

Effect of exogenous polyamines on coconut (*Cocos nucifera* L.) embryogenic callus multiplication

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ABSTRACT

Major bottlenecks of coconut *in vitro* culture are poor plant regeneration rate, severe browning and premature necrosis of cultured tissue, and heterogeneous response of individual palms and explants to *in vitro* culture conditions. Among them, tissue browning is a common and often severe problem in coconut *in vitro* culture systems which results in death of explant/ callus ultimately. This experiment was carried out to enhance the *in vitro* multiplication of coconut, which is a highly recalcitrant species to *in vitro* culture through exogenously added polyamines in the media. The polyamines are important for *in vitro* cell division, cell growth and to delay senescence. In the present study, unfertilized ovary derived calli were cultured on Y3 basal medium supplemented with sucrose (5%), 2,4-D, phytigel (3 gL⁻¹), activated charcoal (2.5 gL⁻¹), and polyamine. Three polyamine types (0.1mM spermine, 1.0 mM putrescine and 0.5mM spermidine) were tested in combination with two 2,4-D concentrations (0.30 and 0.60 mM) in order to enhance coconut *in vitro* multiplication. All the cultures were incubated in dark at 26±2 °C. The embryogenic structures, embryogenic callusing, non-embryogenic callusing, and browning were recorded separately for each treatment after five weeks from the culture establishment. The polyamine treatments did not have significant effects on frequencies of embryogenic structures, embryogenic callus, non-embryogenic callus and browned callus formation at the initial stages of coconut somatic embryogenesis irrespective of the tested 2,4-D concentrations. Furthermore, the results indicated that decreased 2,4-D levels have significantly reduced browning, resulting 44.79 % browning frequency (0.8-fold lesser browning) in media supplemented with 0.30 mM 2,4-D. However, the potential effects of exogenously added polyamines at the initial stages of coconut somatic embryogenesis could be delivered during the latter stages of the somatic embryogenesis as previously reported in other experiments. Thus, continuous subculture may be necessary.

Keywords: Polyamines, Browning, *Cocos nucifera*, *In vitro*, Calli

INTRODUCTION

The coconut palm (*Cocos nucifera* L.) is one of the major plantation crops and plays an important role in the economy of tropical and sub-tropical regions of the world. It has a tremendous potential as a multipurpose crop, which provides foods, beverages, building, and raw materials for households. Unfortunately, there are major issues with the coconut industry including low production and productivity of coconut plantations due to abiotic and biotic stresses, and scarcity of improved planting materials. Therefore, there is an increasing need for coconut crop improvement. Coconut is mainly a cross-pollinated palm, only propagated by seeds resulting heterogeneous population. *In vitro* propagation is the only tool, which produces true-to-type plants facilitating the production of high-quality planting materials with superior traits. Although *in vitro* propagation protocols of coconut have been developed and improved over the past few decades, an efficient micro propagation protocol is yet to be developed. The success has been limited due to slow and low plant regeneration rate, intensive browning of cultured tissues (due to oxidation of polyphenols), premature necrosis of explanted tissues and the heterogeneous response of both individual palms (within the same cultivar) and individual explants (within the same palm) (Nair et al. 1999). Thus, it is important to improve the existing *in vitro* propagation protocols to achieve an efficient mass propagation protocol for coconut.

There are many studies on evaluating different factors affecting *in vitro* performance of coconut such as the genotype, type of explants, media composition, and culture conditions (Oropeza et al., 2018; Perera et al.,

