

Original Research Article

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## Studies on *in vitro* Culture of Coconut var. Chowghat Orange Dwarf through Direct Organogenesis

D.S. Sushmitha, R. Renuka\* and D. Chandrakala

Plant Tissue Culture Laboratory, Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore – 641003, Tamil Nadu, India

\*Corresponding author:

### ABSTRACT

#### Keywords

Coconut, Direct organogenesis, TDZ, Sliced mature embryo, Shoot regeneration, Chowghat Orange Dwarf

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Coconut has a wide range of applications from culinary to industrial areas. Loss of coconut trees due to the devastating effect of deadly diseases and the natural aging of the plants demand rejuvenation of orchards with elite seedlings. A conventional breeding program is not sufficient to meet the growing need for healthy planting materials. However, *in vitro* culture studies can pave the way for mass multiplication. In this study, the effect of TDZ on sliced mature embryo explants through direct organogenesis of coconut var. Chowghat Orange Dwarf (COD) was investigated. The treatment Y3 + 150µM TDZ recorded highest shoot induction (90.91 %) and shoot regeneration (72.73 %). A higher concentration of TDZ reduced the frequency of shoot organogenesis. Well-grown shoots were transferred to rooting media and plantlets were subjected to primary hardening.

### Introduction

Coconut (*Cocos nucifera* L.) is one of the important plantation crops in the tropical region. Coconut is popularly known as “tree of life” as all the parts of the tree is playing a major role in the traditional, cultural and economic activities of human life. One of the major product of coconut is coconut oil and it is rich in short chain fatty acids (Berger and Ong, 1985) and is the best cooling oil next to olive oil. Apart from oil, market demand is

increasing rapidly for other products such as packed coconut water, coconut milk products, coconut jelly from makapuno type nuts, virgin oil and other products (Lao, 2009). Although nuts have immense importance, the commercial value for other parts of the palm is gaining equal importance. Fibrous coir from the mesocarp of the nuts has good water retention capacity and is utilized in agriculture and gardening. Now a days, coco-biodiesel has become an alternative to combat fuel crisis. Blending coco-biodiesel with

diesel fuel improves the quality and reduces the emission of nitrous oxide and sulfur oxide. Use of coconut fronds for roofing, brooms and coco wood is well known. Coconut wine which is known as “mnazi” in Kenya (Muhammed *et al.*, 2013) “taadi” in North India, “thatikallu” in Andhra Pradesh and Telangana, “kallu” in Tamil Nadu and Kerala, is another product of the tree. Coconut plays a significant role in maintaining ecological balance and provides substantial benefits to the environment. It helps in soil conservation in plain, wind-prone areas and coastal areas. Palms can be planted along the border of agriculture land where it acts as windbreaks and canopy provides shelter to the shade-loving species (Orolfo, 1991). Coconut cultivation can alleviate nutrition balance, improve the livelihood of people in rural areas, provide employment through small scale industries and also acts as a supporting system of income (Molla *et al.*, 2004).

Coconuts are grown in over 86 countries across the globe. According to FAOSTAT (2017) survey, Indonesia (18.98 million tonnes) is the world's largest producer of coconut followed by Philippines (14.04 million tonnes), India (11.47 million tonnes), Sri Lanka (2.58 million tonnes), and Brazil (2.34 million tonnes). Now a days, total production of coconut is declining gradually due to aged plants, phytosanitary threats, infectious diseases such as lethal yellowing in America (Arellano and Oropeza, 1995), lethal diseases in Africa (Eden-Green, 1995), cadang-cadang in Asia (Hanold and Randies, 1991). Apart from the external threats, availability of healthy and elite planting material have become scarce as seeds are the only mode of propagation (Renuka *et al.*, 2018). Chowghat Oragne Dwarf (COD) variety is cultivated across South India for tender nuts. On an average, the tree would produce 53 nuts per year and each nut contains an average quantity of 351 ml water.

The demand for tender coconuts and packaged drinks from coconut is increasing as it is one of the world's most versatile natural products and has the highest concentration of electrolytes which makes it an excellent source of hydration. Micropropagation technique serves as the best tool to meet the growing demand for promising genotypes where there is no option for vegetative propagation mode. Investigations conducted to study the efficiency of different explants revealed that rachilla explants from immature inflorescence (Hornung and Verdeil, 1999) and plumule from zygotic embryos (Saenz *et al.*, 2006) have responded well for *in vitro* culture compared to other explants. Although the formation of callus and embryogenic structures were observed when rachilla explants were used, further development into plantlets was hindered (Blake, 1990).

