Effect of Abscisic Acid as a Cryoprotectant on the Survival of Cryopreserved Plumules and Mature Zygotic Embryos of Coconut (*Cocos nucifera* L.)

B.K.A. BALASURIYA[1], H.D.D. BANDUPRIYA[2] and S.C. FERNANDO[2]

[1] *Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP), Sri Lanka.*

[2] *Coconut Research Institute, Bandirippuwa Estate, Lunuwila, Sri Lanka.*

ABSTRACT

In the present study, available protocols for cryopreservation of plumules and zygotic embryo explants of coconut were tested for the effect of Abscisic acid (ABA) as a cryoprotectant. In the first experiment, plumules were excised from mature zygotic embryos of Sri Lanka Tall coconut and encapsulated in sodium alginate. The beads were subjected to different pretreatments comprising of 0.75 M sucrose alone and in combination with 3 different ABA concentrations (10, 20, and 40 µM) for 72 hours. They were further dehydrated in silica gel for 16 hours, prior to immersing in liquid nitrogen. In the second experiment, zygotic embryos were subjected to the same pretreatments but instead of using 0.75 M sucrose, 0.5 M sucrose was used and pretreated for 120 hours. Prior to freezing in liquid nitrogen, the embryos were subjected to desiccation by exposure to silica gel for 15 hours.

A very high survival rate (90 %) was observed in unfrozen plumules pretreated with 20 µM ABA. Similar results were observed in the frozen plumules and the highest survival (84 %) was observed in the plumules pretreated with 40 µM ABA. The plumules (frozen and unfrozen) pretreated only with 0.75 M sucrose showed the lowest recovery (2 % and 5 % respectively) while recovery of plumules pretreated with ABA, was much higher when compared to the control. The highest survival (100%) was recorded in embryos pretreated with 40 µM ABA. Both 10 µM and 40 µM ABA treatments were showed 3% recovery while other treatments were not showing any recovery of frozen embryos. The results indicated a significant effect of ABA on survival and recovery of frozen plumules and recovery of frozen embryos.

KEYWORDS: Coconut, Plumule, Embryo, Cryopreservation, Dehydration, Encapsulation

1. INTRODUCTION

The coconut palm (*Cocos nucifera* L.) is one of the most important crops in the tropics. It ensures income for millions of small holders in the area and is also a primary source of food, drink and shelter for millions of inhabitants (Hocher *et. al.*, 2003).

In Sri Lanka, it is important as a subsistence crop and its demand for industrial and commercial purposes are significantly increasing (Jayasinghe, 2001). It occupies 439,000 ha of land and the annual production is about 2562 million nuts. The gross domestic production of coconut is about 2% and it contributes 3% - 4% foreign exchange (Anon, 2003).

Presently, the genetic erosion of coconut palm is increasing due to various reasons such as natural disasters, land fragmentation, diseases, urbanization etc. The rate of erosion was estimated to be around 1% per annum (Everard *et.al.*, 2000). Therefore conservation of coconut germplasm is essential.

Collection and exchange of coconut germplasm is difficult and costly because of weight and size of the nuts. The recalcitrant nature of coconut seed has made their storage difficult. Therefore establishing and maintaining field gene banks in coconut is very important. However, it is costly and subjected to various stresses such as adverse weather conditions, pests and diseases (Hornung *et.al.*, 2001).

In vitro culture of coconut zygotic embryos and plumules would simplify the transport and provide secure storage conditions during collection and conservation of coconut germplasm. Cryopreservation (*i.e.* storage at temperature between -80 and -196 ^{0}C) is an important tool for safe and long term conservation of genetic resources of recalcitrant seeds (Assy –Bah and Engelmann, 1992).

Pretreatment with cryoprotectants are necessary to avoid the lethal damage of embryos by dehydration and subsequent low temperature storage. The most commonly used cryoprotectants are dimethylsulfoxide (DMSO), glycerol, polyethyleneglycol, sugar and sugar alcohols (Chaudhury, 2003). Abscisic acid (ABA) is also known as a stress hormone that can induce freezing tolerance in plants.

It reduces the water content of the tissues which in turn reduces cell injuries caused by intra-cellular and extra-cellular ice crystal formation (Shiota *et.al*, 1999).

