Research Article

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Cryopreservation of coconut plumule using droplet vitrification

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Abstract

In the present investigation, four types of explants from mature zygotic embryos of coconut, *viz*., whole upper cotyledonary region without haustorium, half of the upper cotyledonary region without haustorium, plumule with a portion of radicle and exclusively plumular tissue, were cultured in 12 different media combinations to find a suitable explant which could be regenerated after cryopreservation. Explants were pre-cultured in medium with 0.4 and 0.5 M sucrose for three days followed by dehydration in PVS_3 solution for different durations on a sterile aluminum strip after treating with loading solution. Strips were treated with liquid nitrogen inside a cryoflask until bubbling stopped and quickly transferred to a cryovial and stored for a minimum period of 24 hours in liquid nitrogen. It was observed that plumule alone or with a small portion of outer tissue was ideal for fast *in vitro* growth and recovery of whole plantlets of coconut in a medium supplemented with NAA alone. Addition of glutamine (5 mg L^{-1}), TDZ (1 mg L^{-1}) and NAA (18 mg L^{-1}) aided the vigorous growth of plantlets. In control, the survival rate ranged from 60 to 90 per cent in plumule pre-grown in media containing 0.5 M sucrose after dehydration with PVS_3 for various durations, whereas it was 14 to 75 per cent in cryopreserved ones. Considering the high survival (75%) and regrowth (35%) of cryopreserved plumule in the present study, there is much scope for further improvement of the procedure to find the right combination of factors so as to enhance complete recovery of plantlets without much injury to plumules during cooling and rewarming.

Keywords: Conservation, loading, pre-culture, PVS_3, regeneration, thawing

Introduction

Conservation of various genetic resources in different crops is essential for its sustainable use at present and at a later date. Advancement in cryobiology has led to formulation of effective cryopreservation strategies for complementary long term *in vitro* conservation since it is a cost-effective and a user-friendly technique (Karun *et al*., 2005). Cryopreservation technique involves storage of frozen tissues usually in liquid nitrogen at ultra low temperature (-196 ^{o}C). Optimization of the technique would reduce freezing damage and lead to successful storage of tissues indefinitely (Engelmann, 1997). Coconut, a monotypic species in the genus *Cocos*, is one of the major oil crops of the tropics and is perennial in nature. Coconut accessions grown traditionally in specific locations are known as ecotypes, of which Coconut Genetic Resources Network has recognized 1416 accessions (Batugal, 2005). The precious landraces/ecotypes and other genetic resources, maintained mainly in five International Coconut Genebanks (ICGs), are at risk due to the commercial cultivation of high yielding varieties and vagaries of nature. Conservation strategies in coconut include *in situ* conservation on farm, in remote islands and atolls (Bourdeix *et al*., 2011) or *ex situ* conservation in the form of Field Gene Banks (FGBs) (Sajini, 2012).

Coconut is classified as a recalcitrant seed and conventional seed storage is not feasible. Moreover, FGBs are vulnerable to biotic, abiotic as well as economic factors (Fu, 2017). Hence, a complementary conservation strategy involving a combination of different conservation strategies is necessary for

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optimum sustainable use of genetic diversity. In this scenario, cryopreservation offers a very efficient and promising method for long-term conservation of germplasm (Mandal, 2000).

The most successful explant to be cryopreserved in coconut is the mature zygotic embryo. Bajaj (1984) was first to suggest the possibility of long term conservation of coconut zygotic embryos when overall swelling and elongation of embryos was achieved after freezing at -196 ^{o}C. Wide range in post-thaw recovery of the explant has been reported by various workers depending on the desiccation or vitrification technique (Karun *et al*., 2005; Sajini *et al*., 2006; Sajini *et al*., 2011). The cryo-injury is lethal for most of the embryonic tissue of zygotic embryo during the cryopreservation except for the regenerating plumule comprising of apical dome and a few leaf primordia (Assy Bah and Engelmann, 1992). Plumular tissue is an ideal explant for cryopreservation since it is small in size, with abundant meristematic cells and enhanced potential for regeneration (Alla-N'Nan *et al*., 2008). Cryopreservation of plumular tissue following encapsulation-dehydration procedure revealed the survivability and plantlet regeneration (Hornung *et al*., 2001; Bandupriya *et al*., 2007; Alla-N'Nan *et al*., 2008). Ultrastructural changes on account of encapsulation-dehydration cryopreservation in coconut plumular tissue have also been observed (Alla-N'Nan *et al*., 2014b). Effect of transport and storage conditions prior to the extraction of plumule on cryopreservation has been studied by Bandupriya *et al*. (2010). A recent report indicated droplet vitrification method of cryopreservation of plumule explants of Brazilian Green Dwarf coconut yielding 30 per cent of embryogenic calli upon recovery and cultured for callogenesis (Ledo *et al*., 2019).

Droplet vitrification method facilitates rapid cooling due to direct contact of explants with liquid nitrogen and also between explant and unloading solution during re-warming, thus resulting in high recovery rates of plantlets (Sakai and Engelmann, 2007). The present study was undertaken to optimize the plumular size and media composition for complete recovery of plantlets and to standardize the pre-growth medium and dehydration duration with plant vitrification solution (PVS_3) for development of droplet vitrification protocol for coconut plumule cryopreservation.

