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Multiplication and Differentiation of Ovary Derived Callus of Coconut (*Cocos nucifera* L.) for Higher Embryogenic Potential

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ABSTRACT

Tissue culture is the only possible vegetative propagation method available for Coconut (*Cocos nucifera L.)* as it is naturally propagated only through seeds. Unfertilized ovaries of immature female flowers are considered as the ideal explant for the clonal propagation due to its somatic nature and plant regeneration efficiency. Growth regulators are very essential component in culture media as it enhances callogenesis and differentiation. Also multiplication and differentiation of embryonic callus depends on the quality of the original callus. Therefore, the present study was conducted to evaluate effect of growth hormone combinations, nature of explant and nature of callus on callus induction, multiplication and differentiation. Thin layer slices of ovaries gave significantly higher callogenesis percentage (65.6%) over crushed ovaries indicating a promising method to increase callogenesis of coconut ovary culture. Embryogenic callus multiplication enhanced significantly (70.4%) by adding 9 µM TDZ to multiplication medium. Callus maturation in hormone free medium with two different phytagel percentage (0.3 %, 0.25% w/v) resulted highest embryogenic differentiation producing 58.06% and 66.67% embryogenic callus, respectively. Frilly translucent callus performed better in callus multiplication and embryogenic nature of initial callus contributed significantly to better differentiation.

KEYWORDS: Callogenesis, Coconut, Embryogenic Potential, Ovary

INTRODUCTION

Coconut palm (*Cocos nucifera* L.) is an important commercial plant which is grown in tropical and subtropical climates in the world. It belongs to the family Areaceae and it is the only species in the genus *Cocos*. Unlike in many other trees, it does not show any vegetative propagation thus propagated only through seeds. Since coconut is a cross pollinated palm, it exhibits great variation in selected characters when propagated through seeds. Therefore vegetative propagation of superior palms via tissue culture is the only possible method available to obtain homogeneous planting material.

In vitro propagation of coconut has been studied since late 1970's using different explants like root (Justin, 1978), immature inflorescences (Branton and Blake, 1983; Verdeil et al., 1994), shoot tip (Weerakoon, 2004), tender leaves (Pannetier and Buffard-Morel, 1982; Buffard-Morel et al., 1988), immature zygotic embryo (Karunaratne and Periyapperuma, 1989; Fernando and Gamage, 2000) and plumule (Hornung, 1995; Chan *et al.*, 1998; Fernando *et al.*, 2003). However the response of these somatic tissues to *in vitro* culture was low and inconsistent.

Unfertilized ovary is identified as an ideal explant for somatic embryogenesis of coconut in recent past due to its somatic nature and high regeneration potential (Perera et al., 2007). Suitable culture conditions have been developed for consistent callogenesis from unfertilized ovaries of coconut (Perera et al., 2007). In a protocol developed by Perera *et al.*, (2009), plant regeneration efficiency was determined by incorporating different growth regulators and the recorded callusing and regeneration frequencies were 70% and 76% respectively. Although *in vitro* propagation of coconut ovaries has led to the production of clonal plantlets, there is still lack of an efficient, reliable and rapid regeneration system for this crop.

In the present study, attempts were made to further improve the callogenesis and plant regeneration protocol of coconut using unfertilized ovary explants. Culturing thin cell layer sections of explants has been reported to stimulate callusing in many crops (da Silva Guedes et al., 2011; Benkirane *et al.*, 2000). In this study two physical stages, (ovaries cut in to thin cell layers and crushed ovaries) were tested for callogenesis. Due to the high morphological variation observed in the callus structures, multiplication of embryogenic callus depends on the quality of the original callus. Thus in this study four morphological stages of initial callus were tested for callus

multiplication in two different media. Furthermore, the effect of high phytagel concentration, BAP (6-Benzylaminopurine) and 2ip (2-Isopenyl adenine) were tested for embryogenic differentiation in coconut ovary culture.

MATERIALS AND METHODS

Selection and Sterilisation of Explant

Unfertilized ovaries excised from immature female flowers of -4 maturity stage coconut inflorescences (~ 48 cm) were used as the explants. Stage 0 of coconut inflorescence refers to the youngest open inflorescence thus; the -4 inflorescence will open approximately 4 months later. Coconut inflorescences were collected from adult coconut palms of T x SR (Sri Lanka Tall x SanRamon) and DT (Dwarf x Sri lanka Tall) varieties. Female flowers are at the basal part of the rachilla. Therefore, 4-5 cm long pieces were obtained from the basal part of rachilla under the laminar flow cabinet and disinfected with 2% clorox for 12 min followed by five rinses with sterilized distilled water. Two male flowers, flanking each female flower were removed. Then the perianth segments of female flowers were removed carefully and ovaries were dissected under a stereo binocular. The ovaries were cultured in glass vials containing 15 ml culture media.