In 1980's a cryopreservation protocol was established for the whole zygotic embryo of coconut (Assy-Bah and Engelmann, 1992). It was demonstrated that cryopreservation of mature zygotic embryo was possible with high survival percentage using pre growth desiccation technique, but the number of genotypes tested was limited. The large size of the embryo limits the application of other techniques such as those based on encapsulation. Coconut plumule appears to be an appropriate material for cryopreservation because of its small size (~ 1mm) and structure (presents of many meristematic cells) (Maularie *et.al.*, 2002). In the present study, currently available protocols for cryopreservation of coconut were tested to study the effect of Abscisic acid (ABA) on survival and recovery of cryopreserved plumules and mature zygotic embryos.

2. MATERIALS AND METHODS

2.1 Planting Material

Mature zygotic embryos were collected from 12 to 14 months (after pollination) old nuts of the variety Sri Lanka Tall.

2.2.1. Experiment 1: Cryopreservation of Coconut Plumules.

2.2.1.1 Surface Sterilization of Embryos

Endosperm plugs containing mature zygotic embryos were excised using a cork borer and surface sterilized with 100% Clorox (5.25% NaOCl) for 30 minutes. Embryos were dissected after rinsing 5 times with sterilized water. Dissected embryos were surface sterilized with 30% (v/v) Clorox for 5 minutes and rinsed in sterile water for 5 times.

2.2.1.2 Encapsulation and Sucrose Pretreatment of Plumules

Coconut plumules were excised from the embryos under a stereo microscope. They were cultured in solid Eeuwen's Y_3 medium (Eeuwence, 1976) at least for 3 days in order to check the contaminations and allow the plumules to recover from excision stress. Then they were encapsulated in 3% (W/V) calcium alginate. Encapsulation was followed by the pretreatment of plumules in 0.75 M liquid sucrose medium with different concentrations (0, 10, 20, 40 µM) of ABA, on a rotary shaker (at 90 rpm) at 27 ^{0}C in dark for 72 hours.

2.2.1.3 Dehydration and Freezing

Pretreated beads were rapidly surface dried on sterile filter paper and weighed. Then they were placed in jars containing 40 g of silica gel (dried at 150 ^{0}C for 12 hours) and dehydrated for 16 hours. Untreated control (without any pretreatment or desiccation) and pretreatment control (after sucrose pretreatment with ABA) were included. After dehydration, beads were weighed. Half of the dried beads were transferred to sterile 2 ml polypropylene cryo tubes and directly plunged in to liquid nitrogen.

2.2.1.4 Assessment of Survival and Recovery of Plumules

After a minimum of 2 hours storage in liquid nitrogen, cryo tubes were rapidly rewarmed in a water bath set at 40 ^{0}C for 3 minutes. Then the individual beads were cultured in the recovery medium (Eeuwen's growth medium). The remaining unfrozen beads were also cultured in the same growth medium. Assessment of plumule survival (indicated by any sign of growth such as swelling, development of new leaf primodia and/or callusing) was performed after 2 months of culture. The recovery of plumules (indicated by the ability of plumules to grow into plantlets) was assessed after 2 months in culture (Fig. 3).

2.2.2. Experiment 2: Cryopreservation of Mature Zygotic Embryos of Coconut

2.2.2.1 Surface Sterilization of Embryos

Embryos were removed from endosperm cores and sterilized with 3% (w/v) NaOCl for 5 minutes, followed by rinsing in sterile water for 5 times.

2.2.2.2 Pretreatment and Culturing

Embryos were pretreated in a sucrose solution (0.5 M) supplemented with different concentrations of ABA (0, 10, 20, 40 µM) for 120 hours. An untreated control (embryos which were not subjected to any pretreatment or desiccation) was also included. These embryos were cultured in Eeuwen's Y_3 liquid medium. After sucrose pretreatment, embryos were rapidly surface dried on sterile filter paper and weighed. Then they were dehydrated in jars containing 100 g of silica gel (dried at 150 ^{0}C for 12 hours) for 15 hours and weighed. Half of the dehydrated embryos were cultured in liquid Y_3 medium. The other half was placed in 2 ml cryo tubes and immersed in liquid nitrogen. Embryos were kept in liquid nitrogen for a minimum of 2 hours and cultured in liquid Y_3 medium.

