COCOS, 2023 ***24:*** *13 - 21*
Printed in Sri Lanka
https://doi.org/10.4038/cocos.v24i1.5825

RESEARCH ARTICLE

Enhanced Coconut Somatic Embryogenesis of Unfertilized Ovary Derived Callus through Improved Media Composition

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ABSTRACT

Coconut is an economically demanding plantation crop in Sri Lanka, having an out-breeding nature with no vegetative propagation methods. Propagation by seeds generates heterogenous population and it is not sufficient to fulfill the increasing demand for coconut plantlets. Therefore, *in vitro* propagation is the ultimate approach of producing clones of coconut from the mother palms of interest. Coconut *in vitro* propagation is achieved through somatic embryogenesis and improvements of this process will easily cater the demand for plantlets. Hence, the main objective of the study was to enhance somatic embryogenic pathway of coconut using two different auxins (2,4-Dichlorophenoxyacetic acid [2,4-D] and 3,6-Dichloro-2-methoxybenzoic acid [Dicamba]) at the concentration of 300 µM. These auxins were tested alone and in combination with calcium ionophore (A23187) and 22(S),23(S)-homobrassinolide. Translucent structures or the ear-like structures obtained from culturing of unfertilized ovaries were used for the treatments. After six weeks of the culture establishment number of embryogenic structures per one translucent structure were counted. Each treatment produced embryogenic structures but a significantly greater number of embryogenic structures were developed with 2,4-D containing media than dicamba. The results revealed the potential of using 300 µM 2,4-D with calcium ionophore (A23187) and 22(S), 23(S)-homobrassinolide to improve the somatic embryogenic pathway of unfertilized ovary derived callus of coconut.

Keywords: Calcium ionophore, dicamba, homobrassinolide, *in vitro*, 2,4-Dichlrophenoxy acetic acid

1. INTRODUCTION

Coconut (*Cocos nucifera* L.) is one of the most important commercial and subsistence crop in the tropics and cultivated globally on about 12 million hectares. The demand for the coconut products around the world is growing exponentially (COGENT, 2018). Inadequate coconut production is a major challenge for the most of the coconut growing countries caused by losses due to senile plantations, pests and diseases. Hence, it is necessary to replant the plantations and increase the cultivation area to fulfill the growing demand for coconut products. Seed propagation of coconut is not sufficient for this purpose and also it produces heterogeneous populations. In order to supply good quality planting materials for coconut, *in vitro* clonal propagation is a suitable alternative (Sandoval-Cancino et al., 2016; Solís-Ramos et al., 2012).

In vitro regeneration of coconut is mainly achieved by the somatic embryogenesis. Coconut is highly recalcitrant for *in vitro* regeneration (Pérez-Núñez et al., 2009). Main difficulties encountered in coconut clonal propagation are slow response *in vitro,* high heterogeneity of the behaviour of the tissues *in vitro,* intensive browning of the tissues, high rhizogenesis ability and a low capacity for embryogenesis (Hocher et al., 1999).

Various laboratories in the world have worked for the development of coconut micropropagation using different sources of explants and media formulations (Nguyen et al., 2015; Sáenz et al., 2016). Unfertilized ovary explant from -4 stage inflorescences is one of the prominently used explant type for coconut micropropagation (Perera et al., 2007; 2008). Ovary explants are advantageous as they produce identical palms to the mother palm, compared to the plumule explants (Pérez-Núñez et al., 2006). They were reported to produce embryogenic callus consistently but with low frequency (Perera et al. 2007; 2009).

Commonly used auxin for coconut micropropagation is 2,4-dichlorophenoxyacetic acid (2,4-D) which is an important component in media formulations of coconut somatic embryogenesis (Sáenz et al., 1999). Dicamba (3,6-Dichloro-2-methoxybenzoic acid) is another plant growth regulator and herbicide that shares similarities in structure and activity to 2,4-D. It was used to substitute 2,4-D when inducing callus in some species such as wheat (*Triticum aestivum*) and maize (*Zea mays*), and sometimes considered as superior to 2,4-D (Carvalho et al., 1997).