Preparation of Culture Media

AnalaR grade chemicals were used for preparation of all culture media. All growth regulators were obtained from sigma chemical company (USA). The pH was adjusted to 5.8 before adding 0.1 % (w/v) oven dried charcoal (coconut shell charcoal: Haycarb, Sri Lanka). Phytagel was added at 0.25% (w/v) unless otherwise stated. Culture media were dispensed into vials (15 ml per each vial) and fruit jars (60 ml per each fruit jar) and sterilized by autoclaving at 121°C, 1.1 kg cm^{-3} pressure for 15 min.

Culture Conditions for Callogenesis

For ovary culture CRI 72 medium (Karunaratne and Periyapperuma, 1989) supplemented with 100 µM 2, 4 - dichlorophenoxyacetic acid (2, 4-D) and 9 µM thidiazuron (TDZ) as explained by Perera et al., (2007) was modified by increasing 2, 4-D level to 160 µM. This was used as the callus induction medium and cultures were maintained in the dark for 28°C for 8 weeks.

Effect of Crushing and Slicing of Ovaries on Callus Induction

After disinfection, the ovaries were dissected out carefully under the stereo binocular and half of the ovaries were crushed with the scalpel blade and cultured while the rest were sliced into small thin layers and cultured. The percentage of callus production in each treatment was recorded after 8 weeks of culture initiation.

Effect of TDZ on Callus Multiplication

Callus multiplication was determined in two different media. Embryogenic structures of the initial calli were further dissected under stereo binocular microscope and sub cultured into fresh callus induction medium either containing 9 µM TDZ or without TDZ after 8 weeks of culture initiation. In the same experiment, the effect of initial callus morphology on the multiplication of callus and production of embryogenic structures was studied. For this, the original calli were categorized into four groups according to the morphological stage, callus with ear like translucent structures (Stage 1), callus with frilly like translucent structures (Stage 2), callus with globular untranslucent structures (Stage 3), callus with compact globular untranslucent structures (Stage 4).

All the cultures were maintained in the dark for 28°C for 6 weeks and the number of calli that produce embryogenic structures was recorded. The morphology of the multiplied calli was also recorded.

Effect of Hormone Free Medium, 2ip and BAP on Differentiation of Callus

After callus proliferation phase, well developed calli were transferred into low 2, 4-D medium for 8 weeks and then to callus maturation medium (Y3 medium containing 6% sucrose, 0.9 g/l glutamine) for 8 weeks as described in Perera et al., (2009). Four treatments were tested to improve the efficiency of differentiation (Table 1). The calli were also categorized as, callus with embryogenic structures (category 1), embryogenic structures and hard structures (category 2), shoot like structures (category 3), and globular structures (category 4).

Statistical Analysis

The data were analysed using SAS statistical package (SAS Institute 1999) and Minitab 15. Two sample proportional analysis was conducted on data of crushed and sliced ovaries for callus initiation. Chi-square or maximum likelihood analysis of variance was conducted using CATMOD procedures for the data of callus multiplication, somatic embryogenesis and differentiation experiments.

Table 1. Treatments for callus maturation

Treatment	Description
T1	Y3 + normal phytagel 0.25 % w/v) + without growth regulators
T2	Y3 + high phytagel (0.3 % w/v) + without growth regulators
T3	Y3 + normal phytagel 0.25 % w/v)+ 20 µM 2ip
T4	Y3+ normal phytagel 0.25 % w/v)+ 10 µM BAP

RESULTS AND DISCUSSION

Effect of Crushing and Slicing of Ovaries on Callus Induction

Calli consist of translucent ear like structures were observed in both crushed and sliced ovary cultures after 8 weeks of inoculation. Ovaries cultured as thin slices gave significantly higher callogenesis percentage (65.6%), indicating positive effect of slicing of ovaries for callogenesis (Table 2). The callusing percentage of crushed ovaries is lower and comparable to that reported by Perera *et al.*, (2007). Culturing of thin layer section as explants reported to improve callusing in many crops (Samosir *et al.*, 1998; Benkirane *et al.*, 2000; Steinmacher *et al.*, 2007). According to Benkirane *et al.*, (2000), lager explants maintain normal tissue interactions which could inhibit rapid cell division resulting callus formation. Crushing of explants could result unnecessary damages to tissues leading tissue death.

