Optimization of callus induction in Coconut cv MATAG anther cultures

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Abstract – Anthers of coconut cv MATAG were harvested from unopen male flowers to investigate the optimal conditions affecting callus induction. After disinfection, the anther explants were cultured on full Y3 and modified Y3 media supplanted with varying concentrations of 2,4-D, NAA or/and IAA. Anthers cultured in Y3 medium containing 2,4-D or NAA formed few calluses, none of which proliferated. The best callogenesis was obtained in Y3 supplemented with 2.0 mg/L IAA. The callus obtained was maintained through sub-culturing on the fresh medium containing the plant growth regulator for callus proliferation. A medium containing 2.0 mg/L IAA in combination with 2.0 mg/L 2,4-D promoted good callus proliferation.

Keywords: Anther, callus induction, callus proliferation, *Cocos nucifera*

Introduction

In the past decade, the global demand for coconut has grown by 500%, helped in part by celebrity endorsements of its products, particularly coconut water and virgin coconut oil, touted as "superfoods" to the global market (Sri Lanka, 2017). Malaysia remains one of the top 10 coconut-producing countries in the world, and coconut is the 4th most important crop in the country after oil palm, rubber, and rice. The production of coconut went through a decreasing trend from 2014 until 2016 when its estimated production was 530,000 MT (Rohani, 2017). To promote coconut planting, commercially desirable cultivars have to be made available to planters. A breeding program is essential to introduce cultivars that are genetically uniform and that possess agronomically superior characters. Nevertheless, the coconut palm's long breeding cycle makes this an expensive undertaking. The tissue culture technique of plant propagation was first reported by Guha and Maheshwari who worked on Datura anther culture (Antonietta, 2006; Guha and Maheshwari; 1964). The production of haploid plants, produced through in vitro anther culture, serves as a complementary approach to traditional plant breeding (Savafikan et al., 1999). In this regard, the production of plantlets is feasible via diploid and haploid callus and/or direct regeneration (Sadeghi, 2002).

Talebi et al. (2007) and He et al. (1998) have reported occasions where genotypes that show high callus induction display poor regeneration ability, and vice versa. Hence, thorough investigation is necessary to optimize both these phases of culture. High callus induction, observed in some instances, might be due to higher doses of hormone in the induction medium. While the application of higher doses of auxins can significantly increase callus induction efficiency, such calluses are frequently embryogenic-less and perform poorly in green plant regeneration. Liang (1978) notes that hormone requirement is genotype specific, and the optimum level of auxin in callus induction media requires some degree of compromise between callus induction and regeneration frequency. To date, there have been few reports on coconut anther culture. The aim of this study was to investigate the effects of plant growth regulators, specifically auxins, on in vitro primary callus induction and proliferation in anther cultures of Coconut cv MATAG. This study was aimed at eventually establishing a protocol for rapid clonal propagation of the coconut palm through somatic embryogenesis.

Materials and methods

Cultures were initiated from anthers of *Cocos nucifera* cv MATAG collected from the MARDI Bagan Datok farm in Perak, Malaysia. Inflorescences with male flower buds were harvested and transported in plastic bags to maintain freshness prior to culture in the laboratory. The inflorescences were surface sterilized three times using 100% ethanol for 20 mins. The rachillae- bearing inflorescences were excised from the spadices and the male inflorescence was isolated from the rachillae. Male flowers were teased open to remove the anthers which were then transferred to callus induction medium (as listed in Table 1). This process was conducted in the laminar flow

chamber that had been cleaned with 70% alcohol to reduce the risk of contamination. Each inflorescence was divided into three equal parts lengthwise: bottom, middle and top sections (Fig. 1a), with the anthers from the male flowers of the inflorescence serving as explants to initiate calluses (Fig. 1b,c,f). The anthers were teased open using scalpel blades and forceps (Fig. 1d,e), and then cultured on callus induction medium (Fig. 1g).
The basal medium used for this experiment was Y3 medium (Eeuwens, 1976) containing 60g/L sucrose and 0.1% activated charcoal. The medium was adjusted to pH 5.8 and then solidified with 3 g/L gelrite before autoclaving at 121°C for 20 min. The cultures were maintained in a dark culture room at 25±2°C. For callus induction of the explant, the basal media were supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA) or/and indole-3-acetic acid (IAA) at concentrations of 0, 0.5, 1.0, 5.0, 10.0, 20.0 and 40.0 mg/Lm separately or in combination with one another as shown in Table 1. The rate of callus production after 3-4 months of culture was recorded, following which the calluses were sub-cultured in fresh media for callus proliferation. For the purpose of callus proliferation, IAA, 2,4-D and NAA were used separately in different concentrations, as well as in combination with one another as shown in Table 2. In this procedure, fresh callus from the treatment using 2.0 mg/L IAA was used as starting material. After the cultures had been incubated in darkness for 8 weeks, data on callogenesis (% proliferation) and the occurrence of tissue browning were recorded.

