Research Article

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Maintenance of embryogenic potential of calli derived from embryonic shoot of West Coast Tall cv. of coconut (*Cocos nucifera* L.)

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Abstract

Maintenance of embryogenic potential of calli is important as the totipotency is often lost in a short time *in vitro*. This caters to the need for year round availability of somatic embryos in a regenerable state. In the present study, 14 media combinations, with either 2,4-D or picloram as auxin source, were tested for maintaining embryogenic calli obtained from embryonic shoot explants of coconut. Irrespective of type and concentration of auxins, callusing was observed in all the media combinations. However, high dose of 2,4-D (above 74.6 µM) in the initial medium resulted in intense browning and lesser percentage of callusing. Embryogenic nature of calli could be maintained to a maximum of 21 weeks in medium supplemented with 2,4-D (74.6 µM) and subsequent culturing into higher concentration of 2,4-D (90.4 µM). Gene expression studies carried out using qRT-PCR revealed that genes such as *ECP, GST, LEAFY* and *WUS* were highly expressed in long term embryogenic calli (21 week old) and genes such as *SERK, GLP, WRKY* and *PKL* in initial embryogenic calli (21 days old). The study concludes that coconut plumular calli could be maintained for longer periods without compromising on the embryogenic potential of the calli.

Keywords: Coconut, embryogenic calli, embryonic shoot, gene expression, histology, plumule

Introduction

Coconut (*Cocos nucifera* L.) is a very important crop in tropical areas, providing cash and subsistence to smallholders. India was the first country to start systematic research in coconut in the year 1916 (Swaminathan, 1991). Tissue culture of coconut has been carried out in several countries including UK (Wye College), France (IRHO/ CIRAD), the Philippines (UPLB and PCA), Sri Lanka (CRI) and Mexico (CICY). Coconut tissue culture work was started at the ICAR-Central Plantation Crops Research Institute in early 1980s.

Coconut is a highly recalcitrant species with respect to tissue culture. Several explants such as immature embryos (Gupta *et al*., 1984), immature leaves (Raju *et al*., 1984; Buffard-Morel *et al*., 1992) and inflorescence tissues (Branton and Blake, 1984) have been tried and the results indicate that regeneration is possible, although rather difficult. However, only limited success has been achieved and the protocols lack repeatability (Rajesh *et al.,* 2005). Somatic embryos developed from plumular explants were capable of germination and subsequent development into plantlets, which could be successfully transferred to nursery (Chan *et al.,* 1998). Coconut plumular explants are white in colour, formed by small meristematic cells and consist of the shoot meristem surrounded by leaf primordia (Saenz *et al.,* 2006). Research on plumule culture was initiated at ICAR-CPCRI during 1998 and the potential of using this protocol for increasing the planting material production upto five to ten fold has already been reported (Rajesh *et al.,* 2005, 2014a).

Induction of embryogenic calli is the critical step for the success of plant regeneration and this

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depends on medium condition, growth regulators, seasonal variation *etc* (Rachmawati and Anzai, 2006). Totipotency of cells in embryogenic calli is often lost in a short time. Long-term maintenance of embryogenic calli is very useful in plant tissue culture to facilitate the year round availability of somatic embryos in regenerable state. The embryogenic competency of the calli of *Sorghum bicolor* could be maintained upto 57 weeks using silver nitrate and casein hydrolysate (Pola *et al.,* 2009). Effect of 2, 4-D and picloram for selection of long term totipotent green callus cultures of sugarcane was studied by Maureen and Paul (1990). The study concluded that the regeneration ability of callus cultures could be retained for more than 12 months on medium containing picloram as auxin source, whereas 2,4-D failed to sustain growth of totipotent calli.

During embryogenic induction of cells, there is differential gene expression resulting in synthesis of new mRNAs and proteins. This genetic information, in turn, elicits diverse cellular and physiological responses that are involved in 'switching over' of the developmental programme of the somatic cells (Chugh and Khurana, 2002). Many genes have been implicated to be involved in the gene expression machinery during somatic embryogenesis *viz., SERK* (somatic embryogenesis receptor-like kinase)*, LEAFY* (leafy cotyledon) and *BBM* (baby boom) (Chugh and Khurana, 2002). Analysis of *CnSERK* expression (*SERK* ortholog of coconut) showed that it could be detected in embryogenic tissues before somatic embryo development (Perez-Nunez *et al.,* 2009). Expression studies of somatic embryogenesis-associated genes could be used as proxy for testing the embryogenic potentials in long-term maintained calli. The present study was undertaken with an objective of maintenance of embryogenic potential of calli derived from embryonic shoot explant of West Coast Tall cultivar and expression studies of various genes known to be induced during somatic embryogenesis.

Materials and methods

Plant material

Matured nuts (11-12 months old) were harvested from West Coast Tall (WCT) palms from the farm at ICAR-CPCRI, Kasaragod. Embryos with endosperm (endosperm plugs) were excised from the cut opened nuts using a cork borer and placed in distilled water and sterilized with 0.01 per cent mercuric chloride ($HgCl_2$) for 5 minutes and rinsed thrice with sterile distilled water to remove the traces of $HgCl_2$. Embryos were excised from endosperm plugs, surface sterilized with 20 per cent sodium hypochlorite (NaClO) solution for 20 minutes and subsequently rinsed with sterile distilled water for 5-6 times. Embryonic shoot were scooped from the embryos and were inoculated to callus induction medium.