Materials and methods

Explant selection and surface sterilization

Mature zygotic embryos from West Coast Tall (WCT) cultivar of coconut were separated from endosperm plugs sterilized with 0.01 per cent $HgCl_2$ for three minutes followed by washing 4-5 times with sterile distilled water. Embryos were then subjected to surface sterilization with 20 per cent sodium hypochlorite solution for 20 min and washed thrice with sterile water in a laminar air flow chamber. Five types of explants from mature zygotic embryo were selected to understand the potential of the explants to regenerate. The explants include (i) whole upper cotyledonary region without haustorium; (ii) half of the upper cotyledonary region without haustorium; (iii) plumule with a portion of radicle; (iv) exclusively plumule tissue and (v) whole embryo as control (Fig. 1)

Standardization of regeneration medium

Explants were inoculated in 12 Y3 (Eeuwens, 1978) based media combinations (Table 1).

All media combinations were supplemented with sucrose (30 g L^{-1}), agar (6.5 g L^{-1}) and activated charcoal (1 g L^{-1}). pH of the media were adjusted to 5.75 before autoclaving.

Pre-culturing, loading, vitrification and cryopreservation

The best regenerating explant based on survival and recovery rate was selected for further studies. Plumular tissue were inoculated on to Y3 solid medium with 0.4 or 0.5 M sucrose and kept for three days in dark. Explants were then kept on a sterile aluminium foil strip and treated with 200 µL droplets of loading solution (Y3 with 2 M glycerol and 0.4 M sucrose) for 20 minutes. Explants were then placed on a new sterile aluminium foil strip (5 mm × 20 mm) and treated with 10 µL drops of PVS_3 (Y3 with 50% (w/v) glycerol and 50 per cent (w/v) sucrose) vitrification solution for different time intervals, *viz*., 0, 20, 40, 80 and 100 minutes. After specific time intervals, strips with the explants were exposed to liquid nitrogen and then inserted to the cryovials and later plunged in liquid nitrogen can (Taylor Wharton, USA). Explants were cryopreserved for a minimum period of 24 hours. The thawing and re-warming were carried out by treating with Y3 medium consisting 1.2 M sucrose for 50 minutes at ambient

Fig. 1. Preparation of explants and cryopreservation procedure in coconut. Selection of different explants from the surface sterilized mature zygotic embryo of WCT cultivar for the conservation study; (a) scooping endosperm plug along with zygotic embryo; (b) endosperm plug; (c) splitting endosperm plugs after sterilization with $HgCl_2$ solution (0.01%) for three minutes; (d) surface sterilized embryos using sodium hypochlorite solution (20%); (e) whole upper cotyledonary region without haustorium; (f) half of the upper cotyledonary region without haustorium; (g) plumule with a portion of radicle; (h) exclusively plumule tissue; (i) whole embryo; (j) pre-culturing of plumule; (k) loading solution; (l) PVS_3 treatment; (m) liquid nitrogen exposure; (n-p) thawing and PVS_3 dilution and (q) inoculation in retrieval medium

sterile conditions. Explants were then inoculated on to Y3 medium with 60 g L^{-1} sucrose for three days and later transferred to the retrieval medium. The data generated were analysed for statistical significance in SAS software through Duncans Multiple Range Test (DMRT).

Results and discussion

Shoot emergence in various explants

Zygotic embryos and plumular explants cultured in different media combinations started to swell irrespective of hormone supplementation in the growth media within 7-10 days after inoculation. Shoot emergence was noticed from the upper cotyledonary sheath of the zygotic embryo after 25-30 days. Germination was not uniform in zygotic embryos, while it was uniform in plumular explants which began within 15 days. Significant differences were noticed between the types of explants for shoot development (Table 2). Shoot development was significantly high in plumular tissue alone or

Table 1. Different media combinations used in the present study

Media	NAA (mg L^{-1})	BAP (mg L^{-1})	GA$_3$ (mg L^{-1})	2iP (mg L^{-1})	TDZ (mg L^{-1})	Glutamine (mg L^{-1})
1.	-	-	-	-	-	-
2.	18	-	-	-	-	-
3.	-	10	-	-	-	-
4.	-	10	-	-	-	5
5.	-	10	-	2	-	5
6.	-	10	1	-	-	5
7.	18	-	-	2	-	5
8.	18	-	1	-	-	5
9.	18	-	-	-	-	5
10.	-	10	-	-	1	5
11.	18	-	-	-	1	5
12.	9	-	-	-		5

plumule with a portion of radicle. Removal of outer cotyledonary sheath exposed the plumular tissue for direct contact with the medium, which might have aided in fast and uniform germination. Average shoot length (1.84 cm) was highest in medium devoid of any hormones (Table 2). Non-utilization of growth hormones in coconut embryo culture studies has been reported previously (Assy-Bah, 1986; Rillo and Paloma, 1990). However, significant interaction has been observed between type of explant and NAA for shoot growth. The plumule with a portion of radicle showed maximum shoot length (4.23 cm) in medium supplemented with NAA (18 mg L^{-1}) alone (Table 1; Fig. 2a). The resulting shoots were not vigorous. Ashburner *et al.* (1