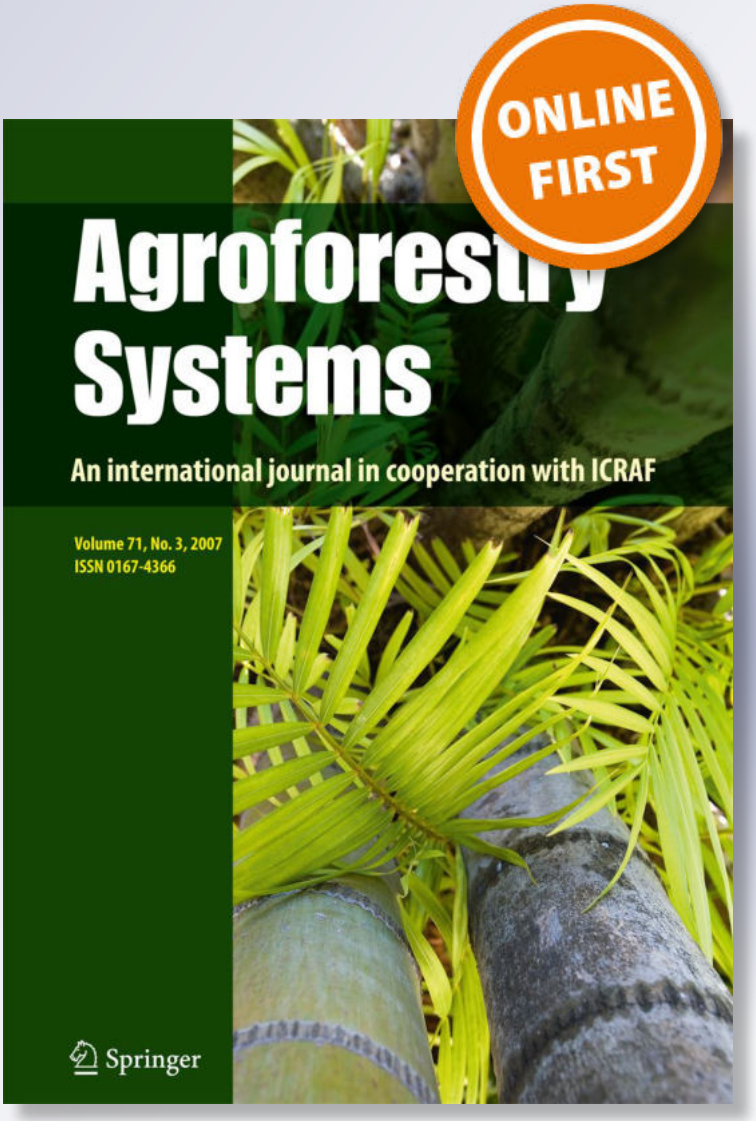
Embryogenesis followed by enhanced micro-multiplication and eco-restoration of Calamus thwaitesii *Becc.: an economic non-wood forest produce for strengthening agroforestry system*

Achuthan Sudarsanan Hemanthakumar, Thankappan Suvarna Preetha, Padmesh Pandaram Pillai & Sooriamuthu Seeni

Agroforestry Systems
An International Journal incorporating Agroforestry Forum

ISSN 0167-4366

Agroforest Syst
DOI 10.1007/s10457-018-0207-9



Springer

Agroforest Syst
https://doi.org/10.1007/s10457-018-0207-9

Embryogenesis followed by enhanced micro-multiplication and eco-restoration of *Calamus thwaitesii* Becc.: an economic non-wood forest produce for strengthening agroforestry system

Achuthan Sudarsanan Hemanthakumar · Thankappan Suvarna Preetha · Padmesh Pandaram Pillai · Sooriamuthu Seeni

Received: 24 January 2017 / Accepted: 5 February 2018

Abstract The present study is focussed on development of a high-frequency micro-multiplication system in *Calamus thwaitesii*, through somatic embryogenesis from immature zygotic embryos cultured in Murashige and Skoog (MS) medium supplemented with 31.68 μM 2,4-dichlorophenoxyacetic acid (2,4-D). The semi-friable calli when cultured in the same medium augmented with 2.22 μM 6-benzylaminopurine (BAP) and 1.07 μM α-naphthalene acetic acid (NAA) induced ~ 12 discrete globular embryoids in 6 weeks. The isolated embryoids in hormone-free media yielded 65% plantlets. Furthermore, embryoids and axenic shoots exhibited maximum shoot induction in medium supplemented with 0.45 μM Thidiazuron (TDZ). The shoot initials after subculture in media supplemented with 1.78 μM BAP and 0.45 μM TDZ produced shoot proliferation followed by elongation in basal medium. The elongated shoots produced roots in media supplemented with 16.11 μM NAA. With this established protocol, ~ 5940 rooted plantlets could be harvested after 40 weeks from a single embryoid. Genetic stability analysis of the plantlets using inter simple sequence repeat markers recorded only 0.05% genetic polymorphism. The plantlets were hardened in a mist house for 8 weeks, and then to 50% shade house for another 16 weeks, and the well-established 6-month-old nursery plants, reintroduced to selected forest segments, exhibited 86% field establishment even after 3 years of observation. Thus, the mass multiplication system developed could be a breakthrough for large-scale multiplication of *C. thwaitesii* to ensure continuous supply of quality planting material to the cottage industry through the development of agroforestry systems. Furthermore, the in vitro culture system developed here can be replicated for research activities related to the long-term–short-term conservation, micro-multiplication and sustainable utilization of rare, endangered, endemic, monopodial/single stemmed rattan palms.

Keywords *Calamus thwaitesii* · Rattan palm · Genetic variability · Embryoids · Eco-restoration

Abbreviations
BAP N_6-benzylaminopurine

A. S. Hemanthakumar
Biotechnology and Bioinformatics Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram 695 562, India

T. S. Preetha (✉)
Department of Botany, University College, Thiruvananthapuram, Kerala, India
e-mail: preethahemanth@yahoo.com

P. P. Pillai
Department of Genomic Science, Central University of Kerala, Kasaragod, Kerala, India

S. Seeni
School of Biosciences, Mar Athanasios College for Advanced Studies, Tiruvalla, Kerala, India

Published online: 08 February 2018

2,4-D	2,4-Dichlorophenoxyacetic acid
IBA	Indole-3-butyric acid
ISSR	Inter simple sequence repeat
MS	Murashige and Skoog
NAA	α-Naphthaleneacetic acid
PGR	Plant growth regulator
TDZ	Thidiazuron

Introduction

Rattans constitute unique and versatile group of spiny climbing palms or canes with solid stem but are fast depleting natural resource in South East Asian countries. It can be seen distributed in forests as well as in rattan gardens, as conserved species. Rattan gardens are an important traditional agroforestry system in Asian countries particularly India, China, Malaysia, etc. They are high-value non-wood forest produce primarily used for making furniture, basket and handicrafts items due to its remarkable aesthetic value. In addition, they are important raw material in cottage industry contributing significantly to the rural economy in the provinces where they occur. As a high-value fashion-proof commodity, it is much sought after for designer trade goods to grace the homes of urbanites the world over. Since the indigenous rattan resources are overexploited and their availability in nature is limited, the existing resources are insufficient to meet the vertical increase in demand. Predictably, the gap between the rate of production and full capacity utilization in the cane processing units has widened and thereby many of the urban units are already closed down owing to shortage of raw material. In this backdrop, micropropagation is considered the only alternative for the large-scale production and supply of quality planting materials. Besides, rattan cultivation areas are abandoned after shifting cultivation, so this system could also be considered as a possible way of rehabilitating unproductive secondary forest and could help stabilize shifting cultivation areas to meet the demand. In the present study, we demonstrate an efficient two-in-one system through somatic embryogenesis followed by direct shoot multiplication from embryoids and axenic shoot cultures for large-scale production and utilization of this economic rattan palm *Calamus thwaitesii*. The plantlets thus multiplied were reintroduced in native forest segments as part of the development of rattan garden. This novel agroforestry system using somatic embryogenesis is not reported earlier in rattan palms, though conventional multiplication through seeds is known (Aminuddin et al. 1992).

