

Original Research Article

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## Influence of Growth Hormones on Initiation of Somatic Embryogenesis in Coconut var. Chowghat Orange Dwarf

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### ABSTRACT

#### Keywords

Coconut, Embryogenic calli, Translucent structures, Embryogenic structures, 2,4-D and kinetin

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Coconut (*Cocos nucifera* L.), is an important perennial crop cultivated across the globe. The demand for quality planting materials is increasing across the world for the establishment of new orchards, rejuvenation of existing orchards and production of quality nuts. Plant tissue culture is an alternate approach for the production of quality planting material. In this investigation, influence of auxin and cytokinin on somatic embryogenesis in coconut is studied using sliced mature embryos as explants in modified Y3 media supplemented with various concentrations of 2,4-D and kinetin. Maximum embryogenic calli and translucent structures were formed in Y3 modified media supplemented with equal concentration of 2,4-D and kinetin (100  $\mu$ M, 200  $\mu$ M and 300  $\mu$ M). Maximum embryogenic structures were formed in Y3 media supplemented with high concentration of 2,4-D (450  $\mu$ M) and with lower concentration of Kinetin (100  $\mu$ M). Further, these embryogenic structures on culturing in media supplemented with cytokinin will form globular embryos.

### Introduction

Coconut (*Cocos nucifera* L.), is an important perennial crop cultivated across the globe in tropics and humid tropics. In India, the coconut palms occupies an area of 2.08 million ha with the production of 23904 million nuts year<sup>-1</sup> and productivity of 11481 nuts ha<sup>-1</sup> ([www.coconutboard.nic.in/Statistics.aspx](http://www.coconutboard.nic.in/Statistics.aspx)). Although India has attained the foremost position globally in production, the productivity of the palm has to be improved.

Limitations in improving productivity of coconut palm are due to its perennial and heterozygous nature. Quality planting materials are the fundamental input for the establishment of new orchards, rejuvenation of existing orchards by replacing diseased, senile and aged palms and the production of quality nuts. Hence, the demand of quality seedlings and nuts are rapidly increasing for establishing plantations.

Natural vegetative propagation is not possible

in crops like coconut where, production of true to type elite planting material becomes a herculean task. Further to add on to the complication, coconut is one of the most recalcitrant species to regenerate under *in vitro* conditions. Earlier, *in vitro* studies in coconut were carried out using a range of explants for the production of plantlets *viz.*, immature inflorescence (Branton and Blake, 1983; Verdeil *et al.*, 1994), tender leaf (Raju *et al.*, 1984; Buffard-Morel *et al.*, 1995), mature embryos (Karun *et al.*, 1999), immature zygotic embryo (Karunaratne and Periyapperuma, 1989; Fernando and Gamage, 2000), plumules (Chan *et al.*, 1998; Fernando *et al.*, 2003; Karun *et al.*, 2004) and unfertilized ovaries (Perera *et al.*, 2007). Hornung and Verdeil (1999) showed that the callusing frequency in explants of leaf and immature inflorescence were very low (20 and 30 per cent respectively) and reported that the results were not consistent. The studies of Chan *et al.*, (1998) and Fernando *et al.*, (2010) revealed that the response of plumule explants under *in vitro* culture through somatic embryogenesis was better in terms of callus formation and embryogenic capacity. Similarly, Hornung and Verdeil (1999) concluded that somatic embryogenesis is viable approach for coconut micropropagation. Perez-Nunez *et al.*, (2006) proved the possibility of producing 35,000-50,000-fold increased somatic embryo formation through secondary somatic embryogenesis and embryogenic calli multiplication using plumule explants.

Somatic embryogenesis in coconut is highly influenced by media components. Samosir *et al.*, (1999) and Fernando and Gamage (2000) reported that manipulation of auxin and ABA levels in the culture medium improved the somatic embryogenesis and plant regeneration. The presence of ABA in combination with high agar concentration induced water stress leading to improvement

in plant regeneration (Fernando *et al.*, 2010). However, the low reproducibility, low efficiency of somatic embryogenesis and poor plant regeneration in coconut compels to explore other strategies to improve somatic embryogenesis in coconut. In this context, the study was proposed to investigate the influence of plant growth hormones *viz.*, 2,4-D and kinetin in culture medium on the formation of callus, translucent structures and embryogenic structures.