Callus induction

Y3 basal medium (Eeuwens, 1976), was supplemented with 30 g L^{-1} sucrose, 1 g L^{-1} activated charcoal and various concentration of 2,4-D (2,4- dichlorophenoxy acetic acid) and picloram, supplemented with and without TDZ (Thidiazuron) (Table 1). A total of fourteen media [MA to MN] were followed to maintain the embryogenic competence of calli. After adjusting the pH to 5.8, the media were autoclaved at 121 °C for 20 min. The excised embryonic shoot explants were inoculated into ICIM (initial calli induction media) and kept for incubation in dark conditions at 27+2 °C.

Table 1. Details of the media combinations with sucrose (30 g L^{-1}), agar (5.8 g L^{-1}) and charcoal (1 g L^{-1}) used for fourteen different protocols

Media	2,4-D (µM)	Picloram (µM)	TDZ (µM)
Control	-	-	-
MA	100	-	-
MB	100	-	4.54
MC	200	-	-
MD	200	-	4.54
ME	74.6	-	-
MF	100	-	-
MG	100	-	4.54
MH	74.6	-	-
MI	-	25	-
MJ	-	25	4.54
MK	-	50	-
ML	-	50	4.54
MM	74.6	-	4.54
MN	74.6	-	4.54

Table 2. Details of media combination used in fourteen different protocols

Protocol	Unit - (μM L^{-1})	A= 2,4-D C=TDZ	Pic=Picloram	HF=hormone free
MA	A100 (30 days)	A1 (10 days)	A1 (10 days)	A1 (10 days)
MB	A100 + C 4.54 (30 days)	A1 + C4.54 (10 days)	A1 + C4.54 (10 days)	A1 + C4.54 (10 days)
MC	A200 (30 days)	HF (10 days)	HF (10 days)	HF (10 days)
MD	A200 + C 4.54 (30 days)	HF (10 days)	HF (10 days)	HF (10 days)
ME	A74.6 (30 days)	A90.4 (10 days)	A90.4 (10 days)	A90.4 (10 days)
MF	A100 (30 days)	HF (10 days)	HF (10 days)	HF (10 days)
MG	A100 + C 4.54 (30 days)	HF (10 days)	HF (10 days)	HF (10 days)
MH	A74.6 (30 days)	A45.2 (10 days)	45.2 (10 days)	45.2 (10 days)
MI	Pic25 (30 days)	Pic10 (10 days)	Pic10 (10 days)	Pic10 (10 days)
MJ	Pic25 + C4.54 (30 days)	Pic10 + C4.54 (10 days)	Pic10 + C4.54 (10 days)	Pic10 + C4.54 (10 days)
MK	Pic50 (30 days)	Pic25 (10 days)	Pic25 (10 days)	Pic25 (10 days)
ML	Pic50 + C4.54 (30 days)	Pic25 + C4.54 (10 days)	Pic25 + C4.54 (10 days)	Pic25 + C 4.5 (10 days)
MM	A74.6 + C4.54 (30 days)	A90.4 + C4.54 (10 days)	A90.4 + C4.54 (10 days)	A90.4 + C4.54 (10 days)
MN	A74.6 + C4.54 (30 days)	A45.2 + C4.54 (10 days)	A45.2 + C4.54 (10 days)	A45.2 + C4.54 (10 days)

Callus maintenance

After one month of culture, the initiated calli were sub-cultured to different concentration of auxins with different subculture duration (Table 2). After 60 days of culture in media supplemented with hormones, the calli were subcultured into hormone-free media at 10 days interval once and 30 days interval twice. Observations were taken periodically for callus initiation, growth and texture of the cultures.

Somatic embryogenesis and regeneration

Proembryos, which were formed from embryogenic calli, were transferred to hormone-free media supplemented with high concentration of charcoal (0.25%) for the maturation of somatic embryos. Y3 medium, supplemented with BAP (22.2 μM), glutamine (34.2 μM), GA_3 (2.8 μM) along with 2,4-D (0.045 μM), was utilized as initial regeneration medium for the germination of somatic embryos. Medium supplemented with BAP (88.8 μM) and IBA (19.6 μM) was used for plantlet recovery.

Statistical analysis

Each treatment was conducted in three replications and designed as CRD. The results obtained were analyzed in a two way ANOVA by using SAS software. Level of significance was represented by CD at 5 per cent.

RNA isolation, cDNA synthesis and RT-PCR analysis

Total RNA from embryogenic calli (control *i.e.* 21-25 days old) and long term maintained embryogenic calli (21 weeks old) was isolated using Nucleospin RNA plant kit (Macherey-Nagel) and integrity, concentration and quality check and DNase 1 (Fermentas) treatment were performed (Fig. 33). Reverse transcription was performed with High Capacity RNA-to-cDNA kit (Life Technologies) using aliquots of total RNA extracted. The cDNA samples were diluted to 20 ng μL^{1}. Amplification was carried out in a reaction mixture containing 2 μL of 10X reaction buffer, 0.2 μL of 10 mM dNTP mix, 1 μM of each forward and reverse primer, 1 μL of synthesized cDNA and 1 U of *Taq* polymerase. RT-PCR reactions were carried out using BIORAD thermal cycler. The cycling program was as follows: 1 min at 94 °C, 30 cycles of 30s at 94 °C, 1 min at 55 °C, 1 min at 72 °C and a 10 min extension at 72 °C, with a negative control (Fig. 34).