2007; Pérez-Núñez et al., 2006). Different types and concentrations of exogenously added chemicals into the basal media were tested previously and among them, polyamines play a major role in many crops. Polyamines are plant bio stimulants, which are considered as a class of growth regulators. They are synthesized during metabolism and important in plant growth, development, and environmental stress tolerance. Polyamines are also found to improve somatic embryogenesis and suppress tissue browning through controlling ethylene production in plant tissue culture. However, the role of polyamines on plant morphogenesis *in vitro* is yet to be understood. In higher plants, polyamines are available in free-form and putrescine (Put), spermidine (Spd), and spermine (Spm) are the main polyamines in plants. Polyamines have been successfully utilized to improve *in vitro* cultures of various plant species including date palm (*Phoenix dactylifera*), oil palm (*Elaeis guineensis*), sugarcane (*Saccharum officinarum*), wheat (*Triticum aestivum*), hazelnut (*Corylus avellane* L.), oats (*Avena sativa*), and coconut (*Cocos nucifera*). Spermidine concentration of 500 mg/l appeared to be the best amino acid used to stimulate steroid biosynthesis in date palm embryos and the highest shoots weight was gained by adding 500 mg/l spermidine in the shooting stage over glutamine, asperagine and spermidine (El-sharabasy et al., 2012). A shoot proliferation medium with 0.5 mM putrescine significantly enhanced somatic embryogenesis and plant regeneration on recalcitrant oats (*Avena* spp) (Kelley et al., 2002). Polyamines also stimulated both shoot elongation and mean number of buds per shoot in hazelnut (*Corylus avellana* L.) cultures (Nas, 2004). Similarly, these polyamines were associated with plant growth increment and the

suppression of tissue browning through controlling of ethylene formation (Apelbaum *et al.*, 1981).

However, incorporation of polyamines into coconut *in vitro* cultures are limited only to plumule and embryo explants with limited success. The present investigation is the first attempt, where polyamines are being incorporated into coconut ovary culture medium and this paper reports the effect of exogenous polyamines (spermine, spermidine and putrescine) on ovary-derived callus multiplication of coconut.

MATERIALS AND METHOD

Immature inflorescences, at the -4 stage (with 0 corresponding to the youngest opened inflorescence), of coconut cultivar dwarf x tall (DT), were harvested. Under aseptic conditions, female flowers, which are attached to the basal part of each rachilla of immature inflorescences, were separated and disinfected using 3% clorox for 10 minutes and

then rinsed four times with sterile distilled water. Then the ovaries were excised by removing two male flowers flanking each female flowers, petals and sepals of the female flower while submerged in an antioxidant solution (sterilized solution composed of 0.15 gL⁻¹ ascorbic acid, 0.1 gL⁻¹ citric acid, and 30 gL⁻¹ sucrose).

All culture media were prepared according to Rivera-Solís *et al.*, 2018. Basal media was prepared with Y3 formulation (Eeuwens, 1976) supplemented with sucrose (5%), phytigel (3 gL⁻¹), and activated charcoal (2.5 gL⁻¹). The pH of the media was adjusted to 5.75 prior to adding phytigel and autoclaving. For the callus induction, unfertilized ovaries were cultured on the media supplemented with 0.6 mM 2,4-D. After incubation in dark at 26±2 °C for 5 weeks, initial calli were developed. Embryogenic structures (3 mm x 3 mm x 3 mm; Fig.1b) isolated from initial calli (Fig.1a) derived from the unfertilized ovary cultures were used as the experimental units.



Figure 1: embryogenic callus and embryogenic structures (ES) under the dissecting microscope (a) An embryogenic callus with ES (b) subdivided ES (3 mm x 3 mm x 3 mm) which were used to initiate *in vitro* cultures under different treatments.

The isolated embryogenic structures were cultured in 40 ml screw-capped vials containing 15 ml of callus multiplication medium. The callus multiplication medium was composed of the basal medium supplemented with 2,4-D (0.6 mM and 0.3 mM) and/or filter-sterilized exogenously added polyamines (0.1 mM spermine, 1.0 mM putrescine, and 0.5 mM spermidine) resulting different treatments. The basal medium supplemented with 0.6 mM 2,4-

D was considered as the control (i.e. T1). All the cultures were incubated in dark at 26±2 °C for 5 weeks. The experiment was laid out as two factor factorial, where the two factors are 2,4-D concentration (i.e. 0.3 mM and 0.6 mM) and polyamine type (i.e. spermine, putrescine, and spermidine), on a completely randomized design (CRD). A total number of 224 initial calli were used for the experiment and each treatment was composed of seven replicates.