Brassinosteroids are a class of growth promoting steroidal phytohormones which involved in plant growth and development (Wang et al., 2017). They promote cell division and cell differentiation and are found more active than or synergistic with, auxins such as indole acetic acid (IAA) or naphthalene acetic acid (NAA) (Brosa, 1999). Promising results were obtained by testing 22(S),23(S)-homobrassinolide on initial callus, embryogenic callus and somatic embryo formation in coconut plumule explants (Azpeitia et al., 2003).

It is assumed that calcium ionophores directly facilitate the transport of Ca^{2+} across the plasma membrane. Treatment with A23187 ionophore promoted somatic embryogenesis in wheat and coffee (*Coffea canephora*) cultures (Dedkova et al., 2000; Mahalakshmi et al., 2007; Ramakrishna et al., 2011). Treating embryogenic structure derived from coconut plumules with ionophore A23187 promoted somatic embryogenesis, and

increased in the formation of embryogenic structures per callus, somatic embryos per callus and developing plantlets per callus (Rivera-Solis et al., 2018).

The present study was aimed to improve the coconut micropropagation protocol using different chemical treatments and media supplements including dicamba, calcium ionophore A23187 and 22(S),23(S)-homobrassinolide for unfertilized ovary derived calli. This was the first attempt to use above chemicals on improving somatic embryogenesis of unfertilized ovary derived calli.

2. MATERIALS AND METHODS

2.1. Plant Material

Immature inflorescences, at the -4 stage (with 0 corresponding to the youngest opened inflorescence), of coconut cultivar Dwarf x Tall (DT), were collected to excise unfertilized ovaries. The basal part of rachilla, bearing female flowers were dissected from inflorescence and disinfected with 3% Clorox for 10 minutes and then rinsed four times with sterile distilled water. Ovaries were excised by removing the perianth parts of each female flower while submerging in a sterile antioxidant solution (0.15 gL^{-1} ascorbic acid, 0.1 gL^{-1} citric acid, and 30 gL^{-1} sucrose) under aseptic conditions. Sliced ovaries were cultured on callus induction medium.

2.2. Media Preparation and Culture Conditions

Y_3 media formulation (Eeuwens, 1976) was used as the basal medium supplemented with 5% sucrose, 0.3% phytagel and 0.25% activated charcoal (Rivera-Solis et al., 2018). Medium pH was adjusted to 5.75 before autoclaving for 20 min at 121 ^{0}C. All the cultures were incubated in dark at 27± 2^{0}C.

Initial calli were obtained through 6–8-week incubation of cultured unfertilized ovaries (as explained previously) on callus induction medium, which is Y_3 basal medium supplemented with 600 µM 2,4-D. For the present study, about 2x2x2 mm^3 translucent structures separated from the initial calli, were cultured on different media types and pre-treatments (Table1). Medium I was Y_3 supplemented with 300 µM 2,4-D while medium II was supplemented with 300 µM Dicamba. For T2, T3 and T6 translucent structures were pre cultured on medium III for three days before sub culturing on medium I or medium II. Medium III contained 1 µM 2,4-D and 1 µM 22(S),23(S)-homobrassinolide. For T1, T3, T5 and T7 cultured translucent structures were treated with 10 µL of 1µM calcium ionophore A23187 deposited with a micropipette on the explant (Rivera-Solis et al., 2018).

Table 2: Different media combinations and pre treatments

Treatment	Pre culturing medium/Pre treatment	Initiation / Sub culturing medium
Control	-	Medium I
T1	Medium I + 1 µM Ca ionophore	-
T2	Medium III	Medium I
T3	Medium III + 1 µM Ca ionophore	Medium I
T4	-	Medium II
T5	Medium II + 1 µM Ca ionophore	-
T6	Medium III	Medium II
T7	Medium III + 1 µM Ca ionophore	Medium II

2.3. Statistical Analysis

The number of embryogenic structures that can be proceeded to the somatic embryogenesis per an initial translucent structure were counted from 480 replicates (60 replicates per each treatment). The data were analyzed using SAS statistical software under Poisson regression.

3. RESULTS AND DISCUSSION

The cultured unfertilized ovaries developed initial calli (Figure 1-A) after 6 to 8 weeks. This initial calli further developed ear-like structures (translucent structures). The treatments were started with the translucent structures (Figure 1-B) that were separated from the initial calli. The translucent structures then developed embryogenic callus (Figure 1-C) with small pearly white structures referred to as embryogenic structures (Figure 1-D). The embryogenic structures (about 3x3x3 mm3) were separated from the embryogenic callus and counted for each treatment.