Quantitative RT-PCR

All real-time PCR reactions were performed using the StepOne™ Real Time PCR system (Perkin-Elmer Applied Biosystems) and the amplifications were done using the SYBR Green PCR Master Mix (Applied Biosystems) (Fig. 35). The thermal conditions were as follows: initial

Fig. 1


Fig. 2


Fig. 3


Fig. 4


Fig. 5

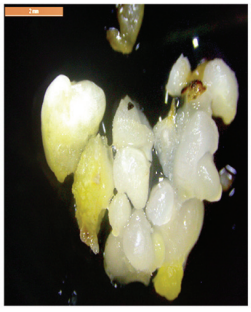


Fig. 6

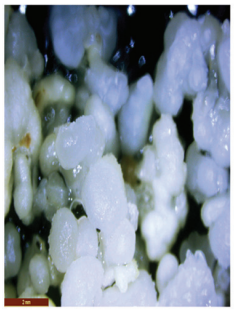
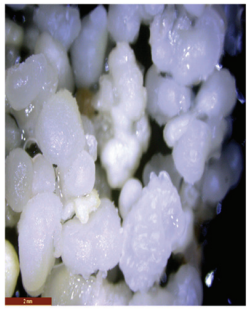


Fig. 7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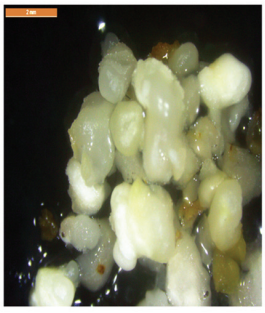


Fig. 9

Fig. 10


Fig. 11

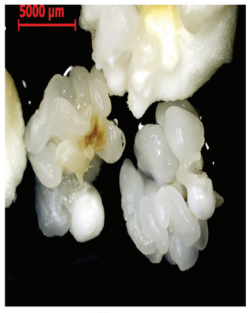


Fig. 12


Fig. 13

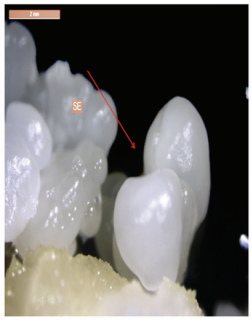


Fig. 14

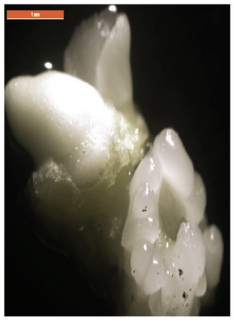


Fig. 15

Fig. 16

Figs. 1-16. Developmental stages of coconut calli derived from embryonic shoot explants. Fig. 1. Callus initiation from embryonic shoot explant after 30 days; Fig. 2. Embryogenic calli after 45 days; Fig. 3. Embryogenic calli after 60 days; Fig. 4. Embryogenic calli after 80 days; Fig. 5. Embryogenic calli after 100 days in ME medium; Fig. 6. Embryogenic calli after 100 days in MH medium; Fig. 7. Embryogenic calli after 120 days in MM medium; Fig 8. Embryogenic calli after 120 days in MN medium; Fig. 9. Embryogenic calli with slight browning after 135 days in ME medium; Fig. 10. Embryogenic calli after 135 days; Fig. 11. Multiplication of embryogenic calli after 150 days; Fig. 12. Multiplied embryogenic calli showing the initiation of pro-embryo after 150 days; Fig. 13. Formation of pro-embryo from embryogenic calli; Fig. 14. Initiation of somatic embryo; Fig. 15. Maturation of somatic embryo; Fig. 16. Germination of somatic embryo