Table 1: Different combinations and concentrations of 2,4-D and polyamines in callus multiplication media

Treatment	2,4-D concentration (mM)	Polyamine concentrations (mM)		
		Spermine	Putrescine	Spermidine
T1 (Control)	0.60	-	-	-
T2	0.60	0.10	-	-
T3	0.60	-	1.00	-
T4	0.60	-	-	0.50
T5	0.30	-	-	-
T6	0.30	0.10	-	-
T7	0.30	-	1.00	-
T8	0.30	-	-	0.50

The number of embryogenic structures, embryogenic calli, non-embryogenic calli, and browned calli were recorded after five weeks from culture establishment. Then embryogenic structure formation, embryogenic callusing, non-embryogenic callusing, and browning frequencies were calculated separately for each treatment. The embryogenic structure formation frequency was calculated as follows:

$$\text{Embryogenic callusing frequency (\%)} = \frac{\text{Number of embryogenic calli}}{\text{Total number of calli}} \times 100$$

Accordingly, the embryogenic structure formation, non-embryogenic callusing, and browning frequencies were also calculated. Data were subjected to analysis of variance (ANOVA) with Minitab 17 statistical software.

RESULTS AND DISCUSSION

The formation of embryogenic structures, embryogenic calli, non-embryogenic calli, and browned calli was evaluated during the initial stages of coconut embryogenesis with the use of two 2,4-D levels(0.6 mM and 0.3 mM) and three polyamine types(0.1 mM spermine, 1.0 mM putrescine, and 0.5 mM spermidine).

Irrespective of the treatment type, all the evaluated *in vitro* stages (i.e. embryogenic

structures, embryogenic calli, non-embryogenic calli, and browned calli) were observed in different frequencies. Embryogenic structure formation frequency of all eight treatments were ranged from 22.76% to 54.45% and embryogenic calli were in 13.81% to 37.82%. Production of non-embryogenic calli and browned calli frequencies of all treatments were ranged from 19.70% to 26.75% and 11.89% to 18.15% respectively (Figure 2) & (Table 2).

Table 2: Effect of 2,4-D and polyamine on callus multiplication of coconut

Treatment		Frequencies			
		ES	EC	NEC	BC
T1 (Control)		29.28	32.59	24.83	13.30
T2		22.76	37.83	21.25	18.16
T3		35.26	24.70	22.50	17.53
T4		35.62	24.23	23.09	17.05
T5		42.54	20.58	19.69	17.19
T6		47.54	13.82	26.75	11.90
T7		35.04	26.94	25.50	12.52
T8		35.13	30.24	22.66	11.97
P-values	2,4-D level	0.668	0.219	0.12	0.026*
	Polyamine	0.992	0.623	0.975	0.181
	2,4-D level*Polyamine	0.991	0.825	0.862	0.533

ES: embryogenic structures; EC: embryogenic calli; NEC: non-embryogenic calli; BC: browned calli
* treatments are significantly different ($P \leq 0.05$)

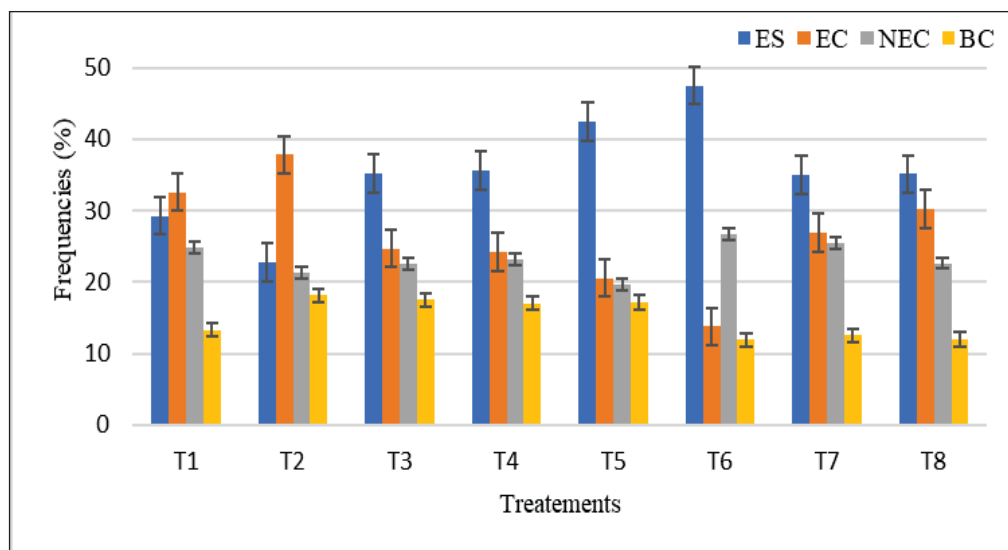


Figure 2: Effect of different types of polyamines and 2,4-D levels on coconut *in vitro* multiplication. ES: embryogenic structures; EC: embryogenic calli; NEC: non-embryogenic calli; BC: browned calli

