Current Horticulture 13(2): 42–47, April–June 2025
DOI: 10.5958/2455-7560.2025.00021.4
Received: 07 December 2023; Accepted 18 January 2025

Standardization of cryopreservation in coconut (*Coconut nucifera*) plumule based on vitrification technique using V-cryomesh

Aparna Veluru [1], Neema Mohamed [2*], Krishna Prakash [3], Fathimath Zaeema M P [1], Sudha R [1] and Muralikrishna KS [1]

[1] *ICAR- Central Plantation Crops Research Institute, Kasaragod, Kerala, India*

Abstract

A technique was tried for cryopreservation of coconut (*Cocos nucifera* L.) plumular regions based on vitrification technique using aluminium V-cryomesh as an anchoring material. Coconut plumular regions, excised from mature zygotic embryos, were pre-cultured in a solid Y3 medium supplemented with 0.2 M to 0.6 M sucrose. After pregrowth, explants were treated with loading solution (Y3 medium with 0.4 M sucrose + 2 M glycerol) and transferred to V-cryomesh and embedded using calcium alginate. Explants adhered to V-cryomesh were subjected to dehydration using PVS3 (40 minutes) and transferred to cryovials for storing in liquid nitrogen. After 48hr of liquid nitrogen treatment, material was thawed at 40°C for 2 minutes and later treated with unloading solution for 30 minutes and explants were inoculated to retrieval media. The optimal protocol involved preculture of plumules for 72 hr on medium with 0.4 M sucrose followed by PVS3 treatment for 40 minutes, rapid cooling and rewarming and treating with unloading solution for 30 minutes. Under these conditions, 33% post thaw recovery and 7% plantlet recovery was observed. Accommodation of smaller sized explants in large number and easy handling were the major advantages of cryomesh. The method can be useful for conservation of valuable coconut germplasm in the form of plumules.

Key words: Liquid Nitrogen, Cryopreservation, Plumule, Vitrification procedure, V-cryomesh

Coconut (*Cocos nucifera* L.) is a significant tropical and monotypic species. Despite being a monotypic species, this palm exhibits substantial variability due to its cross-pollinating nature and high heterozygosity. Given its enormous economic importance, various conservation measures have been implemented to safeguard the genetic diversity of coconut, with the support of the International Coconut Genetic Resources Network (COGENT). According to Coconut Genetic Resources Database (CGRD), coconut germplasm is conserved primarily in field gene banks under *ex situ* conditions across 25 sites in 23 countries, encompassing approximately 1,416 accessions (Engelmann *et al.,* 2005). In addition to field conservation (both *in-situ* and *ex-situ*), cryopreservation presents an ideal alternative for the long-term conservation of coconut germplasm. Research has been conducted on the cryopreservation of coconut genetic resources, focusing on zygotic embryos, plumules, pollen and embryogenic calli (Welewanni *et al.,* 2017). Various protocols including dehydration, encapsulation-dehydration and vitrification have been employed for standardizing cryopreservation techniques for coconut embryos and plumules (Welewanni *et al.,* 2017).

Plumule or embryonic shoots are considered ideal materials for the cryopreservation of coconut germplasm because of their small size and presence of only meristematic cells (Malaurie *et al.,* 2002, Aparna *et al.,* 2023). Unlike other tissues, plumules do not carry pathogens like viruses due to the absence of phloem tissues (Maularie *et al.,* 2002; N'Nan *et al.,* 2008). Additionally, the disintegration of intact cells during the cryopreservation process, which can occur in zygotic embryos were not observed in plumules. The encapsulation dehydration technique has been commonly used for cryopreservation of coconut plumules (Malaurie and Borges, 2001; Hornung *et al.,* 2001; Malaurie *et al.,* 2006, Bandupriya *et al.,* 2007; N'Nan*et al.,* 2008; Bandupriya *et al.,* 2010; Nan *et al.,* 2014). Various studies have reported a post-thaw recovery rate of 20-80% for plumules using this procedure. The recovery of cryopreserved plumules has been improved by adding ABA and ascorbic acids to preculture media containing sucrose (Bandupriya *et al.,* 2007; Malaurie *et al.,* 2006). There is limited knowledge about vitrification-based procedures and use of cryoplate and cryomesh materials for cryopreservation of plumules.Hence study was undertaken to test the effectiveness of cryopreservation using V-cryomesh technique under various sucrose concentrations during preculture.