Results and discussion
The objective of the study was to identify the most effective auxin(s) and concentrations to be added to basic culture medium for successful induction and proliferation of callus from coconut anthers. The effect of auxins on callus induction was investigated using varying concentrations of 2,4-D, NAA and IAA (Table 1) and callus induction success was observed after 3-4 months of culture. Treatment with 2,4-D, IAA and NAA induced calluses in anther cultures with varying levels of efficiency, depending on the concentration of auxins. Overall, the rate of callus initiation for all treatments was very low, being less than 1%. Table 1 shows poor callusing (0.2 to 0.95% in tissue from the middle and top inflorescence segments) in media containing 2-5 mg/L IAA (Fig. 1 h,i,j) . Anthers from the mid-segment of the inflorescence gave the highest success (0.95%), followed by those from the top segment (0.35%). Callus was also observed when the basic medium was supplemented with high concentrations of 2,4-D in the range of 10-40 mg/L. whereby 0.1 to 0.2% callus initiation was observed. Treatment with NAA at a concentration of 40 mg/L also elicited the production of callus at a very low rate (0.1%). Following sub-culture, all the calluses from this treatment turned brown after 4 to 8 months, and no plantlet was obtained (Fig. 1k,l,m)

The findings of the study showed that callus induction rate was affected by the type of auxin used and its concentration. Various auxin types and concentrations are required to obtain efficient yields of embryogenic structures in crop species (Han et al., 2000). Among the auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) is the most widely used in anther culture studies (Ball et al., 1993). Optimum concentration of growth regulators for the induction of androgenesis varies, depending on the species (Custodio et al., 2005). According to Shane et al. (1993), 2,4-D is superior to IAA when added to the anther culture induction medium. While addition of 2,4-D reduced plant regeneration, it substantially increased green plant percentage at a 0.3-mg l−1 concentration of IAA. Isabel et al. (1995) stated that when auxins were used in isolation, only 2,4-D promoted consistent cell proliferation. The addition of BA to auxin-supplemented media enhanced callus formation, with best results obtained in the presence of IAA, IBA, or 2,4-D for anthers of *Sorbus domestica.*

A study was undertaken to examine callus proliferation as influenced by various auxins (IAA, 2, 4-D and NAA) applied singly or in combination with one another. A combination of 2 mg/L IAA and 2 mg/L 2,4-D gave the highest rate of callus proliferation of 20.0%, with no browning of the tissue observed after 8 weeks (Table 2) (Fig. 1n). Higher concentrations of auxin (5 mg/L IAA+ 5 mg/L 2,4-D) lowered the callus proliferation rate to 10.5%. Treatment with 5 mg/L IAA or a combination of 2 mg/L IAA+2 mg/L NAA decreased the proliferation rate further to 5%.

In all the media tested, callus proliferation did not occur after 8 weeks in the absence of auxins. It was only with the supplementation of auxins that callus induction and proliferation were stimulated. The type of auxin and its concentration were important factors in callus proliferation. In this regard, it is noted that anther cultures on MS medium supplemented with a combination of 2.0 mg/L 2,4-D and 0.5 mg/L BAP show good callus growth with wheat explants (*Triticum aestivum* L.) (Mohammad et al., 2003). According to Yu et al. (2008), 2.4-D is generally the best auxin to use in grasses as it supports and enhances callus induction and facilitate subculture. 2.4-D was

successfully used at a concentration of 10 mg/L of MS medium for callus proliferation in wheat (Benderradji et al., 2012). Excellent callus proliferation in oil palm (*Elaeis guineensis* Jacq.) from leaf explants was observed on media containing 67 mg/L 2,4-D, with 19.8% success recorded after 7 months (Yusnita and Dwi Hapsoro, 2011). Subsequent somatic embryogenesis in oil palm is mostly by indirect embryogenesis (Teixeira et al., 1999; Hilae and Te Chato 2005). In peanut (Yusnita et al., 2006), sugarcane (Khan andKhatri, 2006), rice (Meneses et al., 2005), and *Gentiana straminea* Maxim (Cai et al., 2009), formation of callus and embryogenic cell clusters requires auxin, whereas it can be dispensed with, or its concentrations lowered during their development and maturation.

Conclusion

The results from this study showed that anthers of *Cocos nucifera* L. cv MATAG can be used as explants for callus initiation. Y3 culture medium supplemented with 2 mg/L IAA was suitable for callus induction from anthers. A combination of 2,4-D with another plant growth regulator such as NAA could be considered in future work to increase the quality and quantity of calluses obtained.