The success of the multiplication procedure depends on the formation of embryogenic structures and embryogenic calli. The present study showed that the development of both embryogenic structures and embryogenic calli frequencies was greater than the development of non-embryogenic calli and browned calli frequencies in all the tested treatments.

Somatic embryogenesis is the most promising approach of coconut *in vitro* propagation and it comprises several stages: callus induction, somatic embryo initiation, somatic embryo maturation, shoot development, and rooting. Each and every stage is important for the success of the coconut *in vitro* regeneration protocol (Bandupriya *et al.*, 2016). Consistent and rapid production of calli are important to achieve a successful somatic embryogenic protocol (Perera *et al.*, 2007b). Somatic embryogenesis is usually observed through the callus phase and the production of the embryogenic callus is a primary

requirement for successful plant regeneration (Fernando *et al.*, 2004). Theoretically, an unlimited number of embryos can be produced using a single explant, but it is practically difficult with coconut *in vitro* culture (Parrott *et al.* 1991). However, with coconut plumule explants, a greater number (calculated yield of 98,000 somatic embryos) of somatic embryos were reported when callus multiplication and secondary somatic embryogenesis are incorporated into the micropropagation protocol (Pérez-Núñez *et al.*, 2006). Being a zygotic tissue, the use of plumule explants has the disadvantage of, preventing clonal multiplication of palms with known superior agronomic traits.

In the present study, both embryogenic structures (Fig.3a) and calli could be observed under all the treatments after 5 weeks from culture establishment, by using the unfertilized ovary as the initial explant. The results of this study indicated that embryogenic structures themselves can be used as explants to produce

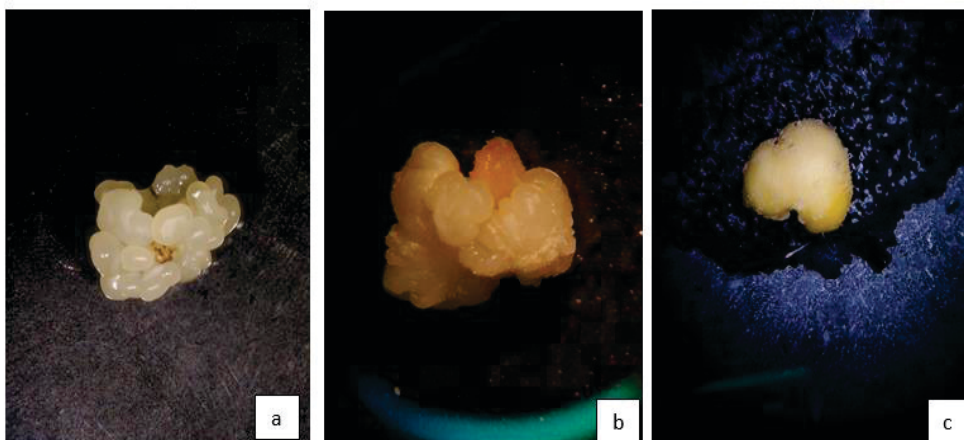


Figure 3: Different developmental stages of coconut ovary culture. (a) embryogenic structures (b) embryogenic calli (c) non-embryogenic calli

the new embryogenic callus leading to greater somatic embryo yield. Embryogenic structures were distinguished from calli by their translucent heart or round shape. Both embryogenic calli (Fig. 3b) and fast-growing non-embryogenic calli (Fig. 3c) were easily distinguishable due to translucent and frilly appearance of embryogenic calli. Secondary somatic embryogenesis significantly contributes to maintain and multiply callus for greater yields and helps to retain embryogenic competence during extended culture times in different species (Stamp and Henshaw, 1987; Baker and Wetzstein, 1995; Schavemaker and Jacobsen, 1995; Martinelli *et al.*, 2001).

