Standardization of in vitro multiplication technique for Areca concinna, an endangered palm species for its conservation and utilization

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Abstract

Areca concinna (Arecaceae) is an endangered plant species endemic to south western Sri Lanka. It is an important palm distributed primarily in Sri Lanka, India, and Southeast Asia and was listed under endangered plants species by IUCN due to habitat loss. Here a protocol was developed for clonal multiplication of *A. concinna.* Clonal propogation is essential for conservation and maintenance of endangered species. Immature inflorescence, immature embryos and mature embryos were tested for callogenesis and subsequent somatic embryogenesis in M72 basal media supplemented with the auxins 2, 4-D and Picloram. Of the three explants, somatic embryos were obtained only from mature embryos. Callus multiplication and somatic embryo formation in M72 basal media supplemented with 2, 4-D were found to be better over Picloram media. Serial transfer of explants from higher to lower concentrations was essential for sustaining the multiplication of callus as well as for induction of somatic embryos. Somatic embryos grown inY3 or 1/2 Y3 basal medium supplemented with BAP (1 mg/L), NAA (0.5 mg/L), and IBA (0.25 mg/L) exhibited shoot initiation and plantlet development. This protocol has its application in rapid multiplication of *A. concinna* palms thereby enhancing the possibility of conservation of the endangered palm.

Introduction

Areca concinna Thwaites or 'Squirrel catechu' is a medium sized palm with low clustering habit bearing attractive lush green leaves, white fragrant flowers and bright red coloured fruits. The palm belongs to genus *Areca* L. and family Arecaceae that comprises of 45 species (Plant List 2013). The plant is most suitable for use as an ornamental palm due to its attractive plant characteristics. It is considered as a wild relative of the only cultivated palm from the Areca genus i.e. *Areca catechu* L. (beetle nut). Similar to beetle nuts, A. *concinna* nuts are also used as masticatory (Murthy and Pillai 1982). Moreover, the species was utilized in hybridization programmes for production of interspecific hybrids with beetle nut. These interspecific hybrids were resistant to *Phytophthora meadii* infections as *A. concinna* exhibited resistance to *P. meadii* (Prathibha et al. 2015). However, because of habitat loss in endemic regions, the palm is included in the IUCN Red list of endangered species in the year 1998 (Johnson 1998). So, it is essential to take-up conservation strategies for preserving these species. In addition to conventional propagation methods, *in vitro* techniques of propagation and conservation have been employed successfully for the conservation of several endangered and threatened plant species like *A. concinna.* In the present study, we report the successful *in vitro* propagation technique of *A. concinna via* somatic embryogenesis.

Materials And Methods

The study was conducted at ICAR-Central Plantation Crops Research Institute, Kasaragod, Kerala during November 2018 to June 2021.

Plant material and culture initiation

Immature inflorescence, matured and immature nut embryosexplants were collected from *Areca concinna* palms during November 2018 and February 2019 from the research field of ICAR-CPCRI. The nuts were washed thoroughly under running tap water for 15 minutes. The calyx was removed from the nuts. These nuts were treated with 0.01% Mercuric chloride for 3 minutes and then rinsed three times with sterile distilled water to remove the traces. In the laminar airflow chamber, husk was separated from the nuts and the kernels were sterilized using 20% sodium hypochlorite for 10 minutes followed by rinsing in autoclaved sterile water for 5 to 6 times. Embryos were carefully excised from the kernels and inoculated in the callusing media. The un-opened immature inflorescences were subjected to flame sterilization and the spathe portion was removed aseptically. The middle portions of rachille were chopped into 2–4 mm pieces and then inoculated in the callusing media.

All the media combinations were uniformly supplemented with 3% sucrose, 0.1% charcoal and 0.8% agar. The pH of the medium was adjusted to 5.7 ± 0.2 with 0.1 N NaOH or 0.1 HCl before autoclaving the media at 10 K Pa for 15 min at 121°C. Inoculated plates were incubated at 27°C under dark condition continuously for three months. Each treatment had 10 zygotic embryos and 3 replications. During dark incubation, the cultures were monitored at weekly intervals. Three months after the first inoculation, first sub culturing was done to medium containing reduced concentration of plant growth hormones.