MATERIALS AND METHODS

The study was conducted at ICAR-Central Plantation Crops Research Institute (CPCRI), Kasaragod, Keral,

***Corresponding author :** neema.m@icar.gov.in
[2] ICAR- Indian Institute of Oil Palm Research, Research Centre Palode, Kerala
[3] ICAR- Indian Agricultural Research Institute, Jharkhand, Gauria Karma, Hazaribag

from November 2021 to February 2023. Treatments (T_1, T_2, T_3, T_4 and T_5) differed in their preculture sucrose concentration (Table 1).

Plant material preparation and inoculation:

Matured nuts (11-12 months old) of West Coast Tall (WCT) variety were selected. Embryos along with endosperm plugs were carefully excised from dehusked and cut open nuts. The endosperm plugs containing embryos were washed under tap water four to five times and treated to 0.01% mercuric chloride for three minutes (Neema *et al.,* 2023). The explant material was subsequently rinsed with distilled water three to four times to remove any traces of mercuric chloride. The washed material was transferred to a laminar airflow chamber where embryos were carefully separated from the endosperm plugs. The separated embryos were washed twice with autoclaved distilled water followed by a 15-minute wash with 20% sodium hypochlorite on a shaker at 100 rpm. After the hypochlorite treatment, embryos were thoroughly rinsed with autoclaved distilled water to eliminate all traces of the chemical. From the sterilized embryos, plumules were carefully excised using a surgical blade and directly inoculated into a preculture media supplemented with 0.2- 0.6 M sucrose, Eeuwen's Y3 basal medium, 5.8 g/L Agar + 1g/L charcoal. The explants were incubated for three days in preculture medium under dark conditions. A complete procedure of plumule separation and inoculation showed in Fig.1.

Pregrowth, vitrification and cryopreservation of coconut plumules

The precultured coconut plumules (in 0.2-0.6M sucrose medium) were placed on cryomesh and encapsulated by pouring a 3% sodium alginate solution over the mesh containing the plumules. The petri plate containing the cryomesh was then flooded with 0.01 M calcium chloride solution. After 15-20 minutes of treatment with calcium chloride solution, plumule material was encapsulated along with cryomesh. The cryomesh containing encapsulated plumules was treated with a loading solution of 'Y3 medium with 0.4 M sucrose + 2 M glycerol' for 20 minutes at room temperature (Fig.2). Following this, the encapsulated material was treated with PVS3 vitrification solution (Y3 basal medium + 50% glycerine + 50% sucrose) in test tubes and incubated for 40 minutes at room temperature. The cryomesh with

Table 1. Different treatment (T_1 to T_5) / methods followed in the experiment

Treatment	Preculture (Sucrose concentration -M)	Preculture duration (hours)	Loading duration (minutes)	PVS3 treatment duration (minutes)	Freezing -196°C duration (hours)	Unloading duration (minutes)
T_1	0.2	72	20	40	48	30
T_2	0.3	72	20	40	48	30
T_3	0.4	72	20	40	48	30
T_4	0.5	72	20	40	48	30
T_5	0.6	72	20	40	48	30

Table.2: Post thaw recovery and plantlet formation of cryopreserved coconut plumules

Treatments	Post thaw recovery , (%) of plumules		Plantlet recovery (%)	
	-LN	+ LN	-LN	+ LN
T_1	76.66±3.33	11.66±1.66	60.00±2.88	4.00±1.00
T_2	73.33±3.33	11.66±1.66	58.33±4.41	6.00±0.57
T_3	76.66±3.33	33.33±3.33	58.33±4.41	7.66±0.66
T_4	73.33±3.33	28.33±1.66	60.00±5.77	6.00±0.57
T_5	75.00±2.88	21.66±4.41	70.00±0.02	5.33±0.33
CD	NS	8.74	NS	2.12
SE(m)	3.24	2.80	4.01	0.66
SE(d)	4.59	3.96	5.67	0.94
CV	7.50	14.45	11.33	19.90

Values represent survival percentage ± SE.
P (survival) < 0.0001; treatment differences were evaluated at 5% probability.
LN: liquid nitrogen