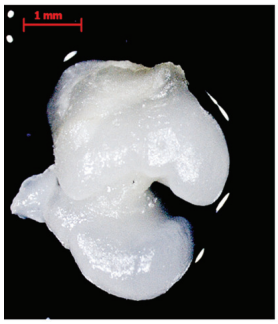


Fig. 17

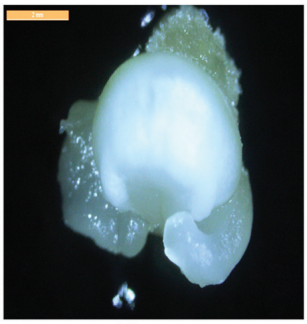


Fig. 18


Fig. 19

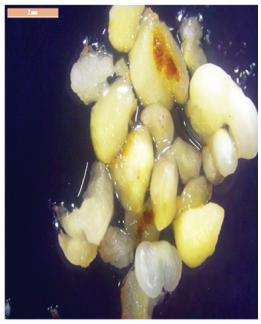


Fig. 20


Fig. 21

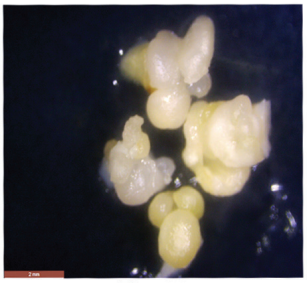


Fig. 22

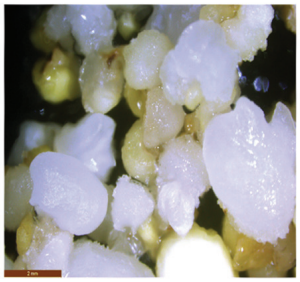


Fig. 23

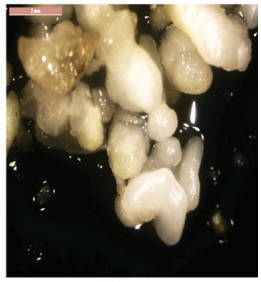


Fig. 24

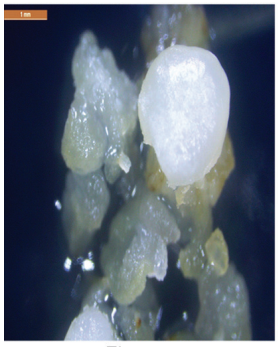


Fig. 25


Fig. 26

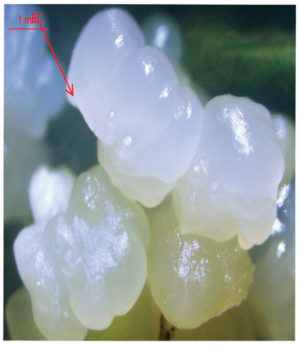


Fig. 27

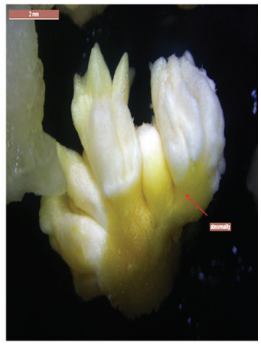
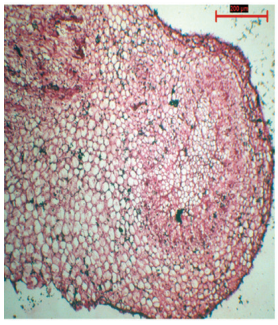


Fig. 28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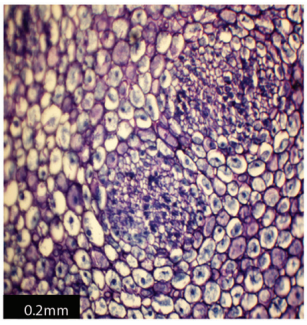

Fig. 30

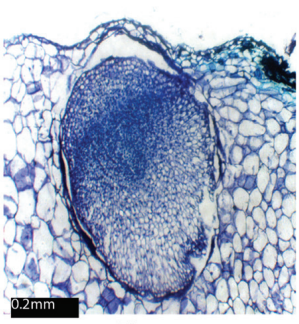

Fig. 31

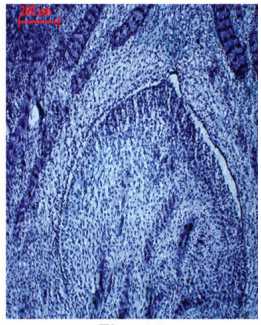
Fig. 32

Figs. 17-32. Comparison of normal and abnormal calli and histological studies. Fig. 17. Embryogenic calli after 30 days; Fig. 18. Abnormal embryogenic calli after 30 days; Fig. 19. Normal embryogenic calli after 60 days; Fig. 20. Abnormal embryogenic calli after 60 days; Fig. 21. Normal embryogenic calli after 80 days; Fig. 22. Abnormal embryogenic calli after 80 days; Fig. 23. Normal embryogenic calli after 120 days; Fig. 24. Abnormal embryogenic calli after 120 days; Fig. 25. Multiplication of embryogenic calli after 135 days; Fig. 26. Formation of compact embryogenic calli; Fig. 27. Proembryo formation after 150 days; Fig. 28. Formation of abnormal meristemoid after 150 days; Fig. 29. Meristematic zone formation; Fig. 30. Formation of meristematic centers; Fig. 31. Pro-embryo formation; Fig. 32. Formation of shoot meristem

Table 3. Effect of fourteen media combinations towards the maintenance of embryogenic competence of the calli. Level of significance was represented by CD at 5 per cent

Media	30 days	45 days	60 days	80 days	100 days	120 days	135 days	150 days
MA	0.97±0.03	14.23±1.73	17.20±1.81	16.67±1.98	12.30±1.48	5.47±0.97	1.67±0.53	2.2±0.58
MB	1.00±0.00	14.03±1.71	13.47±2.34	14.63±1.63	9.67±2.00	5.07±0.83	0.67±0.26	0.87±0.26
MC	1.00±0.00	12.80±1.58	10.97±1.82	10.57±0.57	6.20±0.17	4.13±0.62	0.8±0.05	1.10±0.05
MD	1.00±0.00	14.87±1.09	15.57±0.40	13.77±1.67	9.50±0.51	3.37±0.13	0.53±0.03	0.87±0.12
ME	1.00±0.00	7.80±0.05	14.27±0.21	24.97±0.30	26.73±0.49	31.00±0.90	38.03±1.2	47.9±1.74
MF	1.00±0.00	13.33±2.11	13.07±0.59	9.3±0.85	4.87±1.42	2.60±0.9	3.83±2.76	0.50±0.00
MG	1.00±0.00	14.37±1.08	14.93±1.20	12.20±1.42	7.67±1.11	3.67±0.57	1.00±0.29	0.87±0.26
MH	1.00±0.00	10.07±0.08	14.90±0.45	16.17±0.61	17.47±2.51	18.47±1.99	16.37±1.0	15.23±0.9
MI	1.00±0.00	10.67±0.21	15.7±0.61	18.93±0.48	19.40±0.70	15.63±2.58	10.93±3.1	10.1±2.78
MJ	1.00±0.00	9.57±0.42	15.27±0.69	14.87±2.03	10.0±2.61	8.6±2.32	4.2±1.94	3.67±1.83
MK	1.00±0.00	14.77±0.64	20.13±1.92	15.73±0.52	11.63±1.68	7.47±1.40	1.87±0.16	1.47±0.26
ML	1.00±0.00	9.90±0.23	17.33±0.96	15.13±1.70	14.83±2.55	7.77±1.54	1.63±0.4	1.43±0.21
MM	1.00±0.00	6.70±0.14	18.23±0.03	2.27±0.34	28.2±0.76	31.60±0.23	31.6±0.83	33.5±1.89
MN	1.00±0.00	8.00±0.05	16.47±0.60	23.6±0.83	29.57±0.26	31.17±0.54	38.0±1.03	44.80±0.8