Standardization of the inoculation media

For standardizing the inoculation media for callusing, four different media combinations were evaluated (Table 1). Observations on explant response, browning, callusing and regeneration were evaluated regularly in the initial media as well as subsequent sub culturing media.

Table 1
Inoculation media types and its hormonal compositions

Media	Plant growth chemicals				
	Basal media	2,4-D (mg/L)	Picloram (mg/L)	2-iP (mg/L)	BAP (mg/L)
M1	M72	-	48.29	-	-
M2	M72	25	-	-	-
M3	M72	-	36.21	-	3
M4	M72	15	-	3	-
(2, 4-D: 2, 4-dichlorophenoxyacetic acid; 2-ip: 2-isopentenyl adenine;					
BAP: 6- benzylaminopurine)					

Callusing and somatic embryogenesis

Callusing was observed only in mature zygotic embryos of *A. concinna* whereas no response was seen in immure embryos and inflorescence cultures. Mature embryos gave rise to callus on two media combinations M2 and M4 (Table 3), but formation of somatic embryos from the calli was noticed only from media combination M2 (Table 1). Formation of somatic embryos from the calli was noticed during the first subculture of calli (generated from M2 media) to M72 basal media with 1/10th of initial 2, 4-D concentration. The somatic embryos formed were transferred to hormone free Eeuwen's Y3 basal media for its germination and subsequent development. For somatic embryo development, transferred embryos were exposed to the white LED lights (100 µmol m^{-2} s^{-1}) for a photoperiod of 16 h light and 8 h dark. The temperature and RH were maintained to 27 ± 1°C and 80% respectively.

In vitro **development of shoots and roots**

Germinated somatic embryos were transferred to half and full-strength Eeuwen's Y3 basal medium supplemented with 1 mg/L of BAP, 0.5 mg/L NAA (1-Naphthaleneacetic acid), and 0.25 mg/L of IBA (Indole-3-Butyric acid).

Potting of plantlets

After 12 weeks of culturing in root and shoot induction media, plants with 2–4 leaves and sufficient number of roots were potted into a soil mixture containing sand: soil: coir pith in the ratio 3:1:1.

Statistical analysis

Analysis of variance was performed over the complete randomized design trials using R software Version 4.1.3 (R Core Team 2022) by applying rules of arc sine data transformation (Parsad 2005), since response measured in percentage. Significance of differences between treatment means (Fisher-LSD test) was determined by using "agricolae" package in R (Mendiburu 2020).

Results And Discussion

Explant response for induction of embryogenic callus

To identify the inoculation media for callusing, four different hormonal combinations were evaluated (Table 1). Enlargement of the inoculated zygotic embryos was noticed after 1–2 weeks of dark incubation. The enlarged zygotic embryos gave rise to white translucent calli after two months of initial inoculation on the same callusing medium (Fig. 1B), while some of them were germinated and gave rise to the shoot initials on same media. Observations taken during first sub culturing revealed that, callus formation was seen only in two media combinations i.e. M2 and M4. Maximum response for callusing i.e. 23.33% was observed in M2 than M4 (3.3%) (Table 3). Response for callusing was found to be better with 2, 4-D alone as compared to the 2, 4-D + 2-iP together. 2, 4-D has been used successfully in tissue culture of diverse plant species especially for induction of embryogenic calli and somatic embryos in both monocot and dicot plants (Ammirato 1978). Embryogenic callus could be obtained in different