## **Materials and Methods**

Twelve months old nuts were harvested from Chowghat Orange Dwarf (COD) palms free from pest and diseases with high yield. The cultivar was maintained at Coconut Research Station, Aliyar Nagar, Tamil Nadu Agricultural University, situated at 10<sup>0</sup> N latitude and 77<sup>0</sup> E longitude, at an altitude of 260 m above mean sea level. One of the preferred cultivar for tender coconut in Tamil Nadu is COD.

## **Embryo isolation**

Coconut mature embryos were isolated from 12 months old nuts. The fibrous mesocarp was removed and nuts were cut transversely with a machete. One portion of the broken nut containing eyes was used for embryo isolation. The endosperm cylinder containing the embryo was removed using a tender coconut opener. Endosperm cylinders were stored in 1 per cent sodium hypochlorite for transport to the laboratory.

## **Sterilization of endosperm cylinders and embryos**

Under aseptic conditions, the endosperm cylinders enclosing the embryos were washed in sterile distilled water and were transferred to 70% ethanol for 3 min and rinsed with sterile distilled water, followed by 4% sodium

hypochlorite solution wash for 20 min and rinsed with sterile distilled water. The embryos excised from the endosperm cylinders were subjected to 0.6% sodium hypochlorite solution for 10 min and rinsed with sterile distilled water thrice.

### **Culture medium and conditions**

The sterilised embryos were cultured in the modified Y3 medium supplemented with sucrose (4.5 %), activated charcoal (0.25%), agar (0.8%) combined with growth hormones *viz.*, 2,4-D and kinetin (kin) each at various concentration (150  $\mu$ M, 300  $\mu$ M, 450  $\mu$ M and 600  $\mu$ M). Modified Y3 basal medium (T<sub>1</sub>) served as control for the experiments (Table 1). The media was autoclaved at 15 psi (1kg/cm<sup>2</sup>) at 121°C for 20 minutes. At bearable warmth the media was dispensed into Petriplates and stored until further use. The sterilized embryos were sliced into small pieces and inoculated into the medium. The cultures were incubated at 25±2 °C and relative humidity of 60% under dark conditions for 60 days followed by 16/8 h photoperiod regime (36  $\mu$ mol m<sup>-2</sup>s<sup>-1</sup>). The experiments were replicated sufficiently.

### **Data analysis**

Statistical analysis was performed by adopting Completely Randomized Design (CRD) as per standard procedure of Panse and Sukhatme (1985) and Duncan's Multiple Range Test (DMRT). Analysis was carried out with MS Excel spread sheet and DSAASTAT software.

## **Results and Discussion**

### **Effect of kinetin and 2,4-D on callus initiation**

Initial callusing was observed after 3-4 weeks of inoculation. Highest initial callusing was recorded in T<sub>7</sub> (Y3+300  $\mu$ M 2, 4-D+300  $\mu$ M

kin) (55.55%) followed by T<sub>2</sub> (Y3+100  $\mu$ M 2,4-D+100  $\mu$ M kin) (44.44%). Treatments T<sub>4</sub> (Y3+200  $\mu$ M 2,4-D+200  $\mu$ M kin), T<sub>5</sub> (Y3+300  $\mu$ M 2,4-D+100  $\mu$ M kin) and T<sub>8</sub> (Y3 +450  $\mu$ M 2,4-D+100  $\mu$ M kin), were statistically on par and recorded a callusing percentage of 33.33%. Treatment T<sub>3</sub> (Y3+200  $\mu$ M 2,4-D+100  $\mu$ M kin) recorded 22.22% callusing, while T<sub>6</sub> (Y3+300  $\mu$ M 2,4-D+200  $\mu$ M kin) and T<sub>9</sub> (Y3+450  $\mu$ M 2,4-D+200  $\mu$ M kin) recorded 11.11% of callusing. In control (T<sub>1</sub>) callus initiation was not observed (Table 2).