Materials and methods

Explant types

Zygotic embryos dissected out of immature green fruits (2.16 × 1.74 cm), collected in late November from randomly selected rattan palm *C. thwaitesii* growing in the forests of Kollam district of Kerala, India, were used as explants for the induction of somatic embryoids and subsequent production of plantlets through direct shoot multiplication from embryoids and axenic shoots.

Surface sterilization

Immature green fruits were separated from the rachis, washed in running tap water for 30 min and treated with 0.8% (v/v) Teepol (BDH India Ltd., Bombay) under constant stirring for 10 min. They were again washed thoroughly in running tap water followed by rinsing in distilled water. Surface decontamination of the fruits was done by immersion in 70% ethanol and flaming over a spirit lamp inside the laminar air flow hood. After flaming, the embryos were scooped out of the fruits using a sterile surgical blade and the excised embryos were washed in sterile distilled water to remove remnants of mesocarpic fibres. Finally, the undamaged embryos (1.3 × 0.3 mm) were transferred to full strength Murashige and Skoog media (1962) containing appropriate hormonal regimes. All the cultures were incubated in a culture room maintained at 25 ± 2 °C under 12-h photoperiod and illumination at 50–60 μE m^{-2} s^{-1} provided by cool white fluorescent tubes (Philips India Ltd., Mumbai). Observations on induction of embryogenic callus, embryoid formation and multiple shoot initiation were periodically recorded at 4- to 6-week interval.

Influence of activated charcoal and sugar on embryo development

MS medium supplemented with different concentrations of activated charcoal (0.05–0.3%) and sucrose (1–6%) was tested separately for the various stages of growth and development of embryos.

Induction of somatic embryoids from embryogenic callus culture

For embryogenic callus formation, the immature zygotic embryos were cultured at regular interval of 8 weeks in full strength MS medium supplemented with 4.52–54.30 μM 2,4-D. The white semi-friable calli obtained from the embryos, after 16 weeks of culture, were divided into ~ 500 mg pieces and subcultured to medium supplemented with varied combinations of BAP (0.89, 2.22, 4.44 μM) and NAA (0.54, 1.07, 2.69 μM) to induce the embryoids. After 8 weeks, the embryoids were separated and transferred, in basal medium for another 8–16 weeks for plantlet development. The calli with the remaining young embryoids were subcultured in basal medium through 2–3 cycles of 8 weeks each to facilitate maturation of the embryoids and from them plantlet formation. Embryoids liberated from the callus mass were subjected to free hand sectioning, stained with few drops of 2% aqueous toluidine blue and examined under Nikon SMZ 800 stereomicroscope to analyse the route of embryogenesis.

Induction of direct multiple shoots from somatic embryoids/axenic shoots

Eight-week-old somatic embryoids and 16-week-old embryoid-derived axenic shoots were separated and implanted into MS agar nutrient medium fortified with individual concentrations or combinations of plant growth regulators (PGRs), for multiple shoot induction.

Shoot multiplication, elongation and rooting

Shoot buds induced after 6 weeks from both embryoids and axenic shoots were transferred, either individually or as a group to MS medium supplemented with the combination of BAP (1.78 μM) and TDZ (0.23–0.91 μM) at 6-week interval for further shoot multiplication. Proliferative mass of shoots achieved through subculture passages in multiplication medium was transferred to hormone-free MS medium at regular interval of two passages each of 4 weeks to obtain elongated shoots. The underdeveloped shoots were again transferred to fresh medium devoid of hormones for further shoot elongation.

For in vitro rooting, shoots (4–8 cm long) having the same stage were separated individually or in clusters and transferred either to hormone-free MS media or media fortified with different concentrations of Indol 3-butyric acid (IBA) or NAA.

Hardening and establishment

Plantlets obtained through embryoid and axenic shoot cultures were weaned away from the flasks, washed repeatedly in running tap water to remove traces of agar and treated with 0.1% Dithane M-45 for 5 min in order to avoid microbial infection. They were then transferred to polybags (15 × 20 cm) filled with potting media of pure river sand and maintained in a mist chamber (28 ± 2 °C and 80 ± 5% RH) specially fabricated for rattan palms (M/s Indo-American Exports Ltd., Bangalore) for hardening. The post-transplantation behaviour of the plantlets was periodically monitored and data recorded at regular interval. After 2 months of hardening, the established plants were transferred to the nursery and reared under diffused light and regular watering for a period of 4 months before the plantlets reintroduced to selected forest segments of the Western Ghats.

Eco-restoration

The nursery established plants of 6 months old were transferred to evergreen/semi-evergreen forest segments of Southern Western Ghats region for field performance study followed by the development of agroforestry system. They were planted near the forest trees to provide shade and support during their growth and establishment. The undergrowths were first thinned out and pits of 30 cm^3 were made at 5-m spacing in the forest floor and were filled with forest soil and leaf mould mixture (1:1). The plants were carefully removed from the poly bags and transplanted into the pits retaining the soil around the roots. Planting was done during south-west monsoon rains. Thick canopy of trees provided shade during their

early establishment. As the forest region is infested with wild animals, the field establishment rate of reintroduced plants was monitored periodically by site visits and data on survival rate of the plants were recorded at regular intervals.

Experimental design and statistical analysis

Each in vitro culture experiment consisted of fifteen replicates, each with three explants per culture vessel and all the experiments were repeated thrice. Data on percentage response, somatic embryogenesis, multiple shoot induction, shoot multiplication rate during subculture passages, percentage of rooting response as well as the mean number of roots were statistically analysed by ANOVA and the means were compared by LSD multiple range test ($p \leq 0.05$) using the computer software SPSS/PC + version 10 (SPSS Inc. 1999).

Genetic variability analysis using inter simple sequence repeat (ISSR) markers

Genomic DNA isolation and ISSR

A total of 13 samples (12 micro-plantlets and 1 mother plant) were used for genetic fidelity analysis. Total genomic DNA from the leaf samples were isolated following Murray and Thompson (1980) method using cetyltrimethylammonium bromide (CTAB). After ethanol precipitation, DNA was resuspended in 100 µL of 1XTE buffer (pH 8.0). The DNA was quantified spectrophotometrically by taking the absorbance at 260 nm. ISSR assay was carried out in 25 µL reaction mixture containing 0.2 mM dNTPs, 10 mM Tris–HCL, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 1.0 U Taq DNA polymerase (Finzymes, Helsinki, Finland), 15 pmol primers (IDT, Coralville, USA) and 50 ng of genomic DNA. The amplification was performed in a thermal cycler (Eppendorf ESP-S) as follows: the initial cycle of 2 min at 93 °C, 2 min at 50–55 °C[1] and 2 min at 72 °C. A total of 35 cycles of 1 min at 93 °C, 1 min at 50–55 °C and 1 min at 72 °C were performed. The last cycle was performed by 10-min extension at 72 °C. Reaction mixture wherein template DNA replaced by distilled water was used as negative control. Amplified products were resolved in 1.40% agarose gel (1XTBE) followed by EtBr staining.