Effect of TDZ on Callus Multiplication

Multiplication of initial calli into embryogenic callus or non embryogenic masses was observed in both media after 6 weeks of inoculation. The calli cultured in media containing 9 µM TDZ and 160 µM 2, 4 D produced significantly higher percentage (70.4%) of embryogenic callus than that resulted in medium without TDZ (42.6%) (Table 3). Perera *et al.*, (2007) also reported stimulation of initial callogenesis of coconut ovaries by incorporation of 9 µM TDZ to callus induction medium. Embrogenic potential of multiplied callus also depends on the stage of the initial callus. Initial calli with frilly translucent structures gave the highest number of embryogenic callus after multiplication. However the effect of stage of the initial calli on embryogenic callus multiplication is not statistically significant.

Table 2. Percentage of callogenesis of crushed and sliced explants

Culture method	Number of ovaries cultured	Percentage of callogenesis (%)
Crushed explants	55	36.4
Thin sliced expalnts	61	65.6

Proportional analysis was significant for treatments at $p \leq 0.05$

Table 3. Percentage of Embryogenic callus percentage with and without TDZ

Medium	No of callus sub cultured	Embryogenic callus Percentage (%)
CRI 72H+ 9 µM TDZ+160 µM 2,4-D	27	70.4
CRI 72H+160 µM 2,4-D	68	42.6
Parameter	**Chi-Square**	**Pr > ChiSq**
Stage	7.16	0.0671
Medium	6.91	0.0086*

Maximum likelihood analysis was significant.
**Treatment significantly different at $p \leq 0.05$ level*

Effect of Hormone free Media, 2ip and BAP on Differentiation of Callus

Highest percentage of embryogenic callus maturation (66.67%) was observed in hormone free medium with low phytagel percentage. In hormone free medium with high phytagel content (0.3% w/v), 58.06% of embryogenic callus maturation was observed. When compared the morphological features of the callus matured in hormone free medium, more globular structures with good regeneration ability were observed in media containing high phytagel percentage (0.3% w/v).

Table 4. Somatic embryogenesis percentage of embryogenic callus in different maturation medium

Media	Morphological stage				
	Category 1	Category 2	Category 3	Category 4	Total
2ip	100%	66.67%	30%	13.33%	30%
BAP	100%	66.67%	50%	15.4%	40%
High phytagel	100%	85.71%	33.33%	50%	58.06%
Normal phytagel	100%	66.67%	81.8%	25%	66.67%
Total	100%	82.35%	51.28%	30.43%	

Parameter	Chi-Square	Pr > ChiSq
Morphological stage	18.37	0.0004***
Medium	9.10	0.0280*

Maximum likelihood analysis was significant.

**** and * - Treatments significantly different at $p \leq 0.001$ level and $p \leq 0.05$ level respectively*

The results of this experiment revealed callus maturation in hormone free medium with high phytagel (0.3% w/v) content favour somatic embryogenesis. According to Hornung and Verdeil, (1999) gradual reduction of 2, 4-D in culture medium induced pro-embryos in coconut plumule derived callus. Maturation of pro-embryos occurs in hormone free medium (Perera et al., 2009). In this study maturation of callus in hormone free medium resulted callus with higher embryogenic potential (58.06% and 66%) confirming above facts. Increase of phytagel content in media further improved differentiation in morphological aspects. Early exposure to cytokine (2ip / BAP), omitting the maturation of pro-embryos at hormone free medium, which has negatively effect on differentiation of callus.

CONCLUSIONS

This study indicates that callus initiation could be enhanced by culturing thin slice of ovaries as explants. Multiplication of embryogenic callus is favoured by incorporation of 9 µM TDZ to multiplication medium and further the embryogenic potential of callus after multiplication depends on the morphological features of initial callus. Callus maturation leading to improved somatic embryogenic differentiation is enhanced by using hormone free medium with high phytagel percentage (0.3% w/v) in callus maturation medium. Incorporation of Growth regulators in maturation medium inhibits differentiation of callus towards somatic embryogenesis.

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