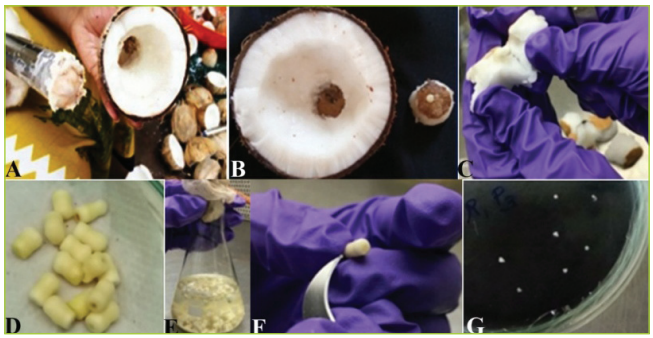

Fig. 1: Extraction of plumule and inoculation; A: extracting endosperm plug using scooper, B. extracted endosperm plug, C: extracting endosperm from the endosperm plug, D: extracted embryo, E: embryo sterilization, F: plumular extraction using sickle blade, G: plumule inoculated into media

Fig. 2: Vitrification and cryopreservation of coconut plumules; (A) vitrification of coconut plumules using V-cryomesh; (B, C, and D) cryopreservation of treated plumules in cryovials

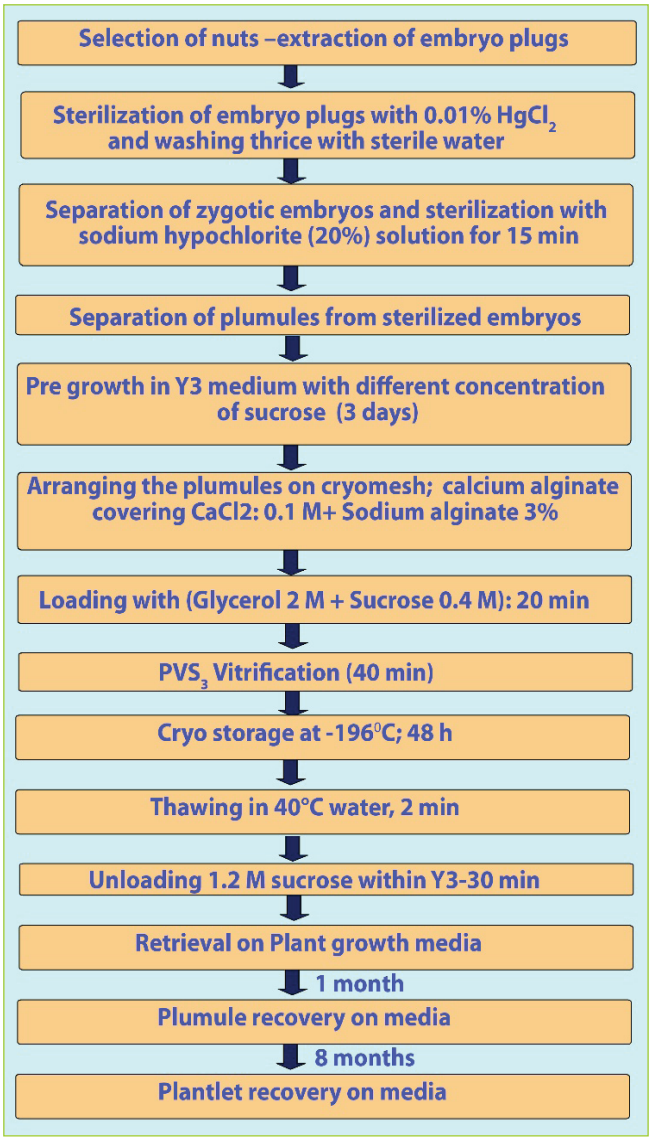

Fig. 3: Flow chart showing cryopreservation of coconut plumules using V-cryomesh technique