Incorporation of polyamines into coconut *in vitro* cultures is limited only to plumule and embryo explants of dwarf coconut varieties during callus induction and proliferation stages (Adkins *et al.*, 1999; Adkins *et al.*, 2001; Rajesh & Karun, 2014). The present study is the first attempt where polyamines are being incorporated into coconut ovary culture medium to enhance callus multiplication and subsequent *in vitro* performance. However, immediate significant

effects of adding polyamines (i.e. 0.10 mM spermine, 1.00 mM putrescine, and 0.50 mM spermidine) in to the media were not observed at the initial stages of somatic embryogenesis. However, coconut plumular explants grown in the media with smaller 2,4-D concentrations (74.66 μ M) and polyamines (100 μ M spermine and 1000 μ M putrescine) resulted in reduced non-embryogenic and rhizogenic portions (unproductive) and enhanced embryogenic portions during monthly subcultures (Rajesh & Karun, 2014). Incorporation of 0.5 μ M spermidine, 7.5 μ M putrescine, and 0.01 μ M spermine into callus induction stage, enhanced the somatic embryogenesis by 52%, 106%, and 176% respectively in coconut mature zygotic embryos of coconuts (Adkins *et al.*, 1998). Therefore, adding polyamines to the basal medium during the initial stages of somatic embryogenesis, may reveal greater effects during latter stages of somatic embryogenesis. Since this article discussed only the effects of exogenously added polyamines during the initial stages of somatic embryogenesis, it will be important to continue the current study in order to observe potential effects of the

polyamines during the latter stages of somatic embryogenesis.

Browning of tissues due to explant deterioration, accumulation and oxidation of phenolic compounds in tissue and culture media is one of the biggest constraints in coconut *in vitro* culture. One of the primary reasons for oxidative browning is higher 2,4-D levels in the media. A major finding of this study was the significant difference in callus browning between two 2,4-D levels. Browning calli frequency was significantly lesser in the media supplemented with 0.30 mM 2,4-D than the 0.60 mM 2,4-D. However, embryogenic structures, embryogenic callusing, and non-embryogenic callusing frequencies were not significantly affected by the type of treatment at the initial stages of coconut somatic embryogenesis. However, using ultra-high levels of 2,4-D (0.60 mM), a successful *in vitro* multiplication protocol was produced using plumule explants of dwarf coconut varieties in Mexico (Pérez-Núñez *et al.*, 2006). The genotype effect could be the primary reason of why severe browning was observed during the current study.

CONCLUSION

This study evaluated the feasibility of developing an *in vitro* plant regeneration

protocol via embryogenic callus multiplication, with use of the unfertilized ovary of coconut as the initial explant. Regardless of the treatments, embryogenic structures, embryogenic calli, non-embryogenic calli, and browned calli were observed in different frequencies. The different polyamine treatments (0.1 mM spermine, 1.0 mM putrescine, and 0.5 mM spermidine) did not show significant effects at the initial stages of coconut somatic embryogenesis. This study also revealed that decreased 2,4-D levels have significantly reduced browning by 0.8-fold, hence the use of 0.30 mM 2,4-D was better than the 0.60 mM 2,4-D in terms of tissue browning. The effect of exogenous polyamines has the potential of increasing callus multiplication at later stages according to previous literature. Therefore, continuous subculture may be essential for development of a successful protocol.

