# GENETIC STABILITY OF COCONUT EMBRYOGENIC CALLI AFTER CRYOPRESERVATION BY ENCAPSULATION-DEHYDRATION TECHNIQUE

**W.W.M.A. Iroshini[1], G.A.U. Jayasekera[1], S.A.C.N. Perera[2], V.R.M. Vidhanaarachchi[3] and H.D.D. Bandupriya*[3]**

*[1]Department of Plant Sciences, Faculty of Science, University of Colombo*

*[2]Genetics & Plant Breeding Division, Coconut Research Institute, Lunuwila*

*[3]Tissue Culture Division, Coconut Research Institute, Lunuwila*

**Corresponding author (email: dbandupriya@yahoo.com)*

## Introduction

Coconut is one of the most important plantation crops in Sri Lanka. The genetic diversity of coconut is lost due to incidence of pest and diseases, human interferences and natural disasters. Therefore, conservation of coconut germplasm is essential for future breeding programmes. However, coconut has a large nut that is sensitive to desiccation, thus it cannot be stored in seed genebanks. Long-term conservation through Cryopreservation is the only safe method available for conservation of such problematic species [1]. Cryopreservation is the conservation of biological material at ultra-low temperatures, usually in liquid nitrogen (LN) at the temperature of -196 °C. The cryopreservation protocols so far developed for coconut have used embryos or plumules which are zygotic tissues [2, 3]. Since coconut is an open pollinated plant, genetic characters of these zygotic tissues may vary from the mother palm. For an efficient long-term conservation method, it is important to maintain the embryogenic competence for long period without genetic alteration. Therefore, experiments were conducted using unfertilized ovary derived embryogenic calli (EC). Further, a successful micropropagation protocol is available for unfertilized ovary [4].

Several techniques are available for cryopreservation of plant tissues. The most critical parameter is removal of intracellular water fraction which is capable of converting to ice crystals during freezing in LN, causing cell injury. Thus, the tissue needs to be dehydrated properly or treated with suitable cryoprotectants prior to rapid cooling in order to prevent cell damage. For the encapsulation-dehydration method, plant material is embedded in alginate beads in order to protect them from dehydration and freezing damage. The beads are pre-grown in a liquid medium enriched with sucrose for several days, partially desiccated using silica gel and rapidly immersed in LN.

While obtaining good survival and recovery, it is very important to determine the genetic fidelity of plants recovered from cryopreservation because some steps in cryopreservation process may presume to be cause genetic instability. Even though genetic variations have been assessed for several crops after cryopreservation, only a one report has been found for coconut [3]. According to Sisunandar et al.[3], cryopreservation of coconut zygotic embryos does not induce molecular changes in

recovered seedlings. Single sequence repeats (SSRs) or microsatellite markers are highly polymorphic and widely used molecular marker for performing genetic stability. Since different techniques include different stress inducing steps, it is important to assess the genetic stability of recovered plants in each cryopreservation technique developed for coconut before using them for large scale storage. Here we report use of EC for the first time for coconut cryopreservation through *in vitro* techniques. The objective of this study was to develop a reliable cryopreservation technique for coconut using unfertilized ovary derived embryogenic callus and assessment of regenerated plants for their genetic fidelity.

**Materials and Methods**

Embryogenic calli were induced from unfertilized ovaries excised from immature female flowers of variety Dwarf x Tall hybrid (DxT), at Tissue Culture Laboratory, Coconut Research Institute of Sri Lanka (CRISL) as described by Perera et al. [4].

*Cryopreservation*

Embryogenic calli were cryopreserved by encapsulation-dehydration method. Calli were first pre-cultured in modified Eeuwens $Y_3$ medium for three days to check contaminations, and then encapsulated in calcium alginate beads. These beads were then pretreated with two sucrose concentrations (0.50 M and 0.75 M) for different durations (24, 48 and 72 h). Then the calli were subjected to dehydration in silica gel for 8, 12, 16 and 20 h. Half of the encapsulated calli were plunged into LN for 2 h while other half was cultured as the dehydration control. Encapsulated calli removed from LN were thawed inside a 40 °C water bath for three minutes and cultured in the $Y_3$ recovery medium. Water loss from alginate beads after dehydration was measured on fresh weight basis. Survival of the EC was recorded after 4 months of culture and they were considered alive when they show any sign of growth. Further development of somatic embryo like structures was taken as an indication of recovery following desiccation alone or desiccation and freezing, as recorded after at least 8 months.

*Sample collection and data analysis*

Treatments were arranged in a Completely Randomized Design (CRD) with 10 replicates per treatment and each treatment was repeated once. Results were analysed by ordinal logistic regression model by Stata (Version 13.1) statistical software to determine treatment effects, where significance was indicated at the 95 % level of probability ($P \leq 0.05$).