### **Effect of kinetin and 2,4-D on formation of translucent structures**

Translucent structures developed from the calli were observed after 45-50 days of inoculation. Maximum percentage of translucent structures (44.44%) were recorded in T<sub>2</sub> (Y3+100  $\mu$ M 2,4 -D+100  $\mu$ M kin), T<sub>4</sub> (Y3+200  $\mu$ M 2,4-D+200  $\mu$ M kin) and T<sub>7</sub> (Y3+300  $\mu$ M 2,4-D+300  $\mu$ M kin) and these treatments were statistically on par. Treatments, T<sub>5</sub> (Y3+300  $\mu$ M 2,4-D+100  $\mu$ M kin) and T<sub>8</sub> (Y3 +450  $\mu$ M 2,4-D+ 100  $\mu$ M kin), were statistically on par and recorded 33.33% of translucent structures. Translucent structures of 22.22 and 11.11 % were recorded in T<sub>3</sub> (Y3+200  $\mu$ M 2,4-D+100  $\mu$ M kin) and T<sub>9</sub> (Y3+450  $\mu$ M 2,4-D+ 200  $\mu$ M kin) respectively. In the treatments T<sub>1</sub>, T<sub>10</sub> and T<sub>11</sub>, callus was not observed and hence there were no translucent structures (Table 2).

### **Effect of kinetin and 2,4-D on formation of embryogenic structures**

After 90 days of inoculation, embryogenic structures developed in media supplemented with 2, 4-D and kinetin. Maximum percentage of embryogenic structures (66.66 %) were recorded in T<sub>8</sub> (Y3+450  $\mu$ M 2,4-D+100  $\mu$ M kin) followed by T<sub>2</sub> (Y3+100  $\mu$ M 2,4-D+100

µM kin) and T4 (Y3+200 µM 2,4-D+200 µM kin) (44.44%). Treatments T3 (Y3+200 µM 2,4-D+100 µM kin), T5 (Y3+300 µM 2,4-D+100 µM kin) and T7 (Y3+300 µM 2,4-

D+300 µM kin) recorded 33.33 % of embryogenic structures and were statistically on par (Table 2 and Fig. 1).

**Table.1** Combinations of 2,4-D and kinetin used in this study

Treatments	Hormonal combinations
T <sub>1</sub>	Y3 Basal
T <sub>2</sub>	Y3 + 100 µM 2,4-D + 100 µM kin
T <sub>3</sub>	Y3 + 200 µM 2,4-D + 100 µM kin
T <sub>4</sub>	Y3 + 200 µM 2,4-D + 200 µM kin
T <sub>5</sub>	Y3 + 300 µM 2,4-D + 100 µM kin
T <sub>6</sub>	Y3 + 300 µM 2,4-D + 200 µM kin
T <sub>7</sub>	Y3 + 300 µM 2,4-D + 300 µM kin
T <sub>8</sub>	Y3 + 450 µM 2,4-D + 100 µM kin
T <sub>9</sub>	Y3 + 450 µM 2,4-D + 200 µM kin
T <sub>10</sub>	Y3 + 450 µM 2,4-D + 300 µM kin
T <sub>11</sub>	Y3 + 450 µM 2,4-D + 450 µM kin