Genetic data analysis

Amplification with each arbitrary primer was repeated 3 times and those primers that produced reproducible and consistent bands were selected for data generation. Reproducible ISSR products were scored against the presence or absence of a fragment. Dice coefficient of similarity defined as $2a/2a + u$, where a is the number of positive matches and u the number of non-matches, was computed using the WINDIST software (Yap and Nelson 1996).

Results and discussion

Selection of explants

Under the climatic and other environmental conditions prevailing in the Western Ghats region, flowering of *C. thwaitesii* occurs during August to September of the year and mature fruits are formed within 8 months after flowering. However, for the isolation and culture of embryos, immature green fruits formed after two and half months of successful pollination and fertilization were preferred than the mature yellow fruits of the later periods. Nearly 80% of the embryos dissected out of the mature fruits were damaged to varied extent and were invariably intolerant to wounding leading to phenolic exudation. Isolation of embryos from immature fruits (2.16 × 1.74 cm) was easily achieved at 95% success rate. The size and the shape of the embryo might show marginal variations depending on the relative maturity of the fruits.

Surface sterilization and embryo germination

As much as 98% infection-free embryos were germinated by the surface sterilization procedure described here. The method followed is akin to embryo culture of few species of rattans (Barba et al. 1985; Hemanthakumar et al. 2013) and seed culture of orchids (Arditti and Ernst 1993). Different nutrient media have been used for embryo culture in palms viz. date palm (Tisserat 1983), oil palm (Corley et al. 1976) and coconut palm (Blake and Eeuwens 1982), while in

[1] Annealing temperature of the primers ranges from 50 to 55 °C for the different primers used in this study.

Rattan palms, full strength MS medium has already been used for the embryo culture of *C. yunnanensis* and *C. obvoideus*. (Chengji and Jiankui 1991). Since salt concentration of MS formulation is quite high, it may be assumed that embryos of rattans in culture prefer high concentrations of salts for their growth and differentiation. It was observed that the reduction of culture initiation period of embryo up to 2 weeks in MS basal medium and three subcultures of 6 weeks each in fresh medium having the same composition was required for the conversion of leafless seedling into fully grown seedlings with normal leaves.

Influence of activated charcoal

In the present study involving embryo cultures of *C. thwaitesii*, supplementation of the medium with 0.1% activated charcoal facilitated large haustoria formation followed by rapid germination and development into seedlings (Hemanthakumar 2010). The formation of haustoria resembled the developmental pattern in nature, but the difference being that they are short lived in vitro. Haustoria formation is also reported in embryo cultures of *Cocos nucifera* (Lopez-Villalobos et al. 2001) and *C. thwaitesii* (Ramanayake 1999). The germination of embryos observed here revealed that the use of activated charcoal at 0.1% (w/v) in the nutrient medium facilitates early germination of *Calamus* embryos presumably by adsorbing phenolic oxidates, low molecular weight inhibitors and breaking physiological dormancy. Activated charcoal is generally used in tissue culture to improve cell growth and development particularly seed germination, somatic embryogenesis, synthetic seed production, protoplast culture, rooting, stem elongation, bulb formation, etc. The effects of activated charcoal on morphogenesis may be mainly due to its irreversible adsorption of inhibitory compounds in the culture medium and substantially decreasing the toxic metabolites, phenolic exudation and accumulation of brown exudates. Furthermore, activated charcoal is involved in a number of stimulatory and inhibitory activities to promote growth, alteration and darkening of culture media, adsorption of vitamins, metal ions, plant growth regulators including abscisic acid and gaseous ethylene (Thomas 2008). Similar observations were also reported in *Fraxinus excelsior* (Meisam Mojarabi et al. 2011) and some other species of rattan palms (Hemanthakumar 2010). The role of activated charcoal in palm tissue culture particularly date palm (Reynolds and Murashige 1979), oil palm (Martin and Rabechault 1976) and coconut palm (Eeuwens 1976, 1978) has been variously interpreted.

Influence of sugar

Concentration of sucrose used in the medium influenced the developmental pattern of cultured embryos in later stages. While 3% sucrose was optimal for normal shoot development in the seedlings, 5% sucrose promoted maximum root formation (Hemanthakumar 2010). It appears that high concentrations of sucrose (5–6%) induced additional root formation and contributed to enhancement of root-related characters and functions though without affecting the shoot characters and function. The results suggest that root and shoot characters are tightly compartmentalized in seedlings of in vitro developing rattan palms. Profuse root differentiation in vitro is an energy-intensive process (Hazarika 2003) which is facilitated by the relatively high concentration of sucrose. The participation of oxidative metabolism in differentiating tissue cultures promoted by the presence of sucrose in the medium is corroborated by the findings of previous workers (Merillon et al. 1984; Wainwright and Scrace 1989).

Induction of somatic embryoids from embryogenic callus culture

Formation of semi-friable calli

In the present study, we found that zygotic embryos cultured in media supplemented with varied concentrations of 2,4-D (9.05–40.72 μM) induced proliferative mass of calli in 8 weeks, which contained compact and semi-friable portions, as well. Interestingly, the friability increased with increasing concentrations of the auxins and a maximum of 70% embryos produced white, semi-friable calli after 16 weeks in media supplemented with higher concentration (31.68 μM) of 2,4-D (Fig. 1a). However, 8–12% of the embryos cultured in the presence of this auxin did not respond to callus formation but changed their colour to yellowish brown or brown during the 16-week culture period. Nevertheless, further increase in the concentration of 2,4-D (45.25 μM) resulted in embryo loss (15–32%) due to browning and necrosis.

