Short Communication

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Initiation of coconut cell suspension culture from shoot meristem derived embryogenic calli: A preliminary study

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ABSTRACT

An attempt was made to establish highly competent embryogenic cell suspension culture in coconut, a species recalcitrant to in vitro culture. Embryogenic calli were initiated from shoot meristem explants of coconut. Y3 medium supplemented with 2.4-D (4.5 μM) and glutamine (34.2 μM) was found to be the best medium to initiate cell suspension. Growth evaluation was done by packed cell volume (PCV) and it was found that maximum growth volume of 9.9% was reached at 200 days of culture initiation. About 52% of viable cells were detected through fluorescent microscopy. Cell aggregation was noticed in Y3 medium supplemented with glutamine (34.2 μM) , malt extract (100mg/l), biotin (40.9 μM) and kinetin (9.3 μM), but further progress could not be achieved. It was also observed that embryogenic calli were not of a friable type, but were associated with densely aggregated cells. Because of its hard nature, we were unsuccessful to obtain high quality cell suspension.

KEY WORDS: KEYWORDS: Coconut, shoot meristem, embryogenic calli, cell suspension

Coconut (*Cocos nucifera* L.) is a woody species, belonging to the family Arecaceae. Since the every part of the coconut tree is made into universally used products, it is popularly known as "tree of life." Since the coconut is a recalcitrant species with respect to tissue culture, only limited success has been achieved, and the protocols lack repeatability (Rajesh *et al.,* 2005; 2014). It is very important to make use of a protocol that successfully solves the major constraints during somatic embryogenesis and its regeneration. It is possible to establish highly competent embryogenic cell suspension cultures to overcome the major bottlenecks during *in vitro* culture (Wang *et al.,* 2002). In a cell suspension, callus is suspended in a liquid callus induction media, containing all required nutrients and elements, to allow for optimal growth which acts to turn all cells into undifferentiated cells (King, 1984). Suspension cultures consisting of proembryonic masses established from calli shows a high competency for somatic embryogenesis (Fki *et al.,* 2003).

In this present study, embryogenic calli initiated from coconut shoot meristem explants were utilized for the establishment of cell suspension cultures. Initially, a date palm suspension culture protocol (Fki *et al.,* 2003) was followed in which 500 mg of coconut embryogenic calli, derived from shoot meristem explants, was weighed and crushed with tissue grinder and inoculated to a full strength Y3 medium supplemented with 2,4-D (4.5 µM), glutamine (34.2 µM), KH_2PO_4 (882.3 µM), adenine (222 µM), and activated charcoal (300 mg/l). About 30 ml of culture media was poured to 150 ml of Erlenmeyer flask. Cultures were kept in a rotary shaker at 120 rpm at 28°C ± 2°C. Sub-cultures were carried out at 7 days intervals 5-6 times. After this, cultures were sub-cultured to a medium containing glutamine (34.2 µM), KH_2PO_4 (882.3 µM), adenine (222 µM), and activated charcoal (300 mg/l). In this stage, cultures were sub-cultured at 20 days intervals. Cell initiation commenced after 75 days of culture incubation, and the cells were observed to be highly vacuolated and elongated. However, in the case of date palm, highly heterogeneous suspension was formed which consists of cells at various stages of differentiation, and large number of somatic embryos were formed in a medium supplemented with 2,4-D (4.5 µM) (Fki *et al.,* 2003). The coconut suspensions were transferred to a medium supplemented with thidiazuron

(4.5 µM) and spermine (50 µM). However, it was noticed that, in this medium, proper cell multiplication was not formed.