However, plumule explants produced callus and somatic embryos which further developed into complete plantlet (Blake and Hornung, 1995). However, a commercial protocol for mass multiplication of coconut is yet to be standardized. Hence, this present study was taken up to study the effect of growth hormone TDZ on the *in vitro* culture of coconut var. COD using sliced zygotic embryos as explants.

## **Materials and Methods**

The present investigation was carried out at the Plant Tissue Culture Laboratory, Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore during academic year 2018-19.

### **Media composition**

Modified Y3 media was prepared and autoclaved at 15 psi pressure and 121 °C

temperature for 20 minutes in a vertical type autoclave. Different concentration of thidiazuron (TDZ) was added after filter sterilization. Media was dispensed into pre-sterilized Petri plates and stored at  $25\pm 2$  °C until inoculation. Treatment T<sub>1</sub> without any hormones served as a control and T<sub>2</sub> to T<sub>4</sub> comprised of TDZ at varied concentration (Table 1).

### **Embryo collection and preparation**

Matured seed nuts were harvested from healthy and elite mother palms. Fibrous mesocarp was removed and nuts were broken open transversely using a machete. Three-eyed half of the nut was selected and the embryo present in the largest eye was isolated using a tender coconut opener. Isolated cylinders were stored in 1% NaOCl until transported to the laboratory (Fig. 1). In the laboratory, endosperm cylinders were washed with Tween-20 solution (1-2 drops of Tween-20 in 100ml distilled water) followed by 2-3 distilled water washes. Under laminar air flow, endosperm cylinders were transferred to a sterile beaker and washed thrice with sterile water. Cylinders were treated with 4% NaOCl for 20 minutes followed by 70% ethanol for 3 minutes. Sterile water washes were given thrice (each for 5 minutes), and later embryos were dissected out using sterile forceps, scalpel and blade. The embryos were sterilized with 0.6% NaOCl for 5 minutes and washed with sterile water thrice. Then, embryos were treated with an antioxidant solution for 15 minutes and stored in the same until inoculated. Each embryo was taken out individually, sliced into four pieces longitudinally and inoculated on Y3 media (Fig. 2).

### **Culture conditions**

Embryo slices were inoculated on modified Y3 media and cultures were maintained at a

temperature of  $25\pm 2$  °C at a relative humidity of 60-70 per cent under darkness. Observations were taken at 15 days interval initially. 45 to 60 days after incubation under darkness, shoot induction was recorded. On initiation of shoots, the cultures were incubated at 16hours photoperiod.

### **Subculturing, *in vitro* rooting and primary hardening**

Shoots were subcultured and maintained in shoot development media until optimum growth was attained. After 180 to 210 days of inoculation, well-grown shoots were transferred into full strength Y3 basal media supplied with 200µM IBA for rooting. After 60 days of incubation in rooting media, plantlets with well-formed shoot and root were transferred into plastic pots containing vermiculite. Essential nutrients were supplied through Hoagland's solution regularly.

### **Statistical analysis**

The data recorded were subjected to statistical analysis by using Completely Randomized Design as per the standard procedure of Panse and Sukhatme (1985) and Duncan's Multiple Range Test (DMRT). The analysis was carried out with the MS Excel spreadsheet and DSAASTAT software.

## **Results and Discussion**

### **Effect of TDZ on shoot induction**

A significant difference was observed among all the treatments used in this study (Table 2). Shoot induction was recorded at 60 days after inoculation and was most influenced by TDZ in the cultures. Among the different treatments investigated for shoot induction, T<sub>2</sub> (Y3 basal + 150 µM TDZ) showed highest shoot induction percentage *i.e.*, 90.91 per cent which was statistically significant followed by

T<sub>1</sub> (Y3 basal) which recorded 72.72 per cent shoot induction. Treatment T<sub>3</sub> (Y3 basal + 300 µM TDZ) recorded 45.45 per cent shoot induction whereas T<sub>4</sub> (Y3 basal + 450 µM TDZ) showed the lowest number of shoots induced *i.e.*, 18.18 per cent. The results clearly indicated that a higher concentration of TDZ is detrimental to shoot induction and lower concentration of TDZ promotes shoot induction.

TDZ was found to be the most effective cytokinin to induce adventitious shoot formation at a lower concentration in a wide range of plant species (Lu, 1993). Shoot organogenesis was successful in different cultivars of pigeon pea when media was supplemented with TDZ (Eapen *et al.*, 1998). Al-Mayahi (2014), showed that the concentration of TDZ at 0.5 mg L<sup>-1</sup> recorded maximum response for *in vitro* bud organogenesis in date palm cv. Hillawi and the research findings of the present study in coconut revealed that TDZ supplemented at 150 µM (33 mg L<sup>-1</sup>) concentration induced maximum shoots (90.91 per cent) through direct organogenesis from sliced mature embryos successfully.