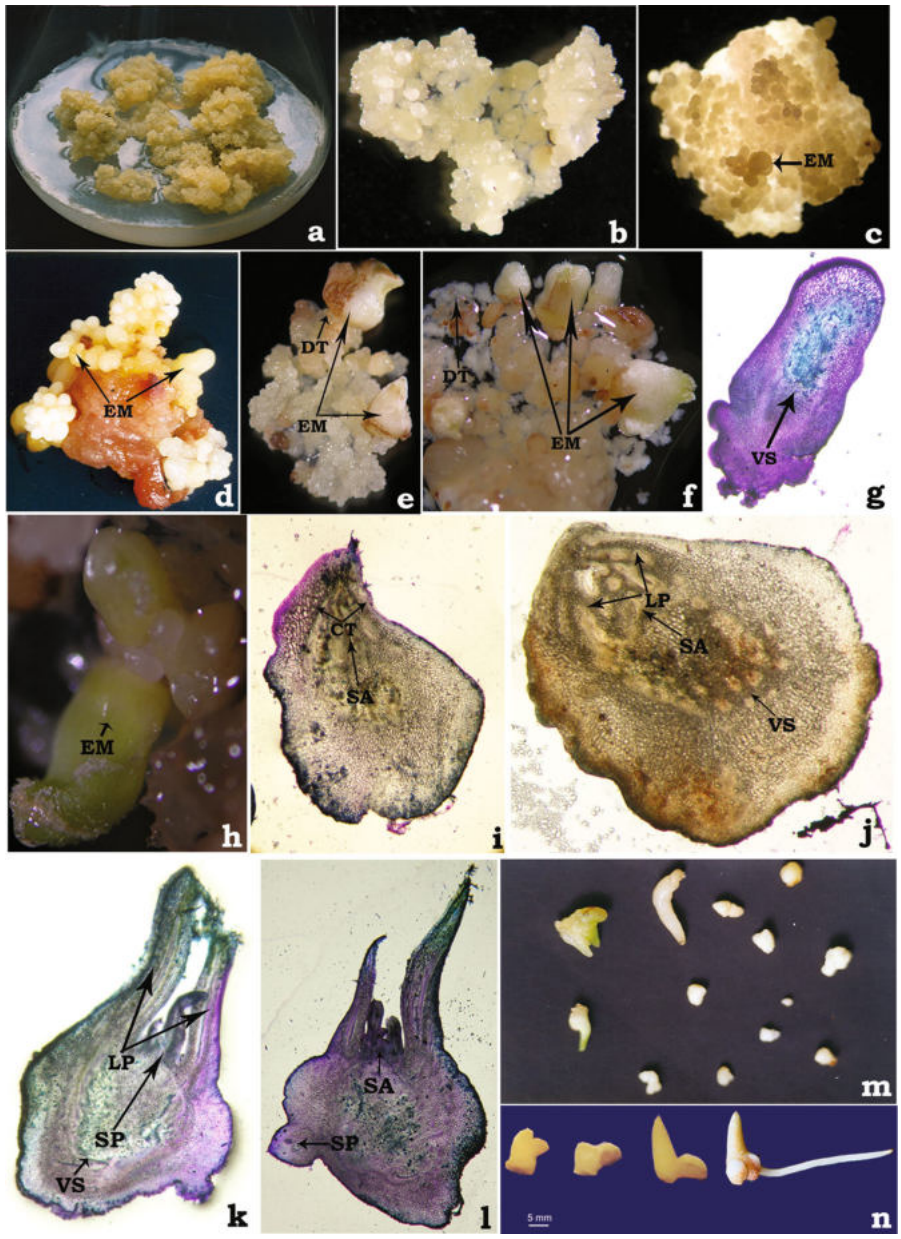


Fig. 1 Various stages of the development of somatic embryos (embryoids) from zygotic embryo calli. **a** Proliferative mass of semi-friable calli in media supplemented with 31.68 µM 2,4-D. **b** Semi-friable calli transformed to embryogenic calli after 4 weeks of culture in media supplemented with 2.22 µM BAP and 1.07 µM NAA. **c** Stereomicroscopic view of globular/spherical bodies of embryoids (EM) over embryogenic calli. **d** Formation of larger embryoid (EM) over the browned callogenic tissue. **e** Independent embryoids (EM) on degenerated callogenic matrices (DT). **f** Embryoids (EM) free from degenerated callogenic tissue (DT) under stereomicroscopic view. **g** LS of globular embryoid under stereomicroscope shows well-differentiated closed vascular system (VS) with shoot apical meristem. **h** Chlorophyll-accumulated lengthwise grown embryoid (EM). **i** LS of embryoids with meristematic dome surrounded by cotyledon (CT) in the shoot apex (SA) under stereomicroscope. **j** LS of embryoid under stereomicroscope shows well-differentiated vascular system (VS) along with leaf primordial (LP) and fully organized shoot apex (SA). **k** Highly differentiated independent closed vascular system (VS) along with leaf primordia (LP) and well-organized shoot apex (SA) observed on LS of toluidine blue-stained mature embryoid. **l** LS of toluidine blue-stained mature embryoid shows well-differentiated shoot apex (SA) and suspensor (SP). **m** Embryoids having different sizes isolated from callogenic tissue. **n** Early stages of development of embryoids

Similar observations were already reported in embryo and tissue cultures of some other rattan palms, where 2,4-D was the most frequently used auxin to produce calli and subsequent normal morphogenesis at lower concentration, within a period of 4–6 weeks (Yusoff 1989; Padmanabhan and Ilangovan 1993; Kundu and Sett 1999; Sett et al. 2002).

Development of the somatic embryos/embryoids

The semi-friable portion dissected out of the embryo callus and subcultured for 8 weeks in the medium containing 31.68 μM 2,4-D continued to proliferate into increasingly semi-friable callus. The calli thus obtained were transformed into embryogenic calli by the subsequent transfer of ~ 500 mg fresh weight to media fortified with 2.22 μM BAP and 1.07 μM NAA in 4 weeks (Fig. 1b) and induced 10–12 white coloured embryoids in 6–8 weeks which reached the first stage of embryoid development. Stereomicroscopic observation showed that certain globular/spherical bodies of embryoids over the embryogenic calli (Fig. 1c) which enlarged with gradual browning of callogenic tissue (Fig. 1d). In the second stage of embryoid development, the callogenic matrices have completely degenerated, and the embryoids became independent (Fig. 1e). After four weeks, stereomicroscopic observation showed that the embryoids were completely free from degenerated callogenic tissues (Fig. 1f). Besides, longitudinal section of globular embryoids showed polarized structure with well-differentiated closed vascular system and shoot apical meristem (Fig. 1g). In the third stage of embryoid development, they gradually grew lengthwise (10–12 mm) and started accumulating chlorophyll after the 6th week (Fig. 1h). Stereomicroscopic observation of the longitudinal section of different embryoids in this stage revealed that the shoot apex consisted of a meristematic dome surrounded by cotyledon (Fig. 1i) and gradually developed into a well-differentiated vascular system along with leaf primordium and a fully organized shoot apex (Fig. 1j). Moreover, the longitudinal section of toluidine blue-stained chlorophyllous mature embryoid displayed under stereomicroscope showed highly differentiated independent closed vascular system along with leaf primordium and well-organized shoot apex (Fig. 1k). Similarly these mature embryoids also showed well-differentiated shoot apex and suspensor in the last stage of embryoid formation (Fig. 1l).

Majority (65%) of 10–12 mm size mature embryoids (Fig. 1m) isolated from the callogenic mass were transferred to MS media, devoid of hormone, developed into well-differentiated shoot system and root system in 4–8 weeks (Fig. 1n) and to independent plantlets in 16 weeks similar to zygotic embryo germination of rattan palms (Hemanthakumar 2010). However, similar observations of somatic embryogenesis were also reported in other economic palms like date palm (Parisa Eshraghi et al. 2005; Sane et al. 2006), oil palm (Scherwinski-Pereira et al. 2010) and coconut palm (Sáenz et al. 2006). In fact, the demonstrated ability to produce plantlets from a single zygotic embryo of *C. thwaitesii* confirms the desirability of using embryogenic rather than indirect organogenetic route otherwise reported in general, and in *C. flagellum* (Kundu and Sett 1999) and *C. tenuis* (Sett et al. 2002) in particular.