Table 1: Effect of 2,4-D, IAA and NAA on induction of callus from anthers obtained from different segments of the inflorescence (Results after 3-4 months)

Auxin treatments (mg/L)			Callus induction (%) and visual assessment* of callus growth after 3-4 months		
2,4-D	IAA	NAA	Top segment	Middle	Bottom
0	-	-	-	-	-
0.5	-	-	-	-	-
1	-	-	-	-	-
5	-	-	-	-	-
10	-	-	-	+ (0.20%)	-
20	-	-	-	+ (0.10%)	-
40	-	-	-	+ (0.10%)	+ (0.10%)
-	0		-	-	-
-	0.5	-	-	-	-
-	1	-	-	-	-
-	2	-	++ (0.35%)	++ (0.95%)	+ (0.25%)
-	5	-	+ (0.20%)	+ (0.30%)	-
-	10	-	-	-	+
-	20	-	-	-	+
-	40	-	-	-	+
-	-	-	-	-	+
-	-	0.5	-	-	-
-	-	1	-	-	-
-	-	5	-	-	-
-	-	10	-	-	-
-	-	20	-	+	-
-	-	40	+ (0.1%)	+ (0.10%)	-
1	2	-	+ (0.10%)	+ (0.20%)	-
5	2	-	-	-	-
10	2	-	-	-	-
20	2	-	-	-	-
1	-	5	-	-	-
5	-	5	-	-	-
10	-	5	-	-	-
20	-	5	-	-	-

*– No callus, + growth almost retarded, + slow growth and browning, + Poor callus

Table 2: Effect of auxins at different concentrations on proliferation of callus cultured on Y3 medium (Results after 8 weeks)

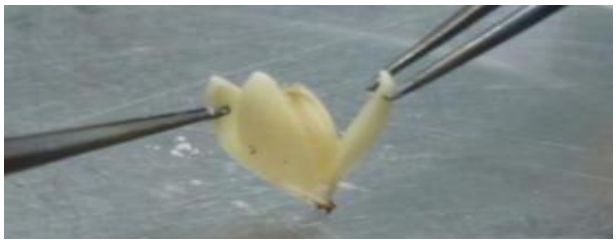
Treatments (mg/L)			Callogenesis (% proliferation)	Browning
IAA	2,4-D	NAA		
0	-	-	0	+++
2	-	-	0	+++
5	-	-	5.0	++
-	2	-	0	+++
-	5	-	0	+++
-	-	2	0	+++
-	-	5	0	+++
2	2	-	20.0	-
2	-	2	5.0	++
-	2	2	0	+++
5	5	-	10.5	+
5	-	5	0	++
-	5	5	0	+++

Callus browning scale: - = Nil; + = <20% browning ; ++ = 30-50% browning; +++ 100%brownin

a

b

c



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e

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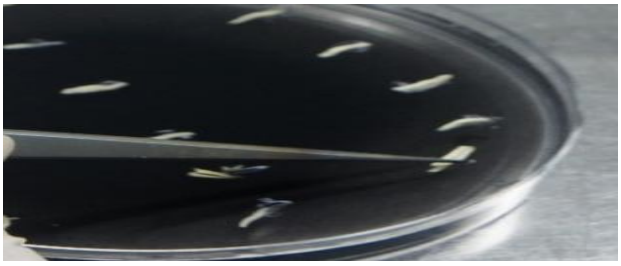

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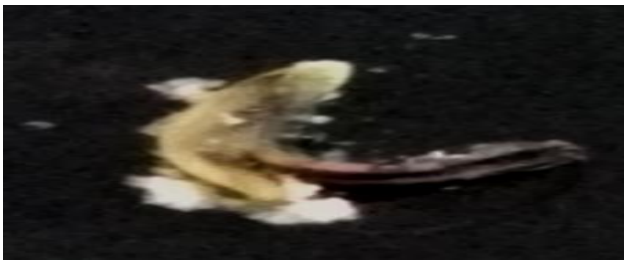

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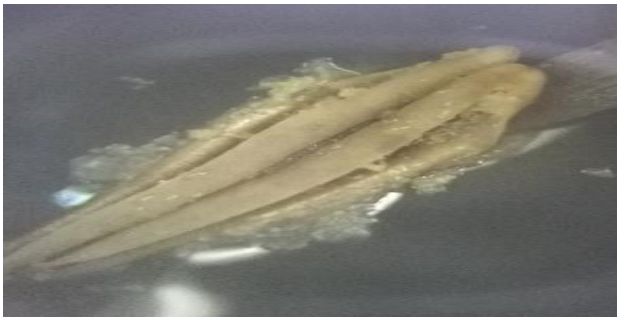

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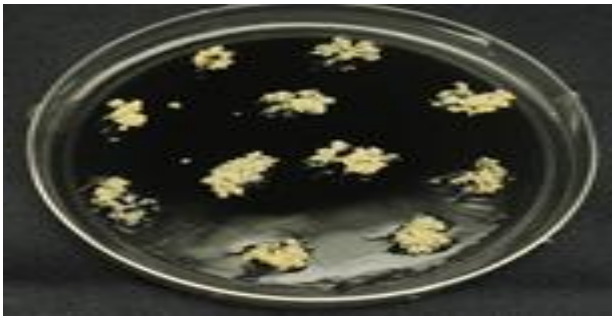

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Figure 1: Figure 1:. Callus induction and proliferation from the anthers of *Cocos nucifera* L. cv MATAG cultured on Y3 media. (a,b) Each inflorescence was divided into three equal parts lengthwise: bottom, middle and top sections, (c) Anther (d,e) Anther wall was broken open, (f) Anther (g) Anther cultured on media after 1 month, (h,i,j), Callus formation from the anther cultured on 2 mg/L IAA, (k,l) No callus produced from these anthers, (m) Anther turned brown and callus proliferated, and (n). Callus proliferation.

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