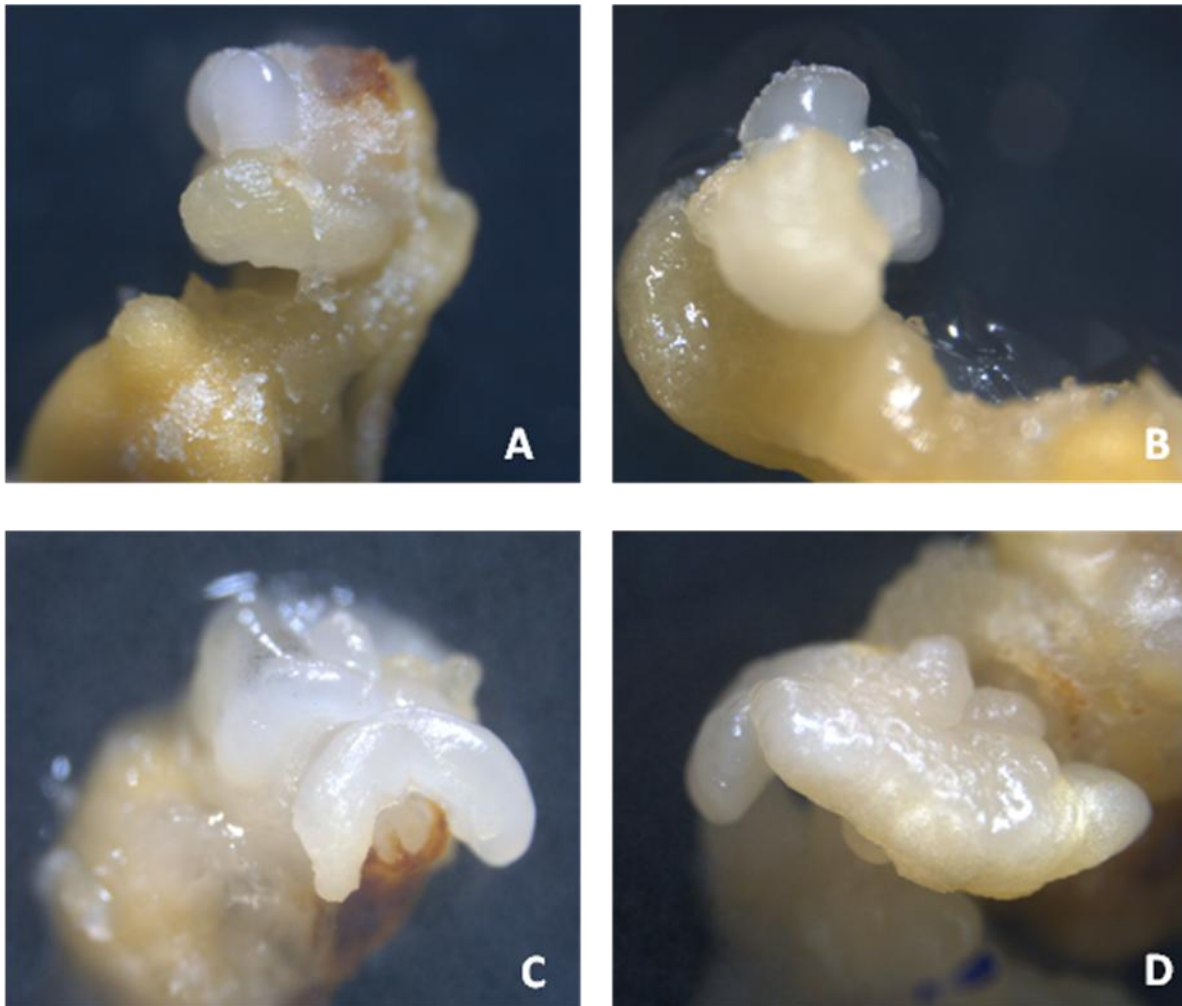
**Table.2** Effect of 2, 4-D and Kinetin on callus initiation, translucent structure and embryogenic structure formation

Treatments	Callus Initiation%	Translucent structure %	Embryogenic structure%
T <sub>1</sub> (Y3 basal)	0.00 (0.71) <sup>f</sup>	0.00 (0.71) <sup>e</sup>	0.00 (0.71)
T <sub>2</sub> (Y3+100 µM 2,4-D+100 µM kin)	44.44 (6.61) <sup>b**</sup>	44.44 (6.61) <sup>a**</sup>	44.44 (6.61) <sup>b**</sup>
T <sub>3</sub> (Y3+200 µM 2,4-D+100 µM kin)	22.22 (4.11) <sup>d*</sup>	22.22 (4.11) <sup>c*</sup>	33.33 (4.91) <sup>c*</sup>
T <sub>4</sub> (Y3+200 µM 2,4-D+200 µM kin)	33.33 (4.91) <sup>c*</sup>	44.44 (5.70) <sup>a*</sup>	44.44 (5.70) <sup>b**</sup>
T <sub>5</sub> (Y3+300 µM 2,4-D+100 µM kin)	33.33 (5.81) <sup>c**</sup>	33.33 (5.82) <sup>b**</sup>	33.33 (5.81) <sup>c**</sup>
T <sub>6</sub> (Y3+300 µM 2,4-D+200 µM kin)	11.11 (2.41) <sup>e</sup>	0.00 (0.71) <sup>e</sup>	0.00 (0.71) <sup>d</sup>
T <sub>7</sub> (Y3+300 µM 2,4-D+300 µM kin)	55.55 (7.40) <sup>a**</sup>	44.44 (5.70) <sup>a**</sup>	33.33 (5.81) <sup>c*</sup>
T <sub>8</sub> (Y3+450 µM 2,4-D+100 µM kin)	33.33 (5.81) <sup>c**</sup>	33.33 (5.81) <sup>b**</sup>	66.66 (6.91) <sup>a**</sup>
T <sub>9</sub> (Y3+450 µM 2,4-D+200 µM kin)	11.11 (2.41) <sup>e</sup>	11.11 (2.41) <sup>d</sup>	0.00 (0.71) <sup>d</sup>
T <sub>10</sub> (Y3+450 µM 2,4-D+300 µM kin)	0.00 (0.71) <sup>f</sup>	0.00 (0.71) <sup>e</sup>	0.00 (0.71) <sup>d</sup>
T <sub>11</sub> (Y3+450 µM 2,4-D+450 µM kin)	0.00 (0.71) <sup>f</sup>	0.00 (0.71) <sup>e</sup>	0.00 (0.71) <sup>d</sup>
SE (d)	1.64	1.97	1.97
CD (0.05)	3.40	4.09	4.09
CD (0.01)	4.62	5.55	5.55