Induction of multiple shoots from somatic embryoids

Among the cytokinins (BAP, 2-iP and Kn) tested for the shoot bud induction in embryoid cultures, 0.23–0.45 μM Thidiazuron (TDZ) was the best to induce maximum percentage and number of shoot bud formation (Fig. 2a). However, 0.45 μM TDZ was the optimal concentration for maximum multiple shoots initiation which produced 6.87 ± 0.23 shoots per embryoid through culture initiation phase (Table 1). Significant portion of the shoots (66–95%) becomes fasciated when concentration of TDZ exceeded 0.91 μM. In addition to individual concentrations of PGRs, combinations were also tried, among them 1.78 μM BAP and 0.45 μM TDZ was the best to obtain 5.17 ± 0.27 shoots per embryoid at 100% efficiency. Overall, the results suggest that 0.45 μM TDZ may be indispensable to obtain justifiable number of shoots from embryoid cultures. However, combination with other cytokinins was necessary to produce non-fasciated shoots. Unlike, many other palms, rattan embryos preferentially responded well in the presence of TDZ and this potential can be exploited for commercial purposes, and in *C. thwaitesii* embryoid cultures, use of TDZ is crucial for successful culture initiation.

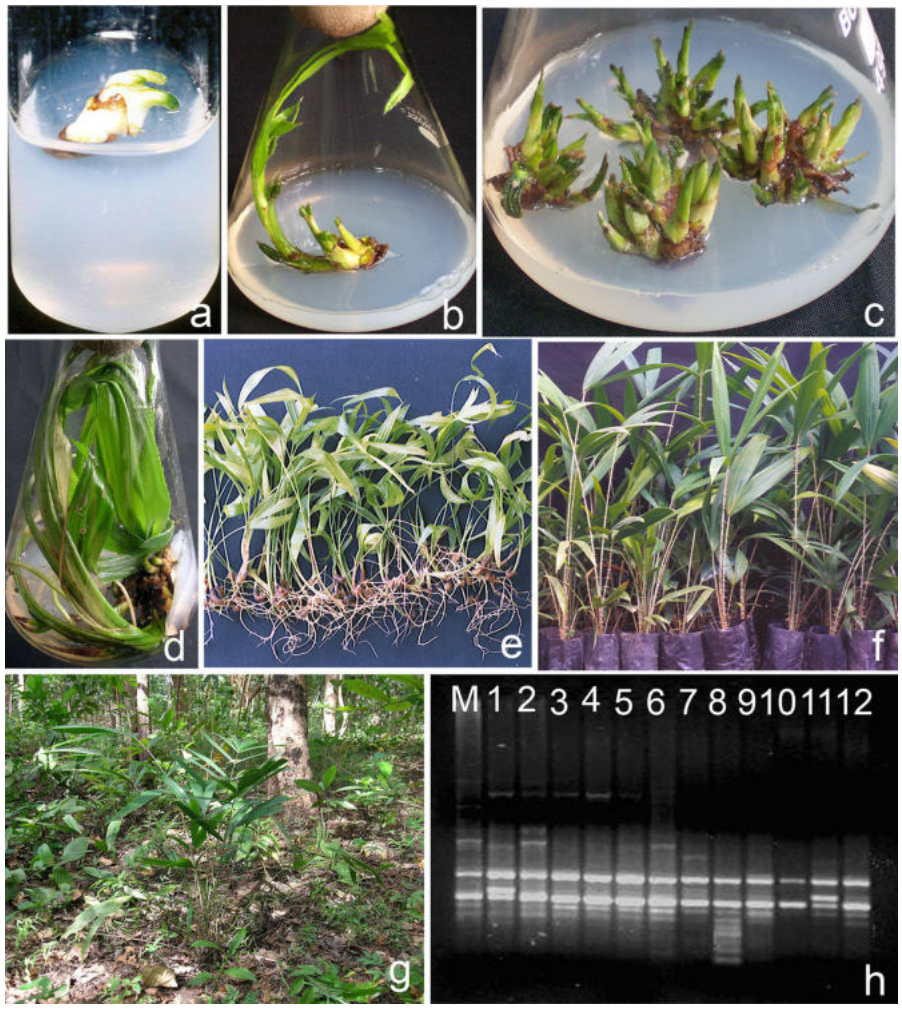


Fig. 2 Micro-multiplication through embryoid and axenic shoots of *C. thwaitesii*. **a** Shoot bud initiation from embryoid in MS medium supplemented with 0.45 µM TDZ. **b** Shoot induction from axenic shoot in MS medium supplemented with 0.45 µM TDZ. **c** Mass multiplication through subculture passages in MS medium supplemented with 1.78 µM BAP and 0.45 µM TDZ. **d** Rooted shoots in MS medium supplemented with 16.11 µM NAA. **e** Deflasked plantlets for hardening. **f** Nursery established plants ready for reintroduction. **g** 3-year-old reintroduced plant in forest segment. **h** ISSR profiling using primer-835 (M mother; 1–12 Micro-plantlets)

Induction of multiple shoots from axenic shoots

Whole shoots devoid of roots, dissected out of 16-week-old plantlet raised through embryoids in MS basal medium, have been used as source of explant for multiple shoot induction. In axenic shoot cultures, the basal part (resident meristem of the shoot tip) responded well with differentiation of buds in 6 weeks (Fig. 2b). Most of the cytokinins except TDZ tested individually were weak and in the order of priority TDZ alone (0.45 µM) or in combination with BAP (1.78 µM) preferred to obtain maximum shoot initiation in terms of frequency and number of shoots. Although shoot buds were formed en masse in the presence of 0.45 µM TDZ, the shoot buds remained more as a proliferating mass than as individual, elongated shoots. Therefore, it is justified that 0.45 µM TDZ is used for direct multiple shoot initiation in axenic seedling-derived whole shoot cultures of *C. thwaitesii*. Hitherto, such individual hormonal concentrations and combinations have been used with certain success for shoot initiation in seedling explant cultures of *Nothapodytes foetida*

Table 1 Direct multiple shoot induction and multiplication from somatic embryoids and axenic shoots in full strength MS medium supplemented with different concentrations and combinations of PGRs