2.2.2.3 Assessment of Survival and Recovery of Embryos

After freezing in liquid nitrogen, cryo tubes were rapidly warmed in a water bath set at 30^{0}C for 10 minutes and the embryos were cultured in liquid Y_3 medium. The survival of embryos (indicate by any sign of growth) was assessed 8 weeks after culture. The recovery of embryos (as measured by the ability of the embryo to grow in to plantlets) was assessed after 8 weeks (Fig. 4).

2.3 *Data Analysis*

Results were analyzed by ANOVA procedure using SAS statistical software package. In first experiment data were taken as the means of three experiments with 10 replicates and data of second experiment were taken as the means of two experiments with 9 replicates.

3. RESULTS AND DISCUSSION

3.1.Experiment 1: Effect of ABA on Cryopreservation of Plumules.

Encapsulation-dehydration is a vitrification-based procedure and comprises the gradual osmotic and evaporative dehydration of plant cells prior to liquid nitrogen exposure (Fang *et. al.*, 2004). Formation of ice crystals within the cells is considered to be a primary source of cryo injury (Fang *et.al.*, 2003). One of the most critical parameters in the cryopreservation procedure is the removal of intracellular water fraction, which is capable of converting to ice crystals during freezing or rewarming (Crowe *et.al.*, 1998). Thus pretreatment of encapsulated plumules in cryoprotectants (e.g. sugars, sugar alcohols, DMSO etc.,) is generally required.

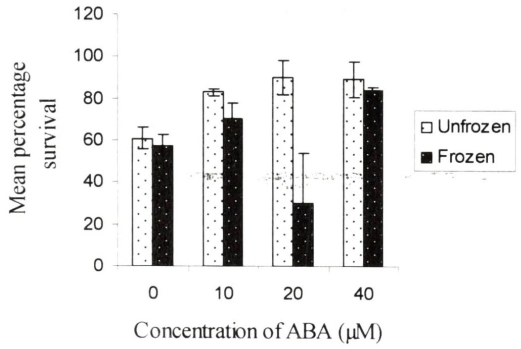
In this experiment, encapsulated plumules were dehydrated for 16 hours in silica gel. The water loss

In this experiment, encapsulated plumules were dehydrated for 16 hours in silica gel. The water loss from plumules was increased with the increase of ABA concentration. Plumules pretreated in ABA-free sucrose solution recorded the lowest water loss (62% on fresh weight basis) whereas the highest water loss (69%) was observed in the plumules pretreated with sucrose in combination with 40 µM ABA.

Table 1: Mean percentage of water loss from plumules pretreated in sucrose and desiccated in silica gel.

Concentration of ABA (µM)	0	10	20	40
Mean percentage of water	62	67	68	69

Fig. 1 and Fig. 2 show the mean percentage of survival and recovery of frozen and unfrozen plumules. The results clearly indicate the positive effect of ABA on survival and recovery of both unfrozen and frozen plumules. A very high survival rate (90 %) was observed in unfrozen plumules pretreated with 20 µM ABA. Similar results were observed in the frozen plumules and the highest survival (84 %) was observed in the plumules pretreated with 40 µM ABA. The plumules (frozen and unfrozen) pretreated only with 0.75 M sucrose showed the lowest recovery (2% and 5 % respectively) while recovery in plumules pretreated with ABA, was much higher when compared to the control. Results showed that ABA enables to increase the survival and recovery rates of frozen embryos by reducing water content of cells.



CV% = 17.2 *P = 0.05*

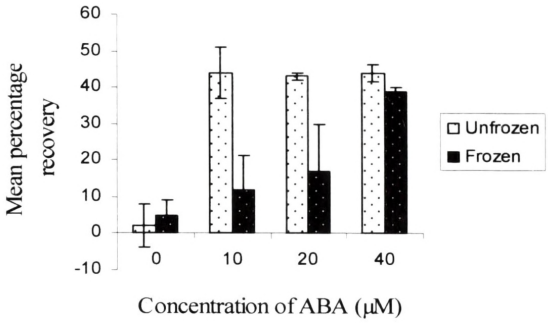
Figure 1: Mean percentage survival of frozen and unfrozen plumules

Freezing in liquid nitrogen has caused a reduction in recovery of plumules. As shown in figure 1 and figure 2, survival and recovery percentages of frozen plumules were lower than that of unfrozen plumules.