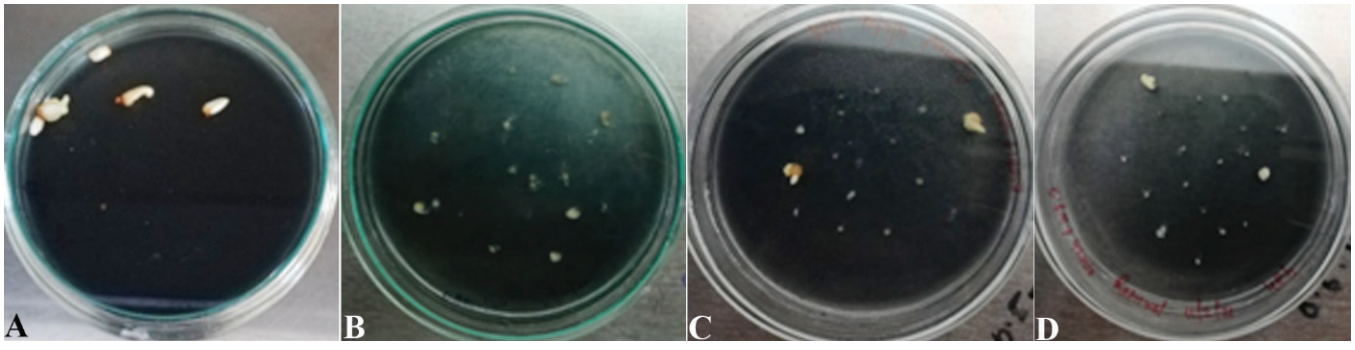

Fig. 4: Post thaw recovery of cryopreserved embryonic shoot portions of coconut (A). control; (B) 0.4 M sucrose with PVS3-40 minutes; (C) 0.5 M sucrose- PVS3-40 minutes; (D) 0.6 M sucrose- PVS3-40 minutes

Fig. 5: Plantlet recovery from the cryopreserved plumules after 8 months of recovery

encapsulated plumules was then transferred to cryovials filled with fresh PVS3 solution and immersed in liquid nitrogen (+LN) for a minimum period of 48 hr. Explants that were not cryostored served as control.

Rewarming and post-cryopreservation recovery

After 48hr of liquid nitrogen storage (+LN), the samples were taken out and then rewarmed in a water bath at 40°C for 2 minutes at room temperature. The cryomesh with explants (+LN/-LN) was placed in an unloading solution (Y3 medium with 1.2 M sucrose) for 30 minutes, with the unloading solution changed every 15 minutes at room temperature. Sodium alginate gel was removed carefully from the explants which were then inoculated onto a recovery medium containingY3 basal medium, 60 g/L sucrose, 1g/L activated charcoal and 5.8 g/L agar. After two weeks of incubation, explants were transferred to Y3 basal media with 30 g/L sucrose, 1 g/L activated charcoal, and 5.8 g/L agar. Once germination occurred, the plumules were transferred to test tubes supplemented with 2 mg/L NAA and 0.5 mg/L IBA in Y3 medium with 30 g/L sucrose, 1 g/L activated charcoal, and 5.8 g/L agar. Plantlets were sub-cultured every two months on the same media prepared in test tubes. Observations on the recovery of plumules (Fig.4) and plantlets (Fig.5) were recorded after one and eight months of culture after changing on to recovery medium. A complete procedure followed the current experiment was showed in flowchart (Fig.3).

RESULTS AND DISCUSSION

The post-thaw recovery of coconut plumules after cryopreservation using V cryo mesh ranged from 11.66% to 33.33% across different treatments. Treatment T_3, with 0.4 M sucrose preculture, showed the highest recovery rate at 33.33%, followed by T_4 (0.5 M sucrose, recovery of 28.33%) and T_5 (0.6 M sucrose, recovery of 21.66%). Lower recovery rates of 11.66% were observed in T_1 and T_2 with 0.2 M and 0.3 M sucrose, respectively. In terms of plantlet recovery, 75% recovery was observed in the control group treated with PVS3 (without liquid nitrogen, -LN). However, for cryopreserved material (+LN), maximum recovery of plumules was 33%, but only 4-7% of the recovered plantlets exhibited healthy growth.

The study demonstrates that the V-cryomesh technique is a viable method for cryopreservation of coconut plumules. The success of technique is significantly influenced by the sucrose concentration in preculture medium. Cryopreservation has been successfully applied to conserve genetic resources in recalcitrant seed species like coconut. Various cryopreservation techniques have been employed for tissues such as immature and mature zygotic embryos, embryogenic calli, and pollen, including air desiccation, pregrowth desiccation, low-freezing, chemical dehydration with programmable-freezing, chemical-freezing, and vitrification (Sisunandar, 2013; Sisunder *et al*., 2014; Karun *et al*., 2014; N'Nan *et al*., 2012; N'Nan, 2003; Sajini *et al.,* 2006; Bajaj, 1984; Chin *et al.,* 1989; Engelmann, 2011; Nguyen *et al*., 2015; Sajini *et al.,* 2011; Cueto *et al*., 2014). However, genetic variability, explant size, and pathogen transmission concerns have made plumules an attractive alternative to zygotic embryos due to their smaller size and meristematic tissue, which is less prone to carry pathogens.