plants by manipulating 2, 4-D concentration and extant of its presence in induction media (Zheng and Konzak 1999). For example, 1 mg/L of 2, 4-D for a week duration is effective for induction of embryogenic calli and subsequent regeneration in rice seed explants (Carsono et al. 2021), whereas the same chemical works only if it's used at 66 mg/L for a duration of eight months to obtain the embryogenic calli from immature inflorescence explants of coconut (Verdeil et al. 1994). Palms are comparatively difficult species to grow *in vitro*. Recalcitrant nature of the palms was explained due to lower response, slower growth rate, excessive browning, and lack of repeatability (Fernando et al. 2010; Shareefa et al. 2019). In the current study we used 2, 4-D and picloram to induce the embryogenic calli. Effectiveness of 2, 4-D was tested in different palms to induce organogenesis and somatic embryogenesis (Sáenz et al. 2018; Zayed et al. 2017; Eeuwens and Blake 1977). Similarly, several researchers demonstrated the positive role of the other synthetic auxin picloram in clonal multiplication of palms like coconut (*Cocos nucifera L.*), arecanut (*Areca catechu L.*), pejibaye palm (*Bactris gasipaes* H.B.K), rattan palm (*Calamus manan* Miquel.) and Canary Island date palm (*Phoenix canariensis*) (Raju 2006; Karun et al. 2004; Valverde et al. 1987; Goh et al. 1999; Huong et al. 1999). Though both the auxins are found to have role in callogenesis and clonal multiplication of palms, response of callusing in *A. concinna* was seen only on induction media supplemented with 2,4-D. When explant sources (mature, immature zygotic embryos and immature explant tissues) of *A. concinna* were compared for the response, only mature zygotic embryos survived and gave rise to callus (3.3–23.33%) on induction media. There was no response for callusing from the other two explants i.e., immature embryos and inflorescence explants (Table 2). Even though browning was observed in all three explant tissues more intense browning was observed in inflorescence explants rather than in embryos. Eventually these browned tissues lost viability by the end of 60th day after inoculation. Oxidative browning is one of the common problems faced in plant tissue culture resulting in lower rates of regeneration or recalcitrance (Laukkanen et al. 2000; Aliyu 2005) or sometimes even leading to the death of explant tissue (Panaia et al. 2000; Tabiyeh et al. 2006). Explant browning and its deleterious effects on palm tissue culture were reported in many studies (EL-Gioushy et al. 2020; Al-Mayahi et al. 2018; Karun et al. 2004). Even though oxidative browning of mature zygotic embryos in all four media combinations ranged from 6.66–11.66% significant differences were not found among the media combinations. However, browning of matured zygotic embryos didn't lead to the death of explant tissue in *A. concinna*.

Table 2
Explant-response for callusing on inoculation media

Explant types	Media combinations			
	M1	M2	M3	M4
Mature embryo	+	++++	+	++
Immature embryos	-	-	-	-
Immature inflorescence	-	-	-	-
(+: low response for callusing; ++: medium response for callusing;				
++++: high response for callusing; -: no response for callusing)				

Somatic embryogenesis and plantlet regeneration

The calli observed on induction media (Table 1, Fig. 1B) was subcultured to Eeuwen's Y3 media containing 2.5 mg/L of 2, 4-D for callus multiplication and somatic embryogenesis. Only 35 to 49% of the calli obtained from inoculation media had showed regenerative capacity (Table 3; Fig. 1C). Somatic embryo formation was observed, when regenerative callus was transferred to hormone free Eeuwen'sY3 media supplemented with 3% sucrose, 0.1% activated charcoal and 0.8% agar (Fig. 1E). To achieve better germination, growth and rooting of somatic embryos, they were transferred to full and half strength Y3 medias supplemented with 1 mg/L of BAP (6-Benzylaminopurine), 0.5 mg/L NAA (1-Naphthaleneacetic acid), and 0.25 mg/L of IBA (Indole-3-Butyric acid) (Table 3). Germination of somatic embryos was witnessed by simultaneous development of shoot and roots (Fig. 1F).

Table 3
Explant response to in inoculation media for callusing and germination

Media	Explant Browning (%)	Callusing (%)	Regenerative callus (%)	Zygotic embryo germination (%)
M1	8.33(2.85a)	0.00(0.00b)	0.00(0.00b)	11.66(3.39a)
M2	10.00 (3.09a)	23.33(4.80a)	8.33(2.85a)	8.66(2.92a)
M3	11.66(3.29a)	0.00(0.00b)	0.00(0.00b)	10.00(3.09a)
M4	6.66(2.54a)	3.33(1.05b)	1.66(0.74b)	30.0(16.64a)
Mean	9.16(2.94)	6.66(1.46)	2.49(0.89)	15.08(6.51)
CV (%)	54.54(28.63)	61.23(68.44)	81.65(72.94)	39.03(156.88)
CD at 0.05	N/A(1.68)	7.80(2.00)	3.90(1.31)	11.25(20.42)
S.Em.	2.88(0.71)	2.35(1.00)	1.17(0.43)	3.39(104.4)
(Figures in parentheses square root transformed values)				