PGRs (μM)		Multiple shoot induction after 6 weeks of culture			
TDZ	BAP	Somatic embryoids		Axenic shoots	
		Percentage response	Mean number of shoots	Percentage response	Mean number of shoots
0.00	0.00	76.67[c]	1.00 ± 0.00[e]	73.33[c]	1.00 ± 0.00[d]
0.09		86.67[b]	1.00 ± 0.00[e]	86.67[b]	2.00 ± 0.00[c]
0.23		96.67[a]	2.33 ± 0.10[d]	70.00[c]	2.50 ± 0.11[c]
0.45		100.0[a]	6.87 ± 0.23[a]	100.0[a]	5.89 ± 0.27[a]
0.91		86.67[b]	3.83 ± 0.24[c]	100.0[a]	3.87 ± 0.17[b]
1.36		90.00[a]	2.83 ± 0.27[d]	96.67[a]	2.67 ± 0.28[c]
1.82		66.67[d]	2.75 ± 0.19[d]	73.33[c]	2.00 ± 0.17[c]
2.27		60.00[d]	2.00 ± 0.16[d]	66.67[d]	1.71 ± 0.16[d]
4.54		56.67[e]	1.00 ± 0.00[e]	56.67[e]	2.00 ± 0.13[c]
9.08		66.67[d]	1.00 ± 0.00[e]	46.67[f]	2.00 ± 0.18[c]
0.23	0.89	70.00[c]	2.33 ± 0.10[d]	66.67[d]	2.50 ± 1.10[c]
0.23	1.78	73.00[c]	2.50 ± 0.11[d]	70.00[c]	2.71 ± 0.13[c]
0.45	0.89	73.00[c]	3.83 ± 0.27[c]	73.33[c]	2.80 ± 0.16[c]
0.91	0.89	76.67[c]	2.50 ± 0.11[d]	73.33[c]	2.80 ± 0.17[c]
0.45	1.78	100.0[a]	5.17 ± 0.27[b]	73.33[c]	5.60 ± 0.23[a]
0.91	1.78	63.33[d]	3.60 ± 0.18[c]	63.33[d]	3.25 ± 0.19[b]

[a]Data represent mean ± SE of 15 replicates repeated thrice, recorded after every 6 weeks of culture. Values followed by the same letter in the superscript do not differ significantly at $p \leq 0.05$ based on ANOVA and LSD t test

(Satheeshkumar and Seeni 2000) and *Areca catechu* (Hsiang-Chih Wang et al. 2003).

Shoot multiplication

Shoot buds obtained from embryoid and axenic shoot cultures when separated and transferred either individually or as a group to media containing selected hormonal regimes induced varied rate of shoot multiplication. The number and frequency of shoot multiplication were dependent on the presence of TDZ either alone (0.45 μM) or in combination with 1.78 μM BAP. The rate of shoot multiplication in TDZ as well as in BAP-TDZ was higher than in culture initiation phase. However, unlike TDZ, simultaneous shoot multiplication and elongation was recorded in BAP-TDZ combination with an average of 5940 shoots/explant at 100% efficiency after three subculture passages at 6-week period in media supplemented with 1.78 μM BAP and 0.45 μM TDZ (Fig. 2c). The multiple shoots obtained when continuously subcultured in media supplemented with TDZ alone resulted in reduced length of the shoots as reported in *Solanum melongena* L. (Magioli et al. 1998). Therefore, the combination of 1.78 μM BAP and 0.45 μM TDZ was preferred as the best PGR combination for shoot multiplication of this species (Table 2).

Shoot elongation and rooting

In the present investigation, shoot elongation was not observed during TDZ-enhanced shoot multiplication phase. Thidiazuron has been shown to induce high rate of shoot/bud proliferation than other purine-based cytokinins in a number of woody species, including *Fagus sylvatica* (Vieitez and San-Jose 1996), *F. orientalis* (Cuenca et al. 2000) and *Salix nigra*, though in many species TDZ-induced buds failed to elongate into shoots (Lyyra et al. 2006). There are many reports on poor elongation of TDZ-induced shoots, which may be consistent with its high cytokinin activity; the concentration and duration of exposure to this compound is critical in this respect (Murthy et al. 1998; Debnath 2005). Shoot elongation was better achieved

Table 2 Shoot multiplication through subculture passages in MS agar medium supplemented with different concentrations and combinations of PGRs

PGRs (μM)		Subculture passages			Average no. of shoots/explant
TDZ	BAP	I	II	III	
0.23		3.00 ± 0.24[d]	04.50 ± 0.11[d]	05.25 ± 0.17[c]	120
0.45		7.87 ± 0.23[b]	09.50 ± 0.11[b]	11.89 ± 0.17[ab]	4158
0.91		4.87 ± 0.17[c]	05.33 ± 0.16[c]	05.83 ± 0.10[c]	300
0.23	1.78	3.71 ± 0.13[d]	05.00 ± 0.19[c]	05.75 ± 0.18[c]	150
0.45	1.78	9.67 ± 0.27[a]	11.60 ± 0.23[a]	12.25 ± 0.19[a]	5940
0.91	1.78	3.25 ± 0.19[d]	05.71 ± 0.13[c]	05.80 ± 0.28[c]	225

[a]Data represent mean ± SE of 15 replicates repeated thrice, recorded after every 6 weeks of culture. Values followed by the same letter in the superscript do not differ significantly at $p \leq 0.05$ based on ANOVA and LSD t test

using liquid or agar medium free of hormones (Singha and Bhatia 1988; Fasolo et al. 1989). Interestingly, irrespective of differential origin of shoot buds (2–3 cm) from source tissue (embryoid, axenic shoot), they all responded alike during the elongation phase. Elongated shoots having maximum 6–8 cm length could be harvested after two subculture passages of 4-week interval, in hormone-free liquid medium. Simultaneous formation of additional shoots during shoot elongation phase may be due to the residual effects of TDZ transferred from the multiplication phase as reported in *Calendula officinalis* (Victório et al. 2012).

In *C. thwaitesii*, shoot elongation and rooting phase was equally long (8 weeks) and root induction was observed on varied length of shoots (4–8 cm) obtained from the same stage of cultures by the transfer of IBA/NAA supplemented media. In fact, among the various concentrations of auxins supplemented individually, 16.11 μM NAA was preferred for better rhizogenic responses at 96% efficiency (Table 3; Fig. 2d). The observed differences in rooting responses may be related to genetic/physiological constitution and also the ability to accumulate auxins endogenously in this species. Similar results were also reported in other species of rattans viz. *C. simplicifolius* (Zhang Fangqiu 1993) and *C. egregius* (Zeng Bingshan 1997). The process of rooting was slow, often taking 2–3 months before the plants get deflasked. Since most of the plants were single rooted, the ability to form only 1–2 roots may be genetically controlled as in certain species of *Calamus* viz. *C. simplicifolius* (Zhang Fangqiu 1993) and *C. egregious* (Zeng Bingshan 1997).

Hardening and establishment

Rooted plants (Fig. 2e) raised through micro-multiplication were established at 96% rate after 8 weeks of hardening in mist house. The hardy nature of the roots greatly helped the plantlets to establish in the sand medium which is in corroboration with the reports on rapid clonal multiplication of *Morinda umbellata* (Nair and Seeni 2002). Since all the rattan palms employed for cultivation are tropical humid species, the high humidity levels (80 ± 5% RH) maintained in the mist house are very much needed for optimal survival and establishment of the plantlets. However, in order to assure optimal growth of the hardened plants, it was necessary to transfer them to nutrient-rich potting medium consisting of sand:top soil:farm yard manure (3:1:1) to facilitate continued growth of the hardened plants in the shade net house (Fig. 2f).