The current study demonstrated that a 0.4 M sucrose preculture provided the best recovery results, aligning with previous studies that identified sucrose concentrations between 0.5 M and 1 M as optimal for the slow drying of encapsulated plumules (Sajini *et al*., 2011). By reducing the sucrose concentration and increasing the incubation time to 72 hours without encapsulation, this study provided new insights into the cryopreservation process. The V-cryomesh technique, which involves direct exposure of plumule tissues to vitrification solutions, offers an advantage over earlier methods that used encapsulation-dehydration. However, longer exposure to vitrification solutions might have contributed to reduced recovery rates and plantlet abnormalities, as previously noted in other studies using encapsulation dehydration techniques (Hornung *et al*., 2001; N'Nan *et al*., 2008).

The use of vitrification-based techniques, particularly with PVS3, has shown promising results in other studies for both zygotic embryos and plumules. Karun et al., (2022) reported a 35% plantlet recovery using droplet vitrification with PVS3, while this study achieved 33% recovery in cryopreserved plumules and 75% recovery

in non-cryopreserved controls. However, the presence of plantlet abnormalities suggests that the plumules were sensitive to prolonged exposure to vitrification solutions. Earlier research by Ledo *et al.*, (2020) and Sajini *et al.*, (2011) indicated that using PVS3 and PVS2 in vitrification can sometimes result in callogenesis instead of plantlet recovery, which aligns with the current study's findings of abnormal growth.

Despite these challenges, study demonstrated that vitrification-based V-cryomesh technique is a viable method for the long-term conservation of coconut plumules. Modifications such as adjusting exposure times to vitrification solutions and incorporating PGRs like ABA into the preculture media may further improve recovery rates and plantlet health.

CONCLUSION

This study demonstrated the feasibility of using the V-cryomesh technique for long-term conservation of coconut plumules. The optimal protocol involved preculturing the plumules for 72 hr in a medium containing 0.4 M sucrose, followed by a 20-minute loading duration. This was followed by PVS3 treatment for 40 minutes, rapid cooling at -196°C, and rewarming at 40°C for 2 minutes. The plumules were then unloaded in 1.2 M sucrose liquid medium for 30 minutes. Under these conditions, 33% post-thaw recovery and 7% plantlet recovery were observed in cryopreserved plumules. The use of V-cryomesh enabled a simplified approach to vitrification, though further adjustments to the protocol could lead to higher recovery rates and healthier plantlet formation.

ACKNOWLEDGEMENTS

The Authors acknowledge the Indian Council of Agricultural Research, New Delhi, India, for providing the necessary funds to carry out the research.

REFERENCES

Aparna, V., Neema, M., Chandran, K.P., Muralikrishna, K.S. and Karun, A., 2023. Enhancement of callogenesis from plumular explants of coconut (Cocos nucifera L.) via exogenous supplementation of amino acids and casein hydrolysate. *Current Horticulture* **11**: 40-3.

Bajaj Y P S. 1984. Induction of growth in frozen embryos of coconut and ovaries of citrus. *Current Science* **53**: 1215–16.

Bandupriya , Fernando SC, Verdeil JL and Malaurie B. 2010. Cryopreservation of encapsulated plumules of coconut: effect of transport/store conditions. *Asia-Pacific Journal of Molecular Biology and Biotechnology* **18**: 135–37.

Bandupriya , Fernando S C, Verdeil JL and Malaurie B. 2007. Effect of abscisic acid on survival and recovery of cryopreserved plumule explants of coconut (*Cocos nucifera* L.). *Cocos* **18**: 45–51.

Chin H F, Krishnapillay B and Hor Y L. 1989. A note on the cryopreservation of embryos of coconut (*Cocos nucifera* L. var. Mawa). *Pertanika* **2***:* 183–86.

Cueto C, Rivera RL, Kim H H, Kong HJ, Baek H J, Sebastian L and Park H J. 2014. Development of cryopreservation protocols for cryobanks of coconut zygotic embryos. *Acta Horticulturae* **1039***:* 297–302.

Engelmann F. 2011. Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cellular & Developmental Biology - Plant* **47**: 5–16.

Engelmann F, Malaurie B, N'Nan O and Borges M. 2005. Status of cryopreservation research in coconut. *In: Coconut Genetic Resources.* Batugal P, Rao VR and Oliver J (Eds.), IPGRI-APO, Serdang, Malaysia, pp. 142–48.

Hornung R, Domas R and Lynch PT. 2001. Cryopreservation of plumular explants of coconut (*Cocos nucifera* L.) to support programmes for mass clonal propagation through somatic embryogenesis. *Cryo Letters* **22:** 211–20.