Relatively equal growth and development of somatic embryos was observed on both the media compositions (Table 4). Around 75% of developed somatic embryos showed proper shoot and root initials. Fully developed plantlets with 2–4 expanded leaves were obtained after 16 weeks of culture in the same medium. Approximately 67% of developed plantlets showed healthy growth in tubes. These healthy plantlets with proper roots were subjected to *in vitro* acclimatization / hardening (Fig. 1G). Plants are transferred to plastic pots containing sand: soil: coir pith (3:1:1) mixture and were covered initially with polyethylene bags and incubated in culture room under white LEDs (200 µmol m^{-2} s^{-1}) for a photoperiod of 16 h light and 8 h dark. The temperature and RH were 27 ± 1°C and 80% respectively. During *in vitro* acclimatization plants were watered with diluted MS macro solution once in a week. After *in vitro* hardening only 35% plantlet recovery was obtained due to poor root development and contamination issues during *in vitro* hardening. After two months of *in vitro* acclimatization, these plantlets were transferred to shade net- house to carry *ex vitro* hardening process.

Table 4
Growth and development of *A. concinna* somatic embryos on germination media

Treatment	Shoot initiation (%)	Rooting (%)	Plantlet survival before hardening (%)	Plantlet survival after hardening (%)
T1	80.00(8.93a)	80.00(8.93a)	71.66(25.86a)	33.33(16.98a)
T2	76.66 (8.75a)	66.66(25.57a)	63.33(42.78a)	38.33(30.15a)
Mean	78.33 (8.84)	73.33(17.25)	67.49(34.32)	35.83(23.57)
CV (%)	10.42 (6.9)	11.13 (122.14)	12.46(61.4)	23.48(81.78)
CD 0.05	NA (2.14)	NA (74.04)	NA(74.05)	NA(67.73)
S.Em.	4.71 (0.37)	4.71 (444.1)	4.85(444.2)	4.85(371.6)
(Figures in parentheses square root transformed values; Compositions of T1 and T2 (somatic embryo germination media); T1:Y3 media + 1 mg/L BAP + 0.5 mg/L NAA + 0.25 mg/L IBA, T2:Y3 media + 1 mg/L BAP + 0.5 mg/LNAA + 0.25 mg/L IBA)				

Summary

In the current study, mature zygotic embryos were used for plantlet regeneration in the endangered palm species, *Areca concinna* through *in vitro* culture. The culture medium and hormonal concentrations played essential role in callogenesis and somatic embryogenesis of the palm. Plantlet regeneration was achieved by gradually reducing the, concentration of hormones during subculture and through constant monitoring of cultures. This protocol could be used for mass multiplication of the palms, thereby acting as a means to safeguard the germplasm of the endangered plant species *A. concinna*.

Abbreviations

2, 4-D: 2, 4-Dichlorophenoxy acetic acid

BAP: N6-benzylaminopurine

IBA: Indole Butyric Acid

LED: Light Emitting Diodes

NAA: Naphthalene Acetic Acid

PGRs: Plant Growth Regulators

Picloram: 4-Amino-3, 5, 6-trichloropyridine-2-carboxylic acid

SE: Somatic Embryogenesis

Y3: Eeuwen's Y3 Basal medium

Declarations

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Author contributions

AV planned and conducted the study MN, KP, AK supervised the experiment. AV wrote the manuscript, SS analysed the experimental data; MN revised the manuscript. All authors read and approved the manuscript

Conflict of interest

The authors declare no conflict of interest.