CD at 5 per cent Protocol 1.29* Days 0.97* Protocol x Days 3.64*

holding stage 52 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95°C for 15 s and a final step at 60 °C for 1 min. All reactions were carried out in triplicate in 48-well reaction plates. The relative quantification in gene expression of somatic embryogenesis receptor-like kinase *(SERK),* leafy cotyledon *(LEAFY), WRKY,* wuschel *(WUS),* extracellular protein (*ECP),* germin-like protein *(GLP),* glutathione-S-transferase *(GST)* and pickle *(PKL)* was determined using the $2\text{-}\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) and normalized to an internal control gene, α-tubulin. The details of the primers used for qRT-PCR analysis (Rajesh *et al.,* 2015) are given in Table 4.

Histological studies

The samples (60, 150, 180 and 250 days old cultures) were fixed in standard fixative Carnoy's 'B' fluid (chloroform-30mL; absolute alcohol-60 mL; glacial acetic acid-10 mL) to localize RNA, DNA, proteins and polysaccharides. Fixed materials were dehydrated serially using alcohol and butanol. A mixture of paraffin wax and bees wax of melting point 58-60 °C, was used for infiltration and embedding. Uniformly thin sections of 10 µm thickness were cut using Leica RM 2145 rotary microtome. Periodic acid Schiff's reagent and toluidine blue stains were used to localize polysaccharides and nucleic acids respectively.

Table 4. Gene-specific primers used in qRT-PCR experiments

Sl. No.	Gene	Sequence (5'-3')
1.	*SERK*	F: GCCGTACATCGAAACTTGCT
		R: GGATCACAATGATCATGCAAA
2.	*GST*	F: CCTGCCTTTGGTGATGTCATA
		R: GAGGCATGGGTGATGAAGTT
3.	*WUS*	F: TGTACTGCCTGCCTGGTTC
		R: CCACCTGATAAGAGGGACGA
4.	*GLP*	F: CCTGGTTAACACCCACAACC
		R: CAGGGTTCGAAGTCACGAAG
5.	*PKL*	F: CCAAGACACATCCTCGACTG
		R: TCCCAGGTGCATTCATCATA
6.	*WRKY*	F: GGATGATTGTGTTCACCCTTG
		R: GCAACCGACAACTAGTGGAGA
7.	*ECP*	F: TATAGGCGAGGCTTTGGAGA
		R: CTGGCAACATTCATGGTACG
8.	*LEAFY*	F:GTGTCCCACCAAGGTTAC
		R: CCCTCCTCAGTGCATTTG
9.	*α-tubulin*	F:CTGGTGTCCTACTGGCTTC
		R: GACCATGATTACGCCAAG

Results

Callus induction

Excised embryonic shoot explants were inoculated into fourteen different media

combinations (Table 1). Slight bulging was observed after 10 days of culture incubation. Media supplemented with 2,4-D (ME, MH, MM and MN) showed frilled type of callus initiation after 25-30 days of culture incubation (Fig. 1). Media supplemented with picloram *i.e.,* MI, MJ, MK and ML also showed the same type of callus initiation (Fig. 2). Some cultures showed slight browning in MA, MB, MC, MD MF and MG which were supplemented with high dose of 2,4-D (Fig. 20). Calli obtained from initial media were chopped and sub-cultured after 25 days of dark incubation.

Callus maintenance

After 25 days of dark incubation, embryogenic calli were chopped finely and inoculated to further media to maintain embryogenic calli (Table 2). Embryogenic calli obtained in the media supplemented with 2,4-D (74.6 µM) were sub-cultured to higher (ME and MM) and lower concentrations of 2,4-D (MH and MN). Subculture duration was followed as mentioned in Table 2 to maintain embryogenic competence of the calli. After 55-60 days of culture incubation, highest percentage of embryogenic calli was observed in ME, MH, MM and MN (Fig. 3, 19) (Table 3). Multiplication of embryogenic calli occured after 80 days (Fig. 4, 21). Number of embryogenic clumps were increased (30-35 nos) after 100 days of culture incubation (Fig. 5, 6). It was observed that ME, MH, MM and MN media were found to be the best media to maintain the embryogenic calli for more than three months (Table 3). It was also noticed that after 120 days of culture incubation, in ME medium, multiplication of embryogenic calli was noticed (Fig. 23) and embryogenic calli which were cultured in MH, MM and MN showed browning and slight compact nature (Fig. 7, 8). During chopping of initial calli, severe browning (with compact nature) was observed in rest of the media *i.e.,* MA, MB, MC, MD MF and MG and calli obtained from these media showed compact structure (Fig. 22). Media supplemented with various concentrations of picloram also showed embryogenic calli formation *i.e.,* MI, MJ, MK and ML (Fig. 17) and few abnormal calli was also noticed (Table 3), (Fig. 18). After 120 days of incubation, embryogenic calli turned to abnormal compact structure (Fig. 24). Only few cultures after 135 days of incubation showed the formation of normal calli (Fig. 25) and majority of the calli showed abnormal development (Fig. 26).