*Assessment of genetic fidelity*

Somatic embryo like structures were collected from cryopreserved and non-cryopreserved EC that treated with 0.75 M sucrose for 72 h followed by dehydration for 20 h. Genomic DNA was extracted by using DNeasy® Plant Mini Kit (QIAGEN®) according to manufacturer's instructions. The purified total DNA was quantified and its quality was determined by agarose gel electrophoresis. DNA was amplified using five SSR marker loci (CNZ04, CNZ10, CNZ12, CAC20 and CAC23). These loci were

selected based on their co-dominant simple inheritance, high polymorphism and reproducibility characters through PCR amplification. PCR amplified DNA fragments were electrophoresed on 6% (w/v) polyacrylamide gel and bands were visualized using silver staining. The banding patterns of the samples were compared with the control and donor palm.

## Results and Discussion

*Effect of different sucrose concentrations on water content of encapsulated ECs after dehydration*

Pretreatment of encapsulated calli using different sucrose concentrations (0.5 M and 0.75 M) for three different durations (24, 48 and 72 h) was undertaken before dehydration in silica gel. Sucrose level had significant effect on the water content of beads after dehydration. Water loss on fresh weight basis was higher in 0.75 M sucrose than in 0.50 M sucrose. However, dehydration did not show a significant effect on water content of encapsulated beads. According to the desiccation curve, there was no difference in water content in different dehydration periods (8, 12, 16 and 20 h; data not shown).

**Table 1**. Water content % (FW) of encapsulated beads after sucrose pretreatment and dehydration. The values correspond to the means ± SE.

| | | Water content % | | | | | |
|---|---|---|---|---|---|---|---|
| **Sucrose Concentration** | | **0.75 M** | | | **0.50 M** | | |
| **Sucrose pretreatment (hrs)** | | **24** | **48** | **72** | **24** | **48** | **72** |
| **Dehydration duration (hrs)** | **8** | 28.12 ± 3.27 | 28.62 ± 3.26 | 28.04 ± 1.48 | 36.9 ± 5.87 | 40.05 ± 4.01 | 38.93 ± 4.27 |
| | **12** | 29.12 ± 3.09 | 29.88 ± 1.13 | 28.99 ± 2.10 | 40.49 ± 3.08 | 42.32 ± 1.60 | 41.40 ± 0.71 |
| | **16** | 27.27 ± 3.09 | 28.58 ± 2.27 | 27.48 ± 1.48 | 37.95 ± 5.08 | 39.38 ± 3.73 | 39.19 ± 2.16 |
| | **20** | 27.29 ± 1.33 | 27.85 ± 1.51 | 30.02 ± 1.16 | 38.96 ± 0.55 | 40.84 ± 2.55 | 40.09 ± 1.35 |

*Effect of dehydration, sucrose concentrations and osmoprotection duration on survival and regrowth of ECs after freezing*

Highest survival and recovery (45 % and 25 % respectively), of EC was achieved by pre-culturing them in 0.75 M sucrose for 72 h and dehydrating for 20 h, followed by rapid LN immersion (Figure 1).

Recalcitrant species do not survive at low moisture levels, below 30–50 % of fresh weight [5]. In this experiment, water content ranged between 25 %– 30 % for 0.75 M sucrose level and at 0.50 M, it was 35 % – 45 % and 0.75 M treated samples showed better survival and recovery. Our data show that sucrose level and sucrose pretreatment duration may be significant factors contribute to the survival rate. Furthermore, 0.75 M sucrose level revealed higher survival rate than 0.5 M sucrose

level. Similarly, higher survival rate and recovery rate were observed when increase the time period of sucrose pretreatment. Moreover, higher recovery rate was observed when increasing the dehydration duration. Therefore, these results suggest that higher survival and recovery rates can be obtained in treatment combinations when 0.75 M sucrose is used for pretreatment for 72 h followed by 20 h dehydration duration prior to LN treatment.