#data in paranthesis is square root transformed data.

\*Significant, \*\* highly significant

**Fig.1** Initiation of somatic embryogenesis in coconut A) Callus initiation, 45 days after inoculation B) Translucent structures, 60 days after inoculation C) Embryogenic structures, 75 days after inoculation D) Embryogenic structures, 90 days after inoculation



The treatments with Y3+ 2,4- D+ kin were analyzed for callus initiation, translucent structures and embryogenic structure formation. Among these, maximum embryogenic callus initiation was documented in treatment T7 (Y3+300  $\mu$ M 2,4- D+300  $\mu$ M kin) (55.55%). These findings are in agreement with Dudits *et al.*, (1991) and Yeung (1995), where influences of exogenously applied auxins *viz.*, 2, 4-D, on the induction of somatic embryogenesis has been well documented. In more than 65% of the protocols, 2, 4-D was applied singly or in combination with other plant growth

regulators (Gaj, 2004). The high efficiency of 2,4-D for induction of embryogenic response found in different *in vitro* systems and plant species indicates a specific and unique character of this plant growth regulator. This synthetic growth regulator and an auxinic herbicide act not only as an exogenous auxin analogue but also as an effective stressor. 2, 4-D brings about different changes in physiology and gene expression of cells and this implicates its possible role as a stress factor triggering embryogenic pattern of development in cultured plant cells (Feher *et al.*, 2003).

Maximum translucent structures (44.44%) were recorded in T2 (Y3+100  $\mu$ M 2,4-D+100  $\mu$ M kin), T4 (Y3+200  $\mu$ M 2,4-D+200  $\mu$ M kin) and T7 (Y3+300  $\mu$ M 2,4-D+300  $\mu$ M kin). While maximum percentage of embryogenic structures (66.66 %) were recorded in T8 (Y3+450  $\mu$ M 2,4-D+100  $\mu$ M kin) followed by T2 (Y3+100  $\mu$ M 2,4-D+100  $\mu$ M kin) and T4 (Y3+200  $\mu$ M 2,4-D+200  $\mu$ M kin) (44.44%). In a similar study Ann *et al.*, (2018), reported maximum callusing followed by development of translucent structures and embryogenic structures in Y3 media supplemented with 450  $\mu$ M 2,4-D + and 200  $\mu$ M BAP. Vidhanaarachchi *et al.*, (2013) reported that on transferring the translucent embryogenic structures of coconut ovary explants to a media with reduced auxin concentration gave nodular structures in the periphery. Further when these structures were subjected to a media free from hormones, these structures enlarged and turned opaque. Sub-culturing of these structures into media containing high cytokinin, they formed globular embryos which showed further development.

This study reveals that maximum embryogenic calli and translucent structures were formed in Y3 modified media supplemented with equal concentration of 2,4-D and kinetin (100  $\mu$ M, 200  $\mu$ M and 300  $\mu$ M) while very high concentration of 2,4-D and kinetin although equal (450  $\mu$ M) does not favour embryogenic callus and translucent structure formation. Maximum embryogenic structures were formed in Y3 supplemented with high concentration of 2,4-D (450  $\mu$ M) with lower concentration of kinetin (100  $\mu$ M). It can be concluded that equal concentration of 2,4-D and kinetin plays a significant role in embryogenic callus initiation and translucent structure formation in coconut and for embryogenic structures formation, media comprising higher concentration of 2,4-D and lower concentration of kinetin are required.

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