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Figures

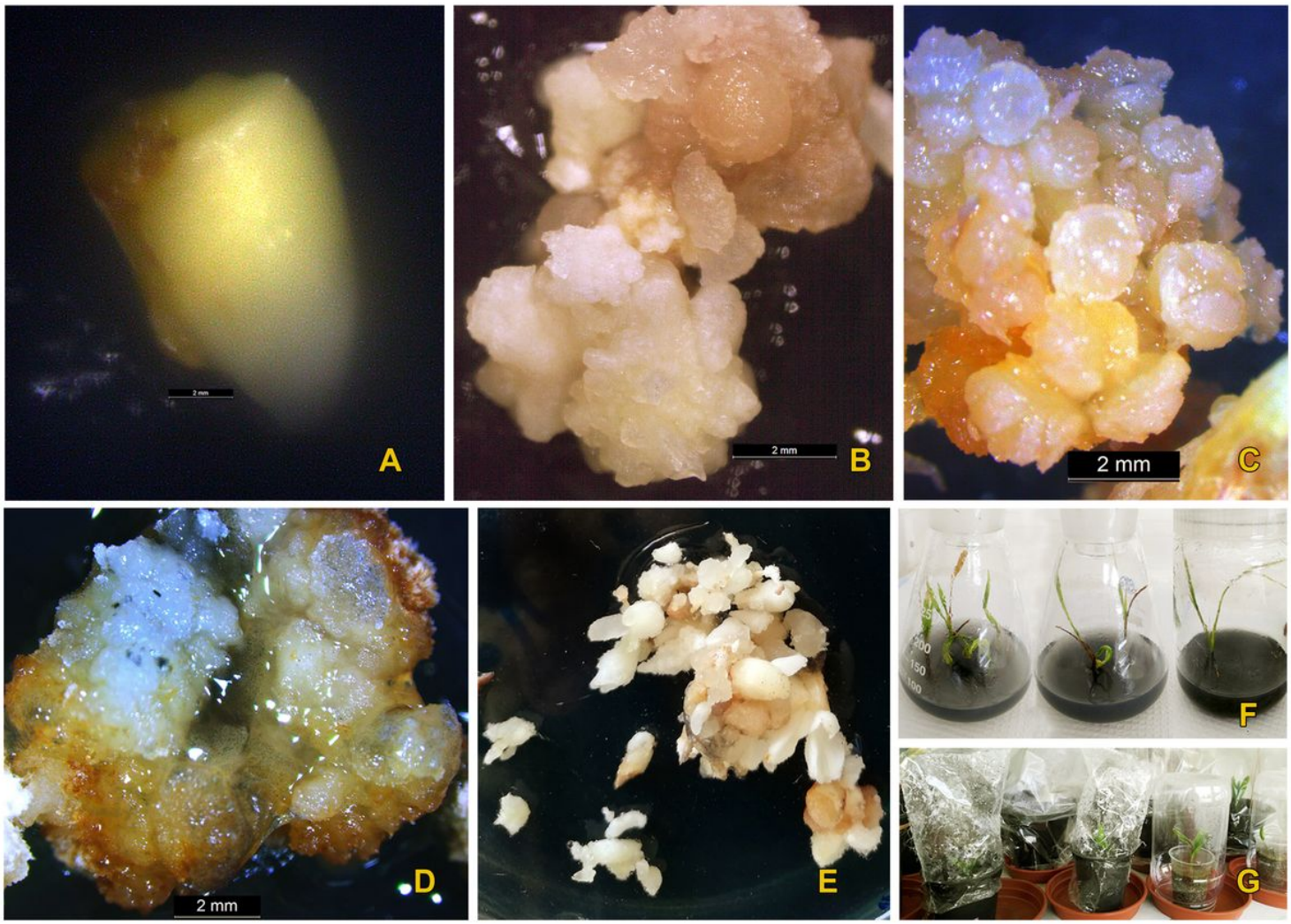

Figure 1

Micropropagation of *Areca concinna* via somatic embryogenesis from mature zygotic embryos; (A).Inoculation of mature zygotic embryos of *A.concinna* in callus induction media, (B). Callus initiation

from zygotic embryos of *A.concinna*, (C). Embryonic calli of *A.concinna* (D). Non-Embryogenic calli, (E). Somatic embryogenesis from the calli, (F). Germination and development of *A.concinna* Somatic embryos, (G). In vitro hardening of somatic embryo derived plants from *A.concinna*