### Effect of TDZ on shoot regeneration

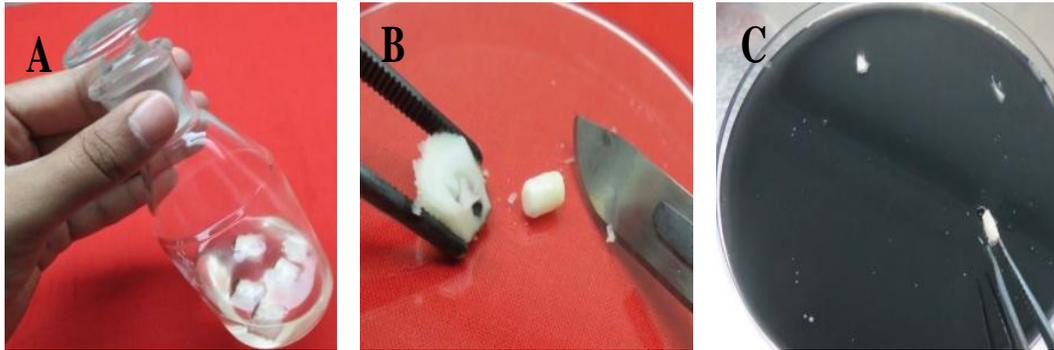
A significant difference was observed among the treatments with respect to shoot regeneration (Table 2). Shoot regeneration was recorded at 120 days after inoculation and the highest percentage was recorded in the treatment T<sub>2</sub> (Y3 basal + 150 µM TDZ) *i.e.*, 72.73 per cent. Treatment T<sub>1</sub> (Y3 basal) recorded 63.64 per cent of shoot regeneration which was followed by T<sub>3</sub> (Y3 basal + 300 µM TDZ) with 36.36 per cent. Lowest shoot regeneration was observed in T<sub>4</sub> (Y3 basal + 450 µM TDZ) *i.e.*, 18.18 per cent. TDZ has a significant effect on shoot regeneration and complete shoot development. These findings are in agreement with Huetteman and Preece (1993) where the effect of TDZ on woody species was well described as TDZ could facilitate micropropagation of many recalcitrant woody species. Maximum shoot regeneration from the embryogenic calli of sugarcane was achieved when MS media supplemented with TDZ (Gallo-Meagher *et al.*, 2000). Lower risk of genetic manipulation in direct organogenesis compared to somatic embryogenesis would be a major advantage in plant regeneration.

Fig. 1. Isolation of embryo along with endosperm from the seed nut



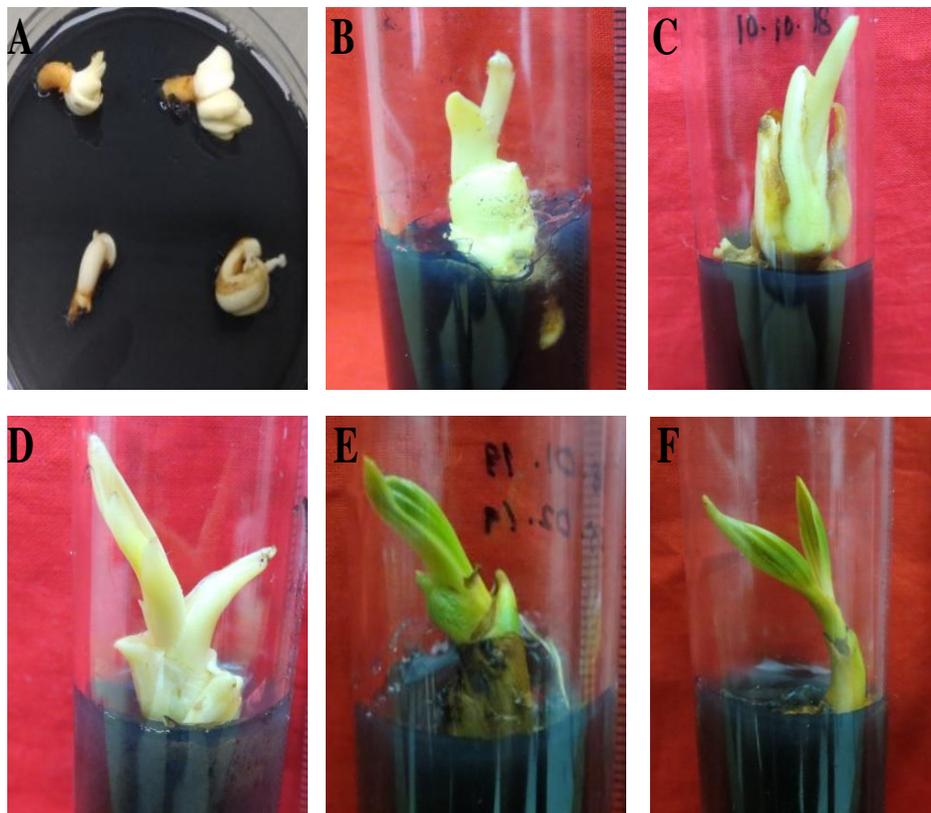
- A. Tender coconut opener and transversely broken nut
- B. Embryo isolation along with the endosperm
- C. Collection of endosperm cylinders in 1% NaCl solution