Eco-restoration

The long period (6 months) required for hardening and nursery establishment was due to the slow growth of the in vitro plantlets. This much long period was also required to observe continuous growth of the slow-growing reintroduced palms. The formation of the first new leaf in the reintroduced/translocated plant itself took nearly 2 months, followed by emergence of 1–2 new leaves within 2–4 months. This kind of encouraging performance of the tissue cultured plants after

Table 3 Rhizogenic response of embryoid and axenic seedling-derived shoots in MS agar medium supplemented with individual concentrations of auxins

Auxins (μM)	Percentage response	Mean no. of roots/shoot	Mean length of root (cm)
IBA 00.00	00.00	0.00 ± 0.00	0.00 ± 0.00
04.92	50.00^{d}	1.33 ± 0.10^{b}	8.17 ± 0.39^{c}
09.84	58.33^{d}	1.43 ± 0.11^{b}	8.52 ± 0.52^{bc}
14.76	83.33^{b}	1.60 ± 0.15^{b}	10.0 ± 0.22^{a}
19.69	66.67^{c}	2.00 ± 0.22^{a}	6.63 ± 0.40^{d}
24.61	58.33^{d}	2.14 ± 0.22^{a}	6.44 ± 0.27^{d}
NAA 05.37	33.33^{e}	1.25 ± 0.10^{b}	9.14 ± 0.34^{b}
10.74	58.33^{d}	1.43 ± 0.16^{b}	8.98 ± 0.41^{bc}
16.11	96.67^{a}	1.64 ± 0.17^{b}	8.84 ± 0.42^{bc}
21.48	83.33^{b}	2.00 ± 0.17^{a}	6.04 ± 0.31^{d}
26.85	66.67^{c}	2.12 ± 0.21^{a}	5.82 ± 0.30^{e}

[a]Data represent mean ± SE of 15 replicates repeated thrice, recorded after every 8 weeks of culture. Values followed by the same letter in the superscript do not differ significantly at $p \leq 0.05$ based on ANOVA and LSD t test

reintroduction is also recorded in species such as Blue Vanda (Seeni and Latha 2000) which is attributed to the best health status of the in vitro-derived plants. Long-term observations on the established plants for 3-year period confirmed 81–86% success rate of the embryoid/axenic shoot-derived plantlets (Fig. 2g). They continued to grow uniformly at the site of reintroduction, indicating the desirability of using these methods for large-scale production, safe handling and rehabilitating unproductive secondary forest to stabilize shifting cultivation areas which could also be helpful to conservation of biodiversity and improve socio-economic welfare of the people.

Genetic variability analysis

Genetic diversity analysis is an inevitable component for successful in vitro propagation and eco-restoration programmes. In the present investigation, genetic variability analysis of in vitro-derived plantlets using ISSR markers showed slight variation from the mother plant. However, similar results were also reported by Bhatia et al. (2008) in leaf explant culture of *Gerbera*. It might be due to such factors as source of explants, mode of regeneration (Goto et al. 1998); media composition, culture conditions (Damasco et al. 1996) and supplementation of plant growth regulators (Martin et al. 2006) resulting in variations. The adventitious buds or well -developed meristematic tissues had low tendency for genetic variation (Rout et al. 1998; Joshi and Dhawan 2007), whereas more clonal variation was recorded in plantlets produced through callus phase as compared to those regenerated from embryogenic tissues (Yang et al. 1999). A very low percentage of genetic polymorphism (0.05%), though negligible, was also noticed by ISSR analysis in embryo callus culture of this rattan palm (Fig. 2h). As rattans are dioecious and open pollinated, seed progeny varies so much that no seedling-derived mature palm is identical to the other. In conservation point of view, nature favours diversity than uniformity and it is effective for the better survival and establishment of reintroduced plants. In this perspective the present study, which developed a high-frequency plantlet production protocol together with enhanced rate of establishment without intensive labour, is suitable for diversity conservation and sustained delivery of quality raw material to rattan industry through the development of agroforestry systems.

Conclusion

Commercially viable high-frequency mass multiplication system successfully demonstrated here is well suited for mass production and supply of quality planting material of this economic rattan palm for agroforestry programmes. Although the analysis of

genetic variability using ISSR markers reveals slight genetic polymorphism of embryo callus cultured *C. thwaitesii,* it will be highly useful for diversity conservation and percentage establishment of micro-plantlets. Thus, an efficient two-in-one system developed would certainly trigger biotechnology-based research in rare, endangered, endemic rattans particularly monopodial rattan palms. Furthermore, the system demonstrated here also would lead to commercial production and better utilization of this economic rattan palm in cottage industry and thereby strengthening the cane industry in India and elsewhere.

Acknowledgements We thankfully acknowledge the Department of Biotechnology (DBT), Government of India, for providing the financial assistance through the research grant (No BT/R&D/08/04/95), the Director, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI) for providing laboratory facilities and the Department of Forests, Government of Kerala extended support for eco-restoration activities.

References

Aminuddin M, Nur Surpadi, Noor Md, Woon WC (1992) Economics of cultivation of large diameter rattan. In: Wan Mohd., Wan R, Dransfield J, Manokaron N (eds) A guide to the cultivation of rattan. Malayan For.Rec. p 35

Arditti J, Ernst R (1993) Micropropagation of Orchids. Wiley, New York, pp 1–682

Barba RC, Patena LJ, Mercado MM, Lorico L (1985) Tissue culture of rattan (*Calamus manillensis*. Wendl.) In: Proceedings of the Second National Symposium on Tissue Culture of Rattan, University Partanian Malaysia

Bhatia R, Singh KP, Jhang T, Sharma TR (2008) Assessment of clonal fidelity of micropropagated gerbera plants by ISSR markers. Sci Hortic 119:208–2111

Bingshan Zeng (1997) Tissue culture of *Calamus egregius*. J. Cent South For Univ 17(4):563–569

Blake J, Eeuwens CJ (1982) Culture of coconut palm tissues with a view to vegetative propagation. In: Rao AN (ed) Proceedings of COSTE\symposium on Tissue Culture of Economically Important Plants, Singapore, pp 145–148

Chengji Zhuang, Jiankui Zhou (1991) Plant regeneration in tissue culture of rattan. Acta Bot Yunnaica 13(1):97–100

Corley RHV, Barrett JN, Jones LH (1976) Vegetative propagation of oil palm via tissue culture. Malaysian International Agricultural Oil Palm Conference, pp 1–8

Cuenca B, Ballester A, Vieitez AM (2000) In vitro adventitious bud regeneration from internode segments of beech. Plant Cell Tiss Org Cult 60:213–220

Damasco OP, Graham GC, Henry RJ, Adkins SW, Smith MK, Godwin ID (1996) Random amplified polymorphic DNA (RAPD) detection of dwarf off-types in micropropagated Cavendish (Musa spp. AAA) bananas. Plant Cell Rep 16:118–123

Debnath SC (2005) A two-step procedure for adventitious shoot regeneration from in vitro-derived lingonberry leaves: shoot induction with TDZ and shoot elongation using zeatin. Hortic Sci 40:189–192

Eeuwens CJ (1976) Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera*) and cultured in vitro. Physiol Plant 36:23–28

Eeuwens CJ (1978) Effects of organic nutrients and hormones on growth and development of tissue explants from coconut (*Cocos nucifera*) and date palms (*Phoenix dactylifera*) cultured in vitro. Physiol Plant 42:173–178

Eshraghi P, Zarghami R, Mirabdulbaghi M (2005) Somatic embryogenesis in two Iranian date palm cultivars. Afr J Biotechnol 4(11):1309–1312. http://www.academicjournals.org/AJB, ISSN 1684–5315

Fangqiu Zhang (1993) A study on Rattan tissue culture. J For Res 6(5):486–492

Fasolo F, Zimmerman RH, Fordham I (1989) Adventitious shoot formation on excised leaves of in vitro grown shoots of apple cultivars. Plant Cell Tiss Org Cult 16:75–87