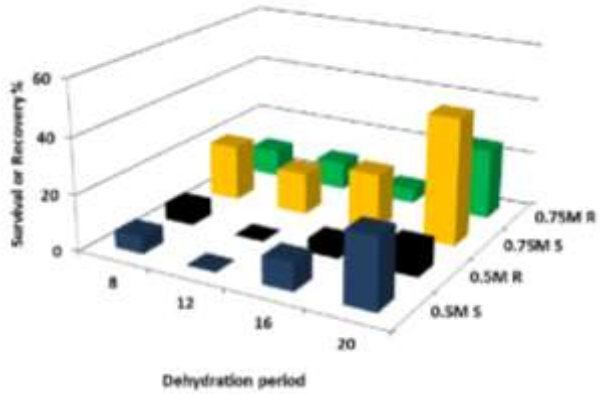



***Figure 1.*** *Survival and recovery percentages for cryopreserved samples treated with 72 hrs of sucrose pretreatment.* ***S*** *Survival %.* ***R*** *Recovery %.*

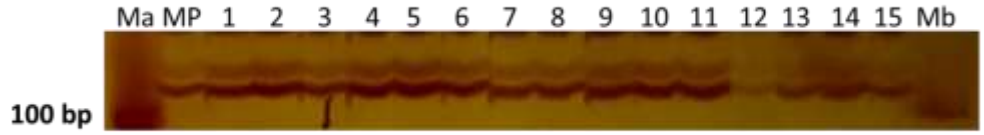
Recovery and survival rates observed in the treatments after dehydration and freezing were very low compared to the untreated controls (100 %). Although good shoot development was recorded for one non-cryopreserved treatment combination after dehydration, overall somatic embryo and shoot development after dehydration of ECs was very low. However, due to recalcitrant nature, 20 % recovery is considered as a commendable achievement in species like coconut [2]. In this study, the highest recovery (25 %) falls within this range and suggest the requirement of further research to develop a reliable cryopreservation technique from coconut EC.

*Assessment of genetic fidelity*

SSR markers were used to evaluate the genetic stability of coconut EC after treatment with encapsulation-dehydration method. The five primers used in the present study were all informative and generated amplicons for the detection of the genetic fidelity of the samples. Each tested SSR primer pair produced clear reproducible bands ranging in size from 100-250 bp. All the samples scored identical alleles with their respective mother palm and untreated controls at all SSR loci analyzed in the current study. Amplification pattern of CNZ04 SSR primer is shown in Plate 1.

At the SSR marker analysis, SSR locus CNZ10 was observed to be heterozygous, while SSR loci CNZ04, CNZ12, CAC20 and CAC23 were homozygous. According to the

amplification patterns, SSR analysis did not show any polymorphisms between cryopreserved samples and untreated controls and with their respective mother palm, suggesting that cryopreservation by encapsulation-dehydration method does




not affect genetic stability of coconut EC.

***Plate 1.*** *Amplification of DNA extracted from non-treated, cryopreserved and non-cryopreserved EC with respective mother palm for CNZ04 SSR primer. Lanes: Ma - 10 bp DNA ladder; Mb - 100 bp DNA ladder; MP - DT-1680 mother palm; 1-5 - untreated EC; 6-10 - non-cryopreserved EC and 11-15 – cryopreserved EC.*

**Conclusions and Recommendations**

DNA banding patterns in any of SSR marker loci showed no evidence of somaclonal variations in tested encapsulation-dehydration method, thus it was proved that visual observations of polyacrylamide gel images for the genetic equality within the cryopreserved and non-cryopreserved samples with respect to untreated controls and mother palm.

**Acknowledgement**

Financial assistances by National Science Foundation of Sri Lanka under the research grant No RG/2014/BT/01.

**References**

[1] F. Engelmann. “Use of biotechnologies for the conservation of plant biodiversity”. *In Vitro Cellular & Developmental Biology – Plant*, vol. 47 (1), pp. 5-16, 2011.

[2] O. N’Nan, V. Hocher, J-L. Verdeil, J-L. Konan, K. Ballo, F. Mondeil. & B. Malaurie. “Cryopreservation by encapsulation-dehydration of plumules of coconut (*Cocos nucifera* L.)”. *CryoLetters*, vol. 29 (4), pp. 339-350, 2008.

[3] Sisunandar, A. Rival, P. Turquay, Y. Samosir, S.W. Adkins. “Cryopreservation of coconut (*Cocos nucifera* L.) zygotic embryos does not induce morphological, cytological or molecular changes in recovered seedlings”. Planta, vol. 232, pp. 435-447, 2010.

[4] P.I.P. Perera, V. Hocher, J-L. Verdeil, S. Doulbeau, D.M.D. Yakandawala & L.K. Weerakoon. “Unfertilized ovary: a novel explant for coconut (*Cocos nucifera* L.) somatic embryogenesis”. *Plant Cell Reports*, Vol. 26 (1) pp. 21-28, 2007.

[5] Y.P.S. Bajaj. “Cryopreservation of plant cell, tissue and organ culture for the conservation of germplasm and biodiversity”. in *Biotechnology in Agriculture and Forestry, Cryopreservation of Plant Germplasm I*, Y.P.S. Bajaj, Ed. Berlin Heidelberg: Springer, 1995, pp. 3–28.