In case of ME medium, number of embryogenic clumps increased after 135 days of culture incubation (38-40) (Fig. 9, 10). Thus, it was found that ME media was more suitable to maintain embryogenic competence of the calli (Table 3). In this condition, calli obtained from the initial media was finely chopped into small pieces and sub-cultured, into increased concentration of 2,4-D *i.e.,* 90.4 µM. Subculture was carried out for every 10 days to same media followed by chopping (Table 2). Later, these embryogenic calli were transferred to hormone-free media and subcultured at 30 days intervals and number of embryogenic clumps was increased (45-50) after 150 days. No further improvement was observed after 150 days and it was also noticed that some of the clumps showed compact nature. Using this protocol, embryogenic competence could be maintained for 21 weeks (Fig. 11, 12).

Somatic embryogenesis and regeneration

Embryogenic calli, which were maintained in ME media, showed pro-embryo formation (Fig. 13, 27) after 30 days, which were again subcultured to hormone-free media supplemented with high concentration of charcoal (0.25%) for maturation of somatic embryos (Fig. 14, 15). Some cultures showed abnormal development of meristemoids (Fig. 28). Germination of the somatic embryos (Fig. 16) occurred in Y3 medium supplemented with BAP (22.2 µM), glutamine (34.2 µM) and GA_3 (2.8 µM) along with 2,4-D (0.045 µM), which was utilized as initial regeneration media. Gradual increase in the concentration of BAP (88.8 µM), IBA (19.6 µM) resulted in the recovery of normal plantlets.

Gene expression studies

In order to analyze the expression patterns of eight genes in 21 days and 21 week old embryogenic calli, cDNA was prepared. RT-PCR reaction was used to amplify the eight genes and a specific single PCR product was obtained using the designed primers (Fig. 34). We then performed qRT-PCR using the designed primers to examine the differential expression of eight genes in both embryogenic and non-embryogenic calli. Amplification plot (Fig. 35) and melt curve analysis indicated that the six primer pairs amplified a