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REFERENCES

1. Adkins, S. W., Samosir, Y. M. S., & Ernawati, A. (1998). Control of ethylene and use of polyamines can optimise the conditions for Somatic Embryogenesis in coconut and papaya. In International Symposium on Biotechnology Tropical and subtropical Species (pp. 459 - 466).
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2. Adkins, S. W., Samosir, Y. M. S., & Godwin, I. D. (1999). Control of environmental conditions and the use of polyamines can optimise the conditions for the initiation and proliferation of coconut somatic embryos. In: Current advances in coconut biotechnology (eds. C. Oropeza, J.L. Verdeil, G.R. Ashburner, R. Cardena, J.M. Santamaria) pp. 1988-1989.
 3. Adkins, S. W., Samosir, Y. M. S., & Nikmatullah, A. (2001). Coconut In vitro ecology : Modification of headspace and medium additives can optimize somatic embryogenesis and plantlet development Coconut (*Cocos nucifera*). *Acta Horticulturae*, v692: 21-31
 4. Aydin, M., Pour, A. H., Haliloglu, K., & Tosun, M. (2016). Effect of polyamines on somatic embryogenesis via mature embryo in wheat. *Turkish Journal of Biology*, 1178-1184.
 5. Bandupriya, H. D. D., Fernando, S. C., & Vidhanaarachchi, Y. R. M. (2016). Micropropagation and Androgenesis in coconut: an assessment of Sri Lankan implication. *COCOS*, 22: 31-47.
 6. Eeuwens, C. J. (1976). Mineral Requirements for Growth and Callus Initiation of Tissue Explants Excised from Mature Coconut Palms (*Cocos nucifera*) and Cultured in vitro. *Physiologia Plantarum*, 23-28.
 7. El-sharabasy, S., Farag, M. A., Safwat, G., & Diab, A. (2012). Effect of Amino Acids on the Growth and Production of Steroids in Date Palm Using Tissue Culture Technique. *Researcher*, 75-84.
 8. Fernando, S. C., Weerakoon, L. K., & Gunathilake, T. R. (2004). Micropropagation of Coconut through plumule culture. *COCOS*, 16: 1-10.
 9. Kelley, R. Y., Zipf, A. E., Wesenberg, D. E., & Govind, C. S. (2002). Putrescine enhanced somatic embryos and plant numbers from elite Oat (*Avena spp.*L.) and reciprocal crosses. *In Vitro Cellular & Developmental Biology*, 508-512.
 10. Nas, M. N. (2004). Inclusion of Polyamines in the Medium Improves Shoot Elongation in Hazelnut (*Corylus avellana* L.) Micropropagation. *Turkish Journal of Agriculture and Forestry*, 189-194.
 11. Oropeza, C., Rodríguez, G., Narváez, M., Sáenz-Carbonell, L., & Rivera-Solís, G. (2018). Addition of ionophore A23187 increases the efficiency of *Cocos nucifera* somatic embryogenesis. *3 Biotech*, 1-10.
 12. Perera, P. I. P., Hoche, V., Verdeil, J. L., Doulebeau, S., Yakandawala, D. M. D., & Weerakoon, L. K. (2007a). Unfertilized ovary: A novel explant for coconut (*Cocos nucifera* L.) somatic embryogenesis. *Plant Cell Reports*, 21-28.
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13. Perera, P. I. P., Hoher, V., Verdeil, J. L., Doubeau, S., Yakandawala, D. M. D., & Weerakoon, L. K. (2007b). Unfertilized ovary: A novel explant for coconut (*Cocos nucifera* L.) somatic embryogenesis. *Plant Cell Reports*, 21-28.
 14. Perera, P. I. P., Vidhanaarachchi, V. R. M., Gunathilake, T. R., Yakandawala, D. M. D., Hoher, V., Verdeil, J. L., & Weerakoon, L. K. (2009). Effect of plant growth regulators on ovary culture of coconut (*Cocos nucifera* L.). *Plant Cell, Tissue and Organ Culture*, 73-81
 15. Pérez-Núñez, M. T., Chan, J. L., Sáenz, L., González, T., Verdeil, J. L., & Oropeza, C. (2006). Improved somatic embryogenesis from *Cocos nucifera* L. plumule explants. *In Vitro Cellular & Developmental Biology - Plant*, 37-43.
 16. Rajesh, M. K., & Karun, A. (2014). Polyamine-induced somatic embryogenesis and plantlet regeneration in vitro from plumular explants of dwarf cultivars of coconut (*Cocos nucifera*). *Indian Journal of Agricultural Sciences*, 84(4): 527-530.
 17. Souza, R., Moura, E. De, Schuabb, A., Santa-catarina, C., & Silveira, V. (2016). Putrescine induces somatic embryo development and proteomic changes in embryogenic callus of sugarcane. *Journal of Proteomics*, 170-179.
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