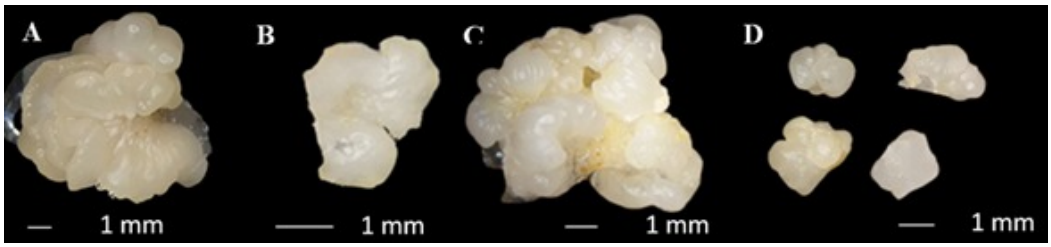

Figure 1: Morphological variations during the formation of embryogenic structures from initial calli. (A) Initial calli; (B) Translucent structures separated from initial calli; (C) Embryogenic callus with embryogenic structures; (D) Embryogenic structures separated from embryogenic calli.

According to the morphological characteristics of embryogenic calli (Figure 2), visible differences were clearly observed under different treatments. With comparison to the samples cultured on media with 2,4-D (Fig. 2 C-T3), all the Dicamba incorporated media produced calli with few embryogenic structures having roots (Fig.2 T4-T7.b). Most of them generated 1-5 germinating somatic embryos on each callus (Fig.2.T4-T7.a). Calli developed after the homobrassinoloide pre-treatment (medium III) produced pure white calli (productive calli) with minimal browning than the control which formed off-white calli. A different morphological characteristic was expressed by ionophore treated calli, which developed curved embryogenic calli into the main base (Fig.2-T1, T3).

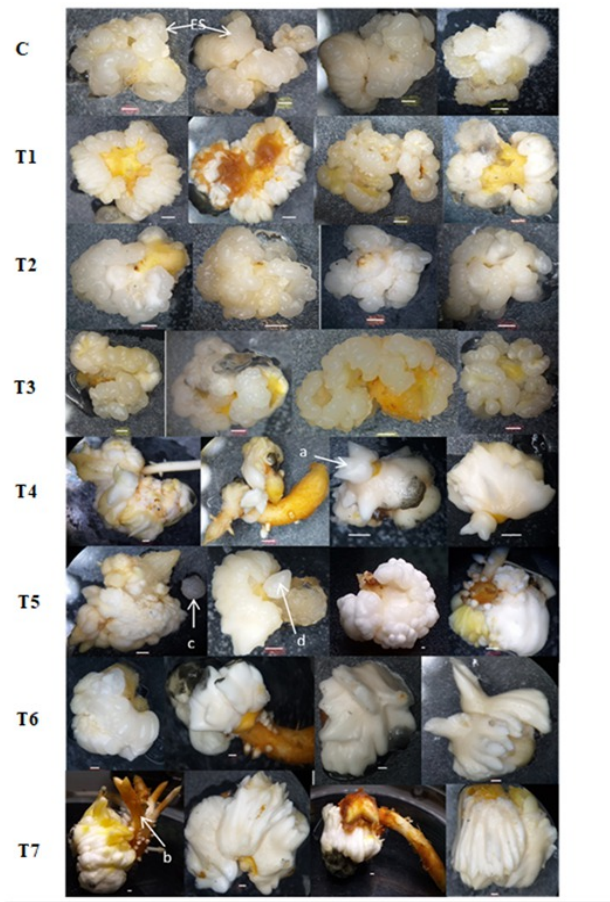

Figure 2: Morphological characteristics of calli after six weeks under different treatments (Bar = 1 mm)
Embryogenic structures (ES); germinating somatic embryos (a); roots (b); globular shaped somatic embryo (c); torpedo shaped somatic embryo (d)

The translucent structures produced varying number of embryogenic structures under each treatment (Table 2). But a significantly greater number of embryogenic structures were obtained with 2,4-D (C, T1, T2, T3) incorporated media compositions rather than dicamba (T4, T5, T6, T7).