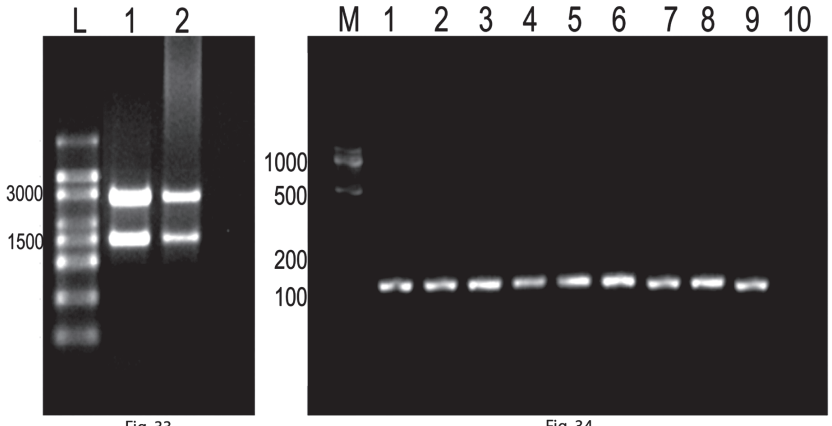

Fig. 33

Fig. 34

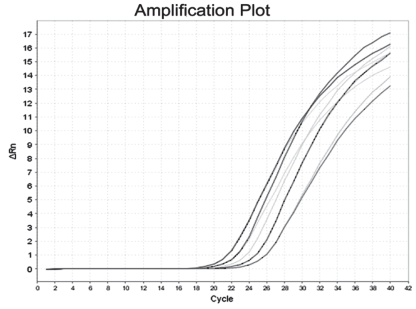

Fig. 35

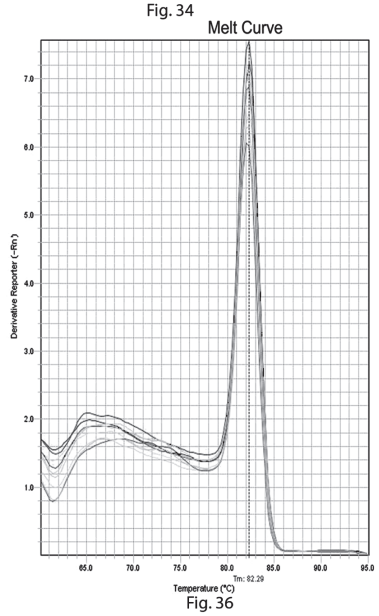

Fig. 36

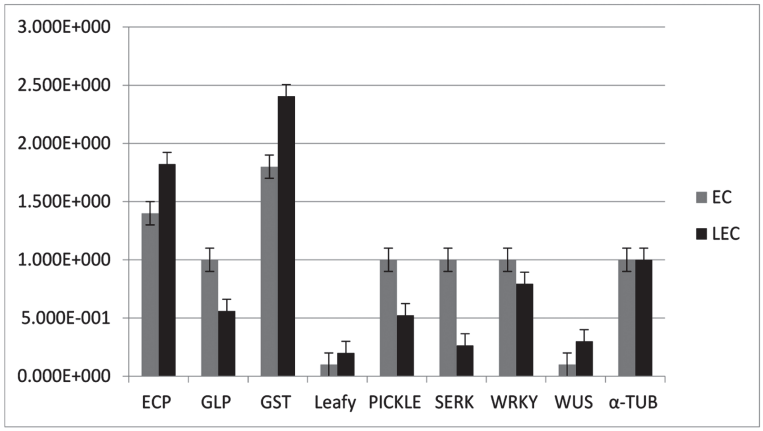

Fig. 37

Figs. 33-37. Real-time analysis of pattern of gene expression in embryogenic calli (EC) and long term embryogenic calli (LEC). Fig. 33. RNA obtained from EC and LEC. High range RNA ladder (L); Fig. 34. RT-PCR gel image of eight genes (Lanes 1-8), α-tubulin (Lane 9) and negative control (Lane 10); Fig. 35. Amplification plot of quantitative RT-PCR analysis of expression of eight genes; Fig. 36. Derivative of melt curve analysis of amplicon of eight genes after SYBR-green real-time assay; Fig. 37. Gene expression level of eight genes in EC and LEC samples

specific PCR product (Fig. 36). It was observed that, the genes such as *ECP, GST, LEAFY* and *WUS* tended to be more highly expressed in long-term maintained embryogenic calli (LEC) than initial embryogenic calli (EC). Expression level of *ECP, GST, LEAFY* and *WUS* were significantly increased by 1.83-fold, 2.39-fold, 0.37-fold and 0.89-fold respectively, in long-term maintained embryogenic calli (LEC) compared to embryogenic calli (EC). With regard to the down-regulated genes, expression level changes of 1.05-fold for *GLP*, *PKL, SERK* and *WRKY* were observed in embryogenic calli (EC) compared with long-term maintained embryogenic calli (LEC) (Fig. 37). α-tubulin real time profiling clearly indicated its stable expression in all the biological samples used for normalizing data.

Histological studies

The histological sections showed that the embryogenic callus was formed by densely stained meristematic cells. Occurrence of a peripheral meristematic zone associated with callus growth was also observed (Fig. 29). Embryogenic calli formed (after 60 days of incubation) showed the formation of meristematic centers (Fig. 30) located at the periphery of the callus. Long-term maintained (150 days old) embryogenic calli showed densely stained actively dividing meristematic cells and meristematic centers. These embryogenic calli formed pro-embryos when transferred to a regeneration media (Fig. 31). These pro-embryos transformed into somatic embryos which shows the presence of shoot meristem consists of actively dividing cells (Fig. 32).

Discussion

The present study describes long-term maintenance of embryogenic calli obtained from coconut embryonic shoot explants. This source of juvenile tissue has been shown to respond well and more rapidly than other explants in terms of callus formation and the development of embryogenic capacity (Hornung, 1995). Since the totipotency of cells in embryogenic calli is often lost in short time, long term maintenance of embryogenic calli cultures is very useful in plant tissue culture to facilitate the year round availability of somatic embryos in a regenerable state (Pola *et al.,* 2009).

In the present study, fourteen different media were tried to maintain the embryogenic competence of the calli obtained from embryonic shoot. Full strength Y3 basal media were supplemented with various concentrations of 2,4-D and picloram with or without TDZ. Out of fourteen media combinations tried, media supplemented with 2,4-D (16.5 mg L^{-1}) showed initiation of calli after 25 days of culture incubation. Initiation of calli was noticed from the perivascular strands of the leaf primordia. The same concentration of 2,4-D in combination with TDZ (1mg L^{-1}) was tried by Rajesh *et al.* (2005). They observed calli initiation after four weeks of culture incubation from perivascular strands of the leaf primordia. In the present study, calli obtained from the initial media were finely chopped into small pieces and subcultured into increased concentration of 2,4-D *i.e.,* 20 mg L^{-1}. Subculture was carried out for every 10 days to the same media. Effect of 2,4-D on callus induction and maintenance on *Sorghum bicolor* was studied by Pola *et al.* (2009). Calli were induced in full-strength MS media supplemented with 2 mg L^{-1} of 2, 4-D and 0.5 mg L^{-1} of kinetin. Three weeks old embryogenic calli were subcultured to same media for embryogenic calli maintenance. Similarly in wheat (*Triticum aestivum* L.), embryogenic calli were maintained in 2,4-D (2 mg L^{-1}) (Rashid *et al.,* 2009; Malik *et al.,* 2003), where as in case of coconut endosperm, embryogenic calli could be maintained in the medium supplemented with 5 mg L^{-1} of 2,4-D with 2 mg L^{-1} of BA (Prakash *et al.,* 1985). However in case of rice, concentrations of 2,4-D higher than 0.5 mg L^{-1} were more appropriate for long-term maintenance of embryogenic calli and to suppress the growth of unwanted tissues (Khayri and Edwin 1995).