Experiments were also conducted to study the effect of different growth regulators on coconut cell suspensions. Eeuwen's Y3 (1976) basal media supplemented with varying concentrations of 2,4-D (0.45-45 µM) and picloram (0.41-41 µM) in combination with 6-benzyle adenine (2.22 µM) were prepared. Sub-cultures were carried out at 7 days intervals to the same medium combination. Cell initiation was observed in all the media combinations. Based on the cell count, it was noticed that medium supplemented with 2,4-D (4.5 µM) showed a better response compared to rest of the media combinations. In oil palm, in medium supplemented with 2,4-D (4.5 µM) in combination with naphthalene acetic acid (NAA) (0.5 µM), the good proliferation of cell suspension was achieved (Tarmizi *et al.,* 2002). Furthermore, in the case of oil palm, fine suspensions, on transfer to media supplemented with 2,4-D (0.45 µM), formed cell aggregates (Palanyandy *et al.,* 2013). In the present study, the suspensions obtained were transferred to a media supplemented with a high concentration of glutamine (34.2 µM). Cell multiplication increased, but cell aggregates were not formed from these cultures. Percentage of round shaped cells was less compared to elongated cells. The proliferation of somatic embryos in date palm was observed in a medium containing 2,4-D (4.5×10^{-7} M), glutamine (6.7×10^{-4} M), and abscisic acid (10^{-5} M) (Zouine *et al.,* 2005).

We have also carried out further experiments to achieve cell aggregation in coconut with high concentrations of 2,4-D (90, 180, 270 µM) (Table 1). Cultures in medium supplemented with 2,4-D (180 µM) developed into the fine suspension. However, very few number of cell aggregates were observed (Figure 1a). Experiments were also conducted to study the effect of different growth supplements viz., full strength Y3 basal media supplemented with sucrose (50 g), glutamine (34.2 µM), malt extract (100 mg/l), biotin (40.9 µM), and kinetin (9.3 µM) (Table 2). Media and cell suspensions were added in 100 ml of Erlenmeyer flask in 3:1 ratio. Cell number and shape of the cells were observed under a microscope. A prominent nucleus was observed inside the cell. Each cell showed high nucleo-cytoplasmic ratio (Figure 1b). Round shaped cells were more in number compared to elongated cells. Based on observations under the microscope, after 30 days of incubation, in 40% of the cultures, cell aggregates were noticed (Figure 1c). In

Table 1: Media combination with high concentration of 2,4-D

Concentration of 2,4-D (µM)	After 45 days	After 30 days	After 30 days
270	180	90	0
180	90	0	0
90	0	0	0

Table 2: Media combination with various growth supplements

Time period	Glutamine (µM)	Malt extract (mg/l)	Biotin (µM)	Kinetin (µM)
Initial inoculation	34.2	100	40.9	9.3
After 15 days	34.2	100	40.5	9.3
After 30 days	34.2	50	20.25	9.3
After 45 days	34.2	50	20.25	4.65
After 60 days	34.2	-	-	-

case of banana suspension cultures, initially Murashige and Skoog liquid medium supplemented with 4.5 µM 2,4-D, 4.1 µM biotin, 100 mg/l malt extract, 680 µM glutamine, and 130 µM sucrose were used to obtain cell aggregates, which later were transferred to regeneration media with same constituents except 2,4-D (Georget *et al.,* 2000). However, it was suggested that in the case of banana, 2,4-D hindered the development of protoderms in the proembryos.

Cell viability was tested with FDA stain and 52% of viable cells were detected by a fluorescent microscope (Figure 1d). Growth evaluation was done by packed cell volume (PCV) tube (Figure 1e). About 500 µl of cell suspension was added to PCV tube and centrifuged for 5000 rpm for 1 min. PCV test was conducted, and data were recorded as a percentage of cell mass of the total centrifuged volume. Maximum growth volume (9.9%) was reached 200 days after suspension culture initiation (Table 3 and Figure 1f). From the initiation time of suspension culture, the lag phase was extended and the highest growth rate observed in the exponential phase (i.e. in 28th week). After 28th weeks, growth deceleration had occurred. In date palm, it was reported that after 8 weeks of culture incubation, growth volume was increased up to 12.1% (Al-Khayri, 2012).