The duration of dehydration is also an important factor affecting the recovery of cryopreserved plumules. Excessive dehydration results cell injury, possibly as a result of membrane stress. Insufficient dehydration results intracellular freezing. These can course injuries due to increase of volume from liquid state. As a result, rupture or reduced the flexibility of the membrane occur (Jayasinghe, 2001).

After pretreatment of plumules in sucrose and ABA medium, post cryopreservation survival and plant regeneration were improved. Accumulation of sucrose inside tissues helps to maintain viability of the tissue during dehydration and cryopreservation by stabilizing cell membrane (Hornung *et.al*; 2001). ABA has been shown to play an important role in plant water balance and adaptation of plants to environmental stress (Seijo, 2000). It has been associated with synthesis of proteins (Seijo, 2000) and compatible solute components which are important in plant stress tolerance (Fang *et.al*; 2004). Dormant buds and seeds of woody plants accumulate higher level of ABA when tissues are exposed to low temperature and water stress (Walton *et.al;* 1988) and it prevents the damage due to low temperature and water stress.

Present study showed that when ABA is combined with sucrose as a cryoprotectant, it induces desiccation tolerance in encapsulated plumules of coconut.



CV% = 19.6 *P = 0.05*

Fig. 2: Mean percentage recovery of frozen and unfrozen plumules

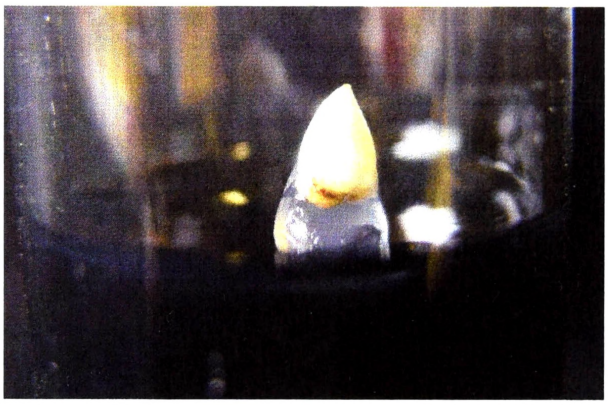


Fig. 3. A recovered coconut plumule after cryopreservation

Fig. 4. A coconut plantlet recovered from cryopreserved mature zygotic embryo.

3.2. Experiment 2: Effect of ABA on Cryopresearvation of Mature Zygotic Embryos.

Successful cryopreservation of coconut mature zygotic embryos was previously reported by Assy-Bah and Engelmann (1992) and Jayasinghe (2001). The survival rates depended on the variety and varied from 33%-93% (Assy-Bah, 1992). Sucrose (0.5M) pretreatment for 120 hours and dehydration for 15 hours were recorded as the optimum conditions for cryopreservation of mature zygotic embryos of local coconut cultivar Sri Lanka Tall and 40% recovery was obtained (Jayasinghe, 2001). Higher recovery rates are needed for cryopreservation of valuable coconut germplasm which is available in limited quantities.

Coconut embryo consists of two distinct parts, the meristemetic pole and the haustorium which have different water contents. The haustorium consists of predominantly highly vacuolated cells (Hornung *et.al*, 2001). Thus suboptimal desiccation could lead to ice nucleation and growth and thereby completely destroying the cell during freezing (Assy-Bah and Engelmann 1992). As mentioned previously, ABA has been shown to play an important role in plant water balance and adaptation of plants to environmental stress (Seijo, 2000).Therefore, in this study ABA was used as a cryoprotectant to further improve the survival rate of cryopreserved embryos reported by Jayasinghe (2001).

The result of this study show that embryos pretreated with 0.5 M sucrose for 120 hours and desiccation in silica gel for 15 hours (control treatment) lost 61 % water. It is comparable with the water loss (63%) reported by Jayasinghe (2001). In this study with the addition of increasing levels of ABA (10-40 µM) to the sucrose solution, the water loss showed a slight increase (63%-65%), (Table 2).