In *Stevia rebaudiana*, embryogenic calli was maintained in half strength MS media in hormone combination of 1.1 mg L^{-1} NAA and 2 mg L^{-1} BAP (Das *et al.,* 2006). In our study, somatic embryogenesis from long-term maintained embryogenic calli was observed in the hormone-free medium supplemented with 2.5 mg L^{-1} of charcoal and regeneration was observed in the media contained BAP (22.2 µM), glutamine (34.2 µM), and GA_3 (2.8 µM) along with 2,4-D (0.045 µM). In case of *Sorghum bicolor* (Pola *et al.,* 2009), regeneration could be observed in the media incorporated with 400 mg L^{-1} of casein hydrolysate

and 10 mg L^{-1} of $AgNO_3$. Effect of 0.1 mg L^{-1} IAA on regeneration of long term preserved embryogenic calli was studied by Rashid *et al.* (2009). In suspension culture of bent grass and Japonica rice, plant regeneration was observed in a long-term cell culture with a medium supplemented with maltose and lactose instead of sucrose (Asano *et al.,* 1994).

In case of media supplemented with picloram, embryogenic calli formation could be observed in second subculture, later it turned to abnormal compact structure. Similarly, during microspore embryogenesis in coconut anther (Perera *et al.* 2009), picloram (in combination with 100 µM 2, 4-D) was not effective in inducing the formation of calli which is in contrast to the result obtained by Han *et al.* (2000) in which picloram (4 µM) was found to be essential for induction and maintenance of calli in cultured anthers of *Lilium* species. The effect of 2,4-D and picloram for selection of long term totipotent green callus cultures of sugarcane was studied by Maureen and Paul (1990). This study concluded that the regeneration ability of callus culture could be retained for more than 12 months on medium containing picloram as auxin source whereas 2,4-D failed to sustain growth of totipotent calli. Calli obtained from endosperm of *Cycas revoluta* (Kiong *et al.,* 2008) were also successfully maintained in 10 µM picloram at normal photoperiod (16 h light, 8 h dark). The calli treated with10 µM picloram that was incubated in 24 h dark condition was found to exhibit less browning effects. However in case of ficus, embryogenic calli were maintained in a medium supplemented with 3 mg L^{-1} of picloram (Anna Ling *et al.,* 2007).

Quantitative RT-PCR data analyses were carried out to study the genes which were expressed during embryogenic calli induction. Alpha-tubulin was used as an endogenous control for the expression analysis of coconut (Rajesh *et al.,* 2014b). The qRT-PCR data analysis revealed that expression of *SERK* gene was significantly increased by 1.05 folds in embryogenic calli than long term maintained embryogenic calli. In an earlier study in coconut (Perez *et al.,* 2009), it was reported that *CnSERK* expression occurred in meristematic centers, where the embryogenic structures are formed, and in turn these structures eventually formed the somatic embryos, thus suggesting that *CnSERK* expression could be used as a marker of cell competence to form somatic embryos in coconut tissues cultured *in vitro*. Expression of *LEAFY* gene was significantly increased by 0.37 folds in long-term maintained embryogenic calli than embryogenic calli. In contrast to these results Ledwon and Malgorgata (2009) reported increased activity of *LEAFY* during somatic embryogenesis compared to other developmental stages in *Arabidopsis*. Expression of *WUS* gene was increased by 0.89 folds in long-term maintained embryogenic calli than embryogenic calli. Similar results were reported by Ying *et al.* (2009) in *Arabidopsis* where the high expression of *WUS* could be observed in the case of embryogenic callus compared to somatic embryos. Expression of *GST* gene was significantly increased by 2.39 folds in long term maintained embryogenic calli than embryogenic calli. However, high expression of *GST* gene was observed in somatic embryo than embryogenic calli in grape (Malabadi *et al.,* 2013). Expression of *ECP* gene was significantly increased by 1.83 folds in long term maintained embryogenic calli than embryogenic calli. However, increased activity of *ECP* was reported during the torpedo stage of somatic embryo in case of carrot and *Arabidopsis* (Chugh and Khurana, 2002). Expression of *PKL, GLP* and *WRKY* genes were significantly increased by 1.05 folds in embryogenic calli than long term maintained embryogenic calli. The presence of GLPs in the extracellular compartment of stage-one somatic embryos was demonstrated by David *et al.* (1996). Expression of *WRKY* in embryogenic calli, in long-term preserved embryogenic calli was comparatively less. Kayum *et al*. (2015) documented the expression and identification of *WRKY* family genes under biotic and abiotic stresses in *Brassica rapa*. It is also reported that, *WRKY* gene family was mainly in response to environmental factors, especially to pressure, imposed by diverse phytopathogens (Ulker *et al.,* 2004).

The histological sections showed that the embryogenic calli was formed by densely stained meristematic cells. Embryogenic calli formed after 60 days of incubation showed the formation of meristematic centers located at the periphery of the callus (Chan *et al.,* 1998). Long term maintained (150 days old) embryogenic calli showed densely stained actively dividing meristematic cells and the presence of meristematic centers. This indicated of

embryogenic capacity and of a multicellular pathway for embryo formation, which is in conformity to the results obtained by Verdeil and Buffard-Morel (1995). In regeneration media, these embryogenic calli formed pro-embryos. These proembryos transformed into somatic embryos which showed the presence of shoot meristem consisting of actively dividing cells. The occurrence of peripheral meristematic zone associated with the callus growth has also been observed in explants of coconut inflorescence (Verdeil *et al*., 1994, 2001).

To conclude, in coconut, embryonic shoot responded well for *in vitro* culturing and produced embryogenic calli. The study also concluded that embryogenic calli obtained from embryonic shoot could be maintained for longer periods (21 weeks) without disturbing the embryogenic potential as confirmed from expression patterns of somatic embryogenesis inducing genes and also through histological studies.

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