The suspension obtained in the present study was transferred to a full strength Y3 semisolid medium (agar 0.75 g/l) to achieve further progress. Suspension and semisolid media were added in various ratios viz., 1:1, 1:2, and 2:1. Much progress was not observed in these cultures. Further manipulation was made in suspension medium by the incorporation of $AdSO_4$ (222 µM) and zeatin (1 µM). In this treatment, the number of cell aggregates increased. However, further development was not achieved. In the case of banana, embryogenic clumps developed into

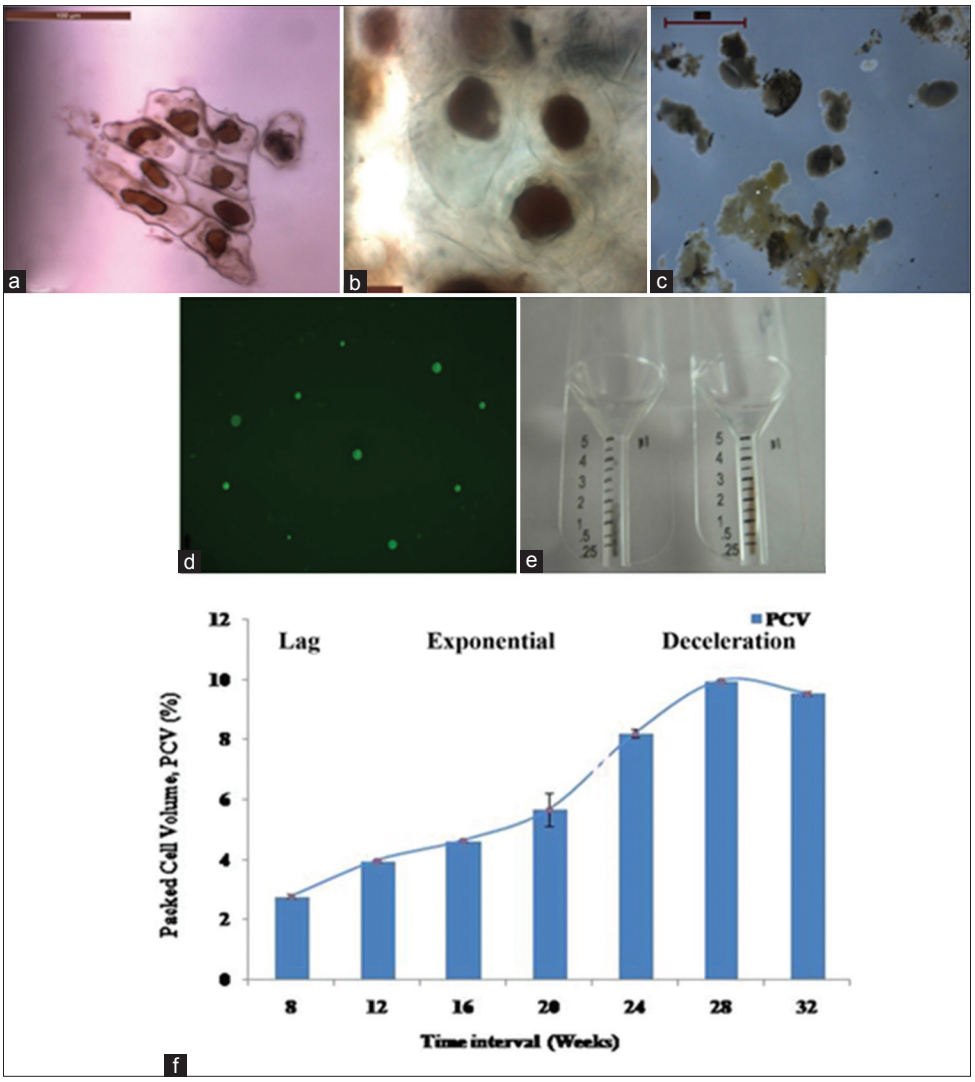

Figure 1: (a) Cell aggregate formation. (b) Cells showing high nucleo-cytoplasmic ratio. (c) Cell aggregates observed under stereo microscope. (d) Viable cells observed under fluorescent microscope. (e) Packed cell volume (PCV) evaluation. (f) The growth curve of coconut cell suspension showing PCV in relation to time (Lag phase, exponential phase, deceleration phase)