Table 2: Mean number of embryogenic structures produced per an initial translucent structure of different treatments

Treatment	Mean number of embryogenic structures per translucent structure (± Standard error)
C	4.33 [a] ±0.68
T1	4.41 [a] ±0.17
T2	4.59 [a] ±0.28
T3	4.55 [a] ±0.27
T4	1.70 [b] ±0.17
T5	1.83 [b] ±0.17
T6	1.80 [b] ±0.17
T7	1.90 [b] ±0.18

[a,b] Different superscripts indicate significant difference ($P \leq 0.05$)

The media with 2,4-D and Dicamba showed significantly different results on formation of embryogenic structures. More callus formation (~4.33 fold) was observed in media with 300 µM 2,4-D while, significantly less callus formation (~1.7-fold) was observed in media with 300 µM Dicamba. In coconut micropropagation, 2,4-D is the prominent auxin used in many protocols (Luis et al., 2012). Dicamba was used in the presence of ascorbic acid and sorbitol during secondary somatic embryogenesis of oil palm *in vitro* propagation, which resulted in 21.55 secondary somatic embryos per one primary haustorium embryo (Te-chato & Hilae, 2007). Dicamba has given promising results in genotype independent somatic embryogenesis from immature zygotic embryos of tropical maize inbred lines (Akoyi et al., 2013). Therefore, more experiments are needed to test the effectiveness of Dicamba for somatic embryogenesis of ovary derived callus of coconut.

Calcium ionophore A23187 has been successfully utilized for somatic embryogenesis of coconut. It was reported to produce 2.8-fold embryogenic structures per callus, 1.5-fold somatic embryos per callus and 1.5-fold developing plantlets per callus. Further, it was observed that the greater expression of CnCERK (Coconut Somatic Embryogenesis Receptor Kinase) gene at initial stages of the culture period under the ionophore treatment (Rivera-Solis et al., 2018). In the present study, significant amount of embryogenic structures were obtained with calcium ionophore treatments when 2,4-D was used as the growth regulator.

The effect of the 22(S), 23(S)-homobrassinolide on initial callus, embryogenic callus and somatic embryo formation in coconut plumule explants were tested and favourable responses were obtained in previous studies. The action of brassinosteroid depends on the exposure time of

the explant to brassinosteroid. Three-day preculture period with brassinosteroid was effective than 7-day period resulting 10.8 somatic embryos per plumule explant in previous studies. The shorter exposure time was found to be more effective since the longer pre culture time might induce brassinosteroid–induced ethylene formation (Azpeitia et al., 2003). Ethylene has inhibitory effects on somatic embryogenesis using coconut zygotic embryo explants (Adkins et al., 1999). Therefore, three-day preculture period was used in the present study for homobrassinolide pretreatment for ovary derived calli but significantly different results were obtained only when they were sub cultured in 2,4-D containing media.

This was the first study of using calcium ionophore, homobrassinolide and dicamba on unfertilized ovary derived calli of coconut cultivar DT. Hence, more studies should be performed using different concentrations and pre culturing time periods to evaluate the effectiveness of these chemicals.

4. CONCLUSIONS

The results indicated morphological variations of the embryogenic calli produced by the different treatments. Pure white calli were observed with the homobrassinoloide pre-culture (medium III) with minimal browning. Number of embryogenic structures were significantly greater in treatments with 2,4-D (C, T1, T2, T3) rather than with dicamba (T4, T5, T6, T7). Further studies are needed using calcium ionophore, homobrassinolide and dicamba since they were reported to produce promising results with plumule explants of coconut. The findings of the present study indicated the potential of using these treatments for somatic embryogenesis of unfertilized ovary derived callus of coconut.

Acknowledgements

Authors wish to acknowledge all the staff of the Tissue Culture Division, Coconut Research Institute for their valuable contribution throughout this investigation and Dr. K. P. Waidyarathne, Principal Research Officer, Plant Physiology Division, Coconut Research Institute for her assistance in performing the statistical analysis of the study.

Conflicts of interest

The authors declare no conflict of interest.

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