Karun A, Sajini K, Muralikrishna K and Rajesh MK. 2022. Cryopreservation of coconut plumule using droplet vitrification. *Journal of Plantation Crops* **50**: 51–59.

Karun A, Sajini KK, Niral V, Amarnath CH, Remya P, Rajesh MK and Engelmann F. 2014. Coconut (*Cocos nucifera* L.) pollen cryopreservation. *Cryo Letters* **35:** 407–17.

Ledo ADSL, Santana FV, Oliveira CAD, Oliveira LARD and Silva AVCD. 2020. Cryopreservation of Brazilian green dwarf coconut plumules by droplet-vitrification. *Ciencia Rural* **50**(1): e20190020.

Malaurie B and Borges M. 2001. Cryopreservation of coconut (*Cocos nucifera* L.) plumules by encapsulation/dehydration. In: *International Workshop on Plant Biotechnology – Plant Breeding and Biotechnology, Ciego de Avila* **16**: 59.

Malaurie B, Bandupriya HDD, Fernando SC and Verdeil JL. 2006. Optimization of the cryopreservation process for coconut plumules. *Proceedings of the BRG* **6**: 449–68.

Malaurie B, Borges M and N'Nan O. 2002. Research of an optimal cryopreservation process using encapsulation/osmoprotection/dehydration and encapsulation/osmoprotection/vitrification techniques on caulinary meristems of coconut (*Cocos nucifera* L.). *Burotrop Bulletin* **20**: 16.

Neema, M., Gupta, A., Gopal, M. and Karun, A., 2023. Basidiomycete (*Pseudolagarobasidium acaciicola*) in coconut (*Cocos nucifera* L.) suspension culture - A report. *Current Horticulture* **11**: 66-8.

N'Nan O, Borges M, Konan JLK, Hocher V, Verdeil JL, Tregear J, N'guetta ASP, Engelmann F and Malaurie B. 2012. A simple protocol for cryopreservation of zygotic embryos of ten accessions of coconut (*Cocos nucifera* L.). *In Vitro Cellular & Developmental Biology - Plant* **48**: 160–66.

N'Nan O, Hocher V, Verdeil JL, Konan JLK, Ballo K, Mondeil F and Malaurie B. 2008. Cryopreservation by encapsulation-dehydration of plumules of coconut (*Cocos nucifera* L.). *Cryo Letters* **29**: 339–50.

Nguyen QT, Bandupriya HDD, López Villalobos A, Sisunandar S, Foale M and Adkins SW. 2015. Tissue culture and associated biotechnological interventions for the improvement of coconut (*Cocos nucifera* L.): a review. *Planta* **242**: 1059–76.

N'Nan OA, Tiécoura K, Bi SG, Verdeil JL and Malaurie B. 2014. Ultrastructural changes during cryopreservation of plumules and embryos of coconut (*Cocos nucifera* L.). *International Journal of Agro and Agricultural Research* **5**: 103–15.

N'Nan O, Borges M, Verdeil JL, Hocher V and Malaurie B. 2003. Are caulinary meristems suitable for cryopreservation of coconut (*Cocos nucifera* L.) with encapsulation/dehydration technique? Histological studies, a way to find the good range of pretreatments. *Burotrop Bulletin* **20**: 17.

Sajini KK, Karun A, Amarnath C and Engelmann F. 2011. Cryopreservation of coconut (*Cocos nucifera* L.) zygotic embryos by vitrification. *Cryo Letters* **32:** 317–28.

Sajini KK, Karun A and Kumaran PM. 2006. Cryopreservation of coconut (*Cocos nucifera* L.) zygotic embryos after pre-growth desiccation. *Journal of Plantation Crops* **34**: 576–81.

Sisunandar. 2013. Germplasm Cryopreservation Conservation: Progress Report on Indonesian Elite Mutant Coconut "Kopyor." In: *Proceedings of the International Conference on Global Resource Conservation and 10th Indonesian Society for Plant Taxonomy Congress.* Brawijaya University.

Sisunandar, Novarianto H, Mashud N, Samosir YM and Adkins SW. 2014. Embryo maturity plays an important role for the successful cryopreservation of coconut (*Cocos nucifera* L.). *In Vitro Cellular & Developmental Biology - Plant* **50**: 688–95.

Welewanni I, Jayasekera A and Bandupriya D. 2017. Coconut cryopreservation: present status and future prospects. *Cord* **32:** 41–61.