Table 3: PCV of various media combinations

Media	30	60	90	120	150	175	200	230
Y3	0.00±0.00	0.00±0.00	0.03±0.03	0.07±0.05	0.10±0.05	0.16±0.05	0.47±0.24	0.46±0.24
Y3+2,4-D (0.1 mg/l)	0.00±0.00	0.93±0.06	1.50±0.05	2.00±0.05	2.63±0.20	3.20±0.27	3.43±0.34	2.97±0.21
Y3+2,4-D (1.0 mg/l)	0.00±0.00	2.77±0.08	3.97±0.03	4.36±0.05	5.67±0.57	8.20±0.14	9.97±0.03	9.53±0.06
Y3+2,4-D (10 mg/l)	0.00±0.00	1.17±0.03	1.47±0.03	2.10±0.14	2.53±0.03	3.50±0.18	3.87±0.03	3.70±0.14
Y3+2,4-D (0.1 mg/l)+BAP (0.5 mg/l))	0.00±0.00	2.53±0.03	3.70±0.05	4.43±0.08	5.87±0.20	7.23±0.34	9.00±0.05	8.43±0.11
Y3+2,4-D (1.0 mg/l)+BAP (0.5 mg/l)	0.00±0.00	1.53±0.03	2.20±0.14	2.87±0.17	3.47±0.21	3.63±0.24	4.03±0.20	3.73±0.21
Y3+2,4-D (10 mg/l)+BAP (0.5 mg/l)	0.00±0.00	2.03±0.03	2.50±0.05	3.00±0.05	3.83±0.06	4.20±0.05	4.90±0.14	4.67±0.13
Y3+Pic (0.1 μM)	0.00±0.00	1.37±0.06	1.90±0.05	2.13±0.12	2.90±0.05	3.07±0.03	3.93±0.03	3.73±0.08
Y3+Pic (1 μM)	0.00±0.00	2.77±0.03	3.67±0.03	4.57±0.03	6.10±0.05	7.73±0.11	9.17±0.03	8.70±0.14
Y3+Pic (10 μM)	0.00±0.00	1.93±0.03	2.20±0.05	2.80±0.05	3.33±0.03	3.73±0.03	4.20±0.10	3.77±0.08
Y3+Pic (0.1 μM)+BAP (0.5 mg/l)	0.00±0.00	1.93±0.03	2.40±0.05	2.87±0.03	3.07±0.03	3.70±0.05	4.17±0.17	3.67±0.08
Y3+Pic (1 μM)+BAP (0.5 mg/l)	0.00±0.00	2.00±0.00	2.40±0.00	3.00±0.00	3.30±0.00	3.83±0.03	4.53±0.03	4.13±0.03
Y3+Pic (10 μM) +BAP (0.5 mg/l)	0.00±0.00	2.37±0.03	3.37±0.18	4.00±0.00	5.23±0.03	6.60±0.09	8.50±0.14	8.23±0.16

Level of significance is represented by CD at 5%. CD at 5%: Media 0.14* Days 0.11* Media×Days 0.37*. PCV: Packed cell volume, BAP: 6-benzyle adenine

somatic embryos by supplementing the media with NAA (1.1 µM), adenine (0.7 µM), kinetin (0.5 µM), and zeatin (0.2 µM) (Cote *et al.,* 2000).

In the case of coconut, embryogenic calli were not friable type and were associated with densely aggregated cells. Because of the hard nature of the embryogenic calli, we

were unsuccessful in obtaining high-quality suspension to initiate somatic embryogenesis. The results of the study revealed that future research should be directed toward initiation of friable calli in coconut.

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