Table 2: Mean percentage of water loss from embryos pretreated in sucrose and desiccated in silica gel.

Concentration of ABA(µM)	0	10	20	40
Mean percentage of water loss	61	63	64	65

The result indicates that pretreatment of embryo in sucrose solution (with or without ABA) and desiccation in silica gel did not have any negative effect on embryo germination. Pretreatment of embryos in sucrose solution (without ABA) and desiccation in silica gel for 15 hours showed a survival of 82% cryopreserved embryos. The survival rates increasing up to 91% and 100% by adding 10µM and 40 µM ABA respectively (Table 3). However the increase was statistically not significant. This may be due to the very low survival rate of embryos observed in 20 µM ABA treatment. The result of this treatment might be due to the contamination of some samples.

Data collected after 8 weeks of culturing showed that above 50% embryos that survived pretreatment in sucrose and desiccation started germination (recovery). Embryos pretreated in sucrose alone did not recover after freezing. This is not in agreement with the results of Jayasinghe (2001) who observed 40 % recovery. Pretreatment of embryos in sucrose in combination with ABA (10 and 40µM) resulted in 3% recovery. Here again results of 20 µM ABA treatment can not be considered due to high contamination of sample (Table 3).

Addition of ABA to the pretreatment solution showed a positive effect on cryopreserved embryo recovery. However the standard deviations of the results are high. It may be due to the low level of replication of the experiment and high contamination of samples.

In the present study, most of the cryopreserved embryos (67 %) turned in to abnormal structures within 8 weeks of culture. This probably explains the complex nature of the coconut embryo, more specifically the haustorium which consists of more parenchymatous tissues with shoot and root meristems. It exhibits differential sensitivity to desiccation and on subsequent freezing (Karun *et. al* 2005).

The results of this study show that ABA has a positive effect on the recovery of cryopreserved embryos. However, the absence of recovered embryos in the control treatment and high variation of results between replicates of the same treatment indicate the necessity of repeating the experiment with more samples in order to come to conclusions

Generally, development of a coconut zygotic embryo needs 12 to 14 months and it is severely affected by the weather conditions. Therefore mature

Table 3. Mean percentage of survival and recovery of frozen and unfrozen embryos.

Concentration of ABA in pretreatment solution (µM)	Mean percentage survival ± SD		Mean percentage recovery ±SD	
	-LN	+LN	-LN	+LN
0	100 ± 0	82 ± 0.5	56 ± 2.7	0 ± 0
10	50 ± 80	91 ± 17.6	34 ± 2.0	3 ± 6.1
20	100 ± 0	4 ± 7.7	62 ± 0.6	0 ± 0
40	100 ± 0	100 ± 0	50 ± 0.7	3 ± 5.6
CV%	**30.3**		**29.4**	
Significance	**P = 0.05**		**P = 0.05**	

SD- Standard Deviation **LN- Liquid Nitrogen**

embryos collected at different time intervals may differ physiologically and biochemically (Verdeil *et al*, 1998 and Rillo *et.al*, 2002). Physiological and biochemical status of an embryo might control its stress tolerance. This might have been a reason for drastically different results (low recovery of embryos after cryopreservation) obtained in this study and study reported by Jayasinghe (2001).

Furthermore, mature zygotic embryos of coconut generally contain high level of cytokinin (Maularie *et.al*, 2002). It has been reported that influence of ABA on freezing tolerance is sometimes counteracted by the presence of cytokinin (Vandarbasshe, 1998).

This might have hindered the expected effect of ABA on cryopreservation of mature embryos of coconut.

3. CONCLUSIONS

The results obtained from the experiment on crypreservation of plumules are encouraging and the study indicated the feasibility of using coconut plumules for effective cryopreservation of coconut germplasm. However, the protocol needs to be refined further to improve the recovery. Once it is refined, the study could be extended to other varieties of coconut. Results of the experiment on cryopreservation of mature zygotic embryos indicate that ABA has a positive effect on survival and recovery of cryopreserved embryos. However it is important to repeat the experiment with an increased number of replicates to confirm the consistency of the results.

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