Fig. 2. Sterilization of endosperm cylinders, dissection, slicing and inoculation



- A. Sterilization of endosperm cylinders with 4% NaOCl
- B. Excision of embryo from the endosperm cylinders
- C. Slicing and inoculation on to media

Fig 3. Shoot induction and growth after various days of inoculation (DAI)



- A. Shoot induction at 30 days after inoculation
- B. Shoot induction at 60 days after inoculation
- C. Shoot growth at 90 days after inoculation
- D. Shoot regeneration at 120 days after inoculation
- E. Shoot development at 150 days after inoculation
- F. Well-developed shoot after 180 days of inoculation

Fig 4. Plantlet under *in vitro* rooting and primary hardening



**Table.1** Treatments used to study direct organogenesis in coconut

Treatment	Hormonal combination
T <sub>1</sub>	Y3 Basal
T <sub>2</sub>	Y3 + 150 µM TDZ
T <sub>3</sub>	Y3 + 300 µM TDZ
T <sub>4</sub>	Y3 + 450 µM TDZ

**Table.2** Effect of TDZ on shoot induction and shoot regeneration in COD

Treatment	Shoot induction percentage	Shoot regeneration percentage
T <sub>1</sub> (Y3 Basal)	72.73 (4.17) <sup>ab</sup>	63.64 (3.65) <sup>a*</sup>
T <sub>2</sub> (Y3 + 150µM TDZ)	90.91 (4.91) <sup>a*</sup>	72.73 (4.17) <sup>a*</sup>
T <sub>3</sub> (Y3 + 300µM TDZ)	45.45 (2.30) <sup>bc</sup>	36.36 (2.09) <sup>ab</sup>
T <sub>4</sub> (Y3 + 450µM TDZ)	18.18 (1.04) <sup>c</sup>	18.18 (1.04) <sup>b</sup>
S.E.D	1.16	1.16
LSD (p<0.05)	2.35	2.33
LSD (p<0.01)	3.14	3.12

# Data in parenthesis is arc sine transformed data.

\*significant

**Table.3** Effect of TDZ on shoot length after 180 days of inoculation

Treatment	Average shoot length at 180 DAI (in cm)
T <sub>1</sub> (Y3 Basal)	1.8
T <sub>2</sub> (Y3 + 150µM TDZ)	1.21
T <sub>3</sub> (Y3 + 300µM TDZ)	1.16
T <sub>4</sub> (Y3 + 450µM TDZ)	0.3
S.E.D	0.73
LSD (p<0.05)	1.48
LSD (p<0.01)	1.98

### Effect of TDZ on shoot length

Shoot length was recorded at every 30 days interval starting from shoot induction stage (Fig. 3). Treatment T<sub>1</sub> (Y3 basal) recorded highest shoot length frequency of 1.8 cm. T<sub>2</sub> (Y3 basal + 150 µM TDZ) and T<sub>3</sub> (Y3 basal + 300 µM TDZ) recorded mean shoot length of 1.21 cm and 1.16 cm respectively. Lowest shoot length frequency of 0.3 cm was recorded in T<sub>4</sub> (Y3 basal + 450 µM TDZ).

However, significant difference was not observed among the treatments in case of shoot length (Table 3). Well-grown shoots were transferred to modified Y3 media containing 200 µM IBA for rooting. After rooting, plantlets were transferred to vermiculite and subjected for primary hardening (Fig. 4).

Bates *et al.*, (1992), revealed that embryo culture of white ash (*Fraxinus americana* L.) showed maximum shoot regeneration and shoot elongation when media supplemented with TDZ. Supply of TDZ (0.005 µM and 0.05 µM) along with BAP enhanced shoot growth and subsequent rooting in *Acer* cultivars (Marks and Simpson, 1994). Greeshma *et al.*, (2018), concluded that the concentration of cytokinin to auxin play a significant role in development and growth of somatic embryos of coconut.

The present research concludes that the TDZ has a significant role in direct organogenesis of coconut and could increase the frequency of shoot induction and shoot regeneration.

In this study, maximum shoot induction and shoot regeneration was documented in Y3 media supplemented with 150 µM TDZ. Further, well-grown shoots were transferred to rooting media and plantlets were obtained.

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