Goto S, Thakur RC, Ishii K (1998) Determination of genetic stability in long-term micropropagated shoots of *Pinus thunbergii* Parl. using RAPD markers. Plant Cell Rep 18:193–197

Hazarika BN (2003) Acclimatization of tissue-cultured plants. Curr Sci 85:1704–1712

Hemanthakumar AS (2010) Studies on embryo and tissue culture of three economically important rattan sps. Thesis submitted to Kerala University

Hemanthakumar AS, Preetha TS, Krishnan PN, Seeni S (2013) Utilization of zygotic embryos of an economic rattan palm *Calamus thwaitesii* Becc. (Arecaceae) for somaplant regeneration and cryobanking. 3 Biotech 3:195–203

Joshi P, Dhawan V (2007) Assessment of genetic fidelity of micropropagated *Swertia chirayita* plantlets by ISSR marker assay. Biol Plant 51(1):22–26

Kundu M, Sett R (1999) Regeneration through organogenesis in rattan. Plant Cell Tissue Org Cult 59:219–222

Lopez-Villalobos A, Dodds PF, Hornung R (2001) Changes in fatty acid composition during development of tissues of coconut (*Cocos nucifera* L.) embryos in the intact nut and in vitro. J Exp Bot 52:933–942

Lyyra S, Lima A, Merkle S (2006) In vitro regeneration of *Salix nigra* from adventitious shoots. Tree Physiol 26:969–975

Magioli C, Rocha APM, de Oliveria DE, Mansur E (1998) Efficient shoot organogenesis of eggplant (*Solanum melongena* L.) induced by thidiazuron. Plant Cell Rep 17:661–663

Martin JP, Rabechault H (1976) Procede de multiplication vegetative de vegetaux et plants ainsi obtenus, French Patent No. 7628361

Martin KP, Pachathundkandi SK, Zhang CL, Slater A (2006) RAPD analysis of a variant of Banana (*Musa* sp.) cv. Grande Naine and its propagation via shoot tip culture. In Vitro Cell Dev Biol 42:188–192

Merillon JM, Rideau M, Chenieux JC (1984) Influence of sucrose on levels of ajmalicine, serpentine and triptamine

in *Catharanthas roseus* cells in vitro. Plant Med 50:497–501

Mojarabi M, Nasr SMH, Jalilvand H, Kooch Y (2011) Effect of activated charcoal, growth supplements and storage on removing dormancy, germination indices and vigour of Ash (*Fraxinus excelsior* L.). Ann Biol Res 2(5):203–212

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Murray HG, Thompson WF (1980) Rapid isolation of high molecular weight DNA. Nucl Acids Res 8:4321–4325

Murthy BNS, Murch SJ, Saxena PK (1998) Thidiazuron: a potent regulator of in vitro morphogenesis. In Vitro Cell Dev Biol 34:267–275

Nair LG, Seeni S (2002) Rapid clonal multiplication of *Morinda umbellate* Linn. (Rubiaceae), a medicinal liana, through cultures of nodes and shoot tips from mature plant. Phytomorphology 52:77–81

Padmanabhan D, Ilangovan R (1993) Surgical induction of multiple shoots in embryo cultures of *Calamus gamblei* Becc. RIC Bull 12:8–12

Ramanayake SMSD (1999) Viability of excised embryos, shoot proliferation and in vitro flowering in a species of rattan *Calamus thwaitesii* Becc. J Hortic Sci Biotechnol 74(5):594–601

Reynolds JF, Murashige T (1979) Asexual embryogenesis in callus cultures of palms. In Vitro 15:383–387

Rout GR, Das P, Goel S, Raina SN (1998) Genetic stability of micropropagated plants of ginger using random amplified polymorphic DNA (RAPD) markers. Bot Bull Acad Sin 39:23–27

Sáenz L, Azpeitia A, Chuc-Armendariz B, Chan JL, Verdeil JL, Hocher V, Oropeza C (2006) Morphological and histological changes during somatic embryo formation from coconut plumule explants. In Vitro Cell Dev Biol 42:19–25. https://doi.org/10.1079/ivp2005728

Sane D, Aberlenc-Bertossi F, Gassama-Dia YK, Sagna M, Trouslot MF, Duval Y, Borgel A (2006) Histocytological analysis of callogenesis and somatic embryogenesis from cell suspensions of date palm (*Phoenix dactylifera*). Ann Bot 98(2):301–308

Satheeshkumar K, Seeni S (2000) In vitro multiplication of *Nothapodites foetida* (Wight.) Sleumer through seedling explant cultures. Indian J Exp Biol 38:273–277

Scherwinski-Pereira JE, da Guedes RS, Fermino PCP Jr, Silva TL, Costa FHS (2010) Somatic embryogenesis and plant regeneration in oil palm using the thin cell layer technique. In Vitro Cell Dev Biol 46:378–385. https://doi.org/10.1007/s11627-010-9279-6

Seeni S, Latha PG (2000) *In vitro* multiplication and ecorehabilitation of the endangered Blue Vanda. Plant Cell Tiss Org Cult 61:1–8

Sett R, Kundu M, Sharma P (2002) Regeneration of plantlets through organogenesis in *Calamus tenuis*. Indian J Plant Physiol 7:358–361

Singha S, Bhatia SK (1988) Shoot proliferation of pear cultures on medium containing thidiazuron and benzylamino purine. Hortic Sci 23:803

SPSS 9 (version 10.0) (1999) Manuals SPSS Inc., Chicago

Thomas TD (2008) The role of activated charcoal in plant tissue culture. Biotechnol Adv 26(6):618–631

Tisserat B (1983) Tissue culture of date palms: a new method to propagate an ancient crop-and a short discussion of the california date industry. Principes 27(3):105–117

Victório CP, Lage CLS, Sato A (2012) Tissue culture techniques in the proliferation of shoots and roots of *Calendula officinalis*. Rev Ciênc Agron 43(3):539–545

Vieitez AM, San-Jose MC (1996) Adventitious shoot regeneration from *Fagus sylvatica* leaf explants in vitro. In Vitro Cell Dev Biol 32:140–147

Wainwright H, Scrace J (1989) Influence of in vitro preconditioning with carbohydrates during the rooting of microcuttings on in vivo establishment. Sci Hortic 38:261–267

Wang H-C, Chen J-T, Wu S-P, Lin M-C, Chang W-C (2003) Plant regeneration through shoot formation from callus of *Areca catechu*. Plant Cell Tiss Org Cult 75:95–98

Yang H, Tabei Y, Kamada H, Kayamo T, Takaiwa F (1999) Detection of somaclonal variation in cultured rice cells using digoxygenin-based random amplified polymorphic DNA. Plant Cell Rep 18:520–526

Yap IV, Nelson RJ (1996) Win Boot: a program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. IRRI Discussion Paper Series No. 14, International Rice Research Institute, Manila, Philippines

Yusoff AM (1989) Shoot formation in *Calamus manan* under in vitro. In: Rao AN, Aziah Mohd. Yusoff (eds) Proceedings of the seminar on Tissue Culture of Forest Species, Forest Research Institute Malaysia and International Development Research Centre, Singapore, pp 45–49