these secondary embryos belong to the single-state type. During long-term maintenance of callus culture, secondary embryogenesis could be obtained from the posterior region of abnormal embryos (Fig. 5*B*) as well as from normal embryos. In general, a similar type of embryos arose closely on a clump of callus and developed an aggregation (Fig. 5*C*,*F*). However, occasionally, normal and abnormal embryos were found on the same clump of callus (Fig. 5*E*).

Callus proliferation: When embryos kept growing, the parent callus continued to reduce the size correspondingly (Fig. 5*F*). However, callus could be formed from nodular masses of the expanding abnormal embryos during long-term subculture on dicamba-containing medium. The proliferation rate could be enhanced by subdivision of nodular masses derived from deformed abnormal embryos. It was found that fused non-grooved abnormal embryos (Fig. 5*C*) were the main source of newly formed callus. After 4 years, the callus consisted from the parent callus, newly formed callus, normal embryos, abnormal embryos and some deformed structures. The importance of dicamba on the induction of embryogenic potential and the improvement of regeneration ability was demonstrated in some plants (Immonen 1996, Plazek *et al.* 1999, Osuna and Barrow 2004, Steinmacher *et al.* 2007), but little is known about the effect on morphological abnormality of somatic embryos. According to our results, dicamba could induce abnormal embryogenesis from callus culture of betel nut palm during long-term culture.

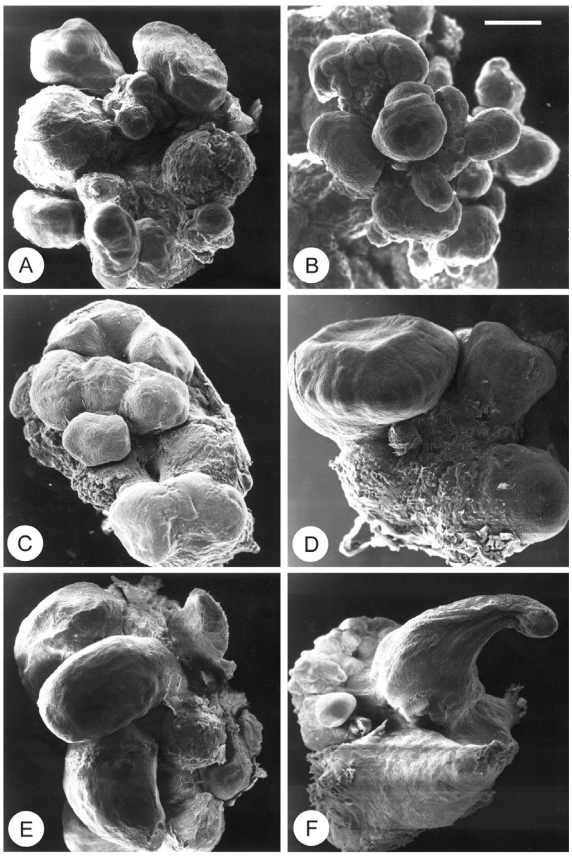

Fig. 5. Scanning electron microscopy of somatic embryogenesis from long-term maintained callus culture of *Areca catechu* (*bar* in upper right refers to all panels). *A* - a cluster of various stages of embryos formed from callus (*bar* = 0.65 mm). *B* - various stages of secondary embryos (*bar* = 0.35 mm). *C* - fused abnormal embryos (*bar* = 0.45 mm). *D* - abnormal embryos from a piece of callus (*bar* = 0.35 mm). *E* - one abnormal embryos and two normal embryos were coexistent (*bar* = 0.65 mm). *F* - normal mature embryo with a secondary globular embryo (*bar* = 0.4 mm).

Conclusion: The present study elucidated morphogenetic events that occur during long-term maintenance of callus culture of betel nut palm. It may provide a model system for studying factors and mechanism affecting callus proliferation, embryogenetic capacity and embryo development.

References

Baker, C.M., Wetzstein, H.: Repetitive somatic embryogenesis in peanut cotyledon cultures. - Plant Cell Tissue Organ Cult. **40**: 249-254, 1995.

Chan, J.L., Saenz, L., Talavera, C., Hornung, R., Robert, M., Oropeza, C.: Regeneration of coconut (*Cocos nucifera* L.) from plumule explants through somatic embryogenesis. - Plant Cell Rep. **17**: 515-521, 1998.

Dawns, C.J.: Biological Techniques in Electron Microscopy. - Barnes and Noble, New York 1971.

Jaligot, E., Rival, A., Beulé, T., Dussert, S., Verdeil, J.L.: Somaclonal variation in oil palm (*Elaeis guineensis* Jacq.): the DNA methylation hypothesis. - Plant Cell Rep. **19**: 684-690, 2000.

Konan, E.E., Durand-Gasselin, T., Kouadio, J.Y., Flori, A., Rival, A.: A modeling approach of the *in vitro* conversion of oil palm (*Elaeis guineensis*) somatic embryos. - Plant Cell Tissue Organ Cult. **84**: 99-112, 2006.

Immonen, A.S.T.: Influence of media and growth regulators on somatic embryogenesis and plant regeneration for production of primary triticales. - Plant Cell Tissue Organ Cult. **44**: 45-52, 1996.

Mainz, B.H., Kerala, V.J.P.: Embryo development in *Cocos nucifera* L.: a critical contribution to a general understanding of palm embryogenesis. - Plant System. Evol. **132**: 91-106, 1979.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 495-497, 1962.

Osuna, P., Barrow, J.R.: Regeneration of black grama (*Bouteloua eriopoda* Torr.) plants via somatic embryogenesis. - *In Vitro* Plant **40**: 299-302, 2004.

Perera, P.I.P., Hocher, V., Verdeil, J.L., Doulbeau, S., Yakandawala, D.M.D., Weerakoon, L.K. Unfertilized ovary: a novel explant for coconut (*Cocos nucifera* L.) somatic embryogenesis. - Plant Cell Rep. **26**: 21-28, 2007.

Plazek, A., Filek, M., Widzony, M.: Improvement of regeneration ability in *Phleum pratense* L. *in vitro* culture by dicamba. - Acta Physiol. Plant. **21**: 397-403, 1999.

Rajesh, M.K., Radha, E., Karun, A., Parthasarathy, V.A. Plant regeneration from embryo-derived callus of oil palm – the effect of exogenous polyamines. - Plant Cell Tissue Organ Cult. **75**: 41-47, 2003.

Sarasan, V., Ramsay, M.M., Roberts, A.V.: *In vitro* germination and induction of direct somatic embryogenesis in 'Bottle Palm' [*Hyophorbe lagenicaulis* (L. Bailey) H.E. Moore], a critically endangered Mauritian palm. - Plant Cell Rep. **20**: 1107-1111, 2002.

Steinmacher, D.A., Clement, C.R., Guerra, M.P.: Somatic embryogenesis from immature peach palm inflorescence explants: towards development of an efficient protocol. - Plant Cell Tissue Organ Cult. **43**: 124-132, 2007.

Verdeil, J.L., Huet, C., Grosdemange, F., Buffard-Morel, J.: Plant regeneration from cultured immature inflorescence of coconut (*Cocos nucifera* L.): evidence for somatic embryogenesis. - Plant Cell Rep. **13**: 218-221, 1994.

Von Arnold, S., Sabala, I., Bozhkov, I., Dyachok, J., Filonoval, L.: Developmental pathways of somatic embryogenesis. - Plant Cell Tissue Organ Cult. **69**: 233-249, 2005.

Wang, H.C., Chen, J.T., Chang, W.C.: Somatic embryogenesis and plant regeneration from leaf, root and stem-derived callus cultures of *Areca catechu*. - Biol. Plant. **50**: 279-282, 2006.

Wang, H.C., Chen, J.T., Wu, S.P., Lin, M.C., Chang, W.C.: Plant regeneration through somatic embryogenesis from zygotic embryo-derived callus of *Areca cathecu* L. (Arecaceae). - *In Vitro* Plant **39**: 34-36, 2002.

Wang, H.C., Chen, J.T., Wu, S.P., Lin, M.C., Chang, W.C.: Plant regeneration through shoot formation from callus of *Areca cathecu* L. - Plant Cell Tissue Organ Cult. **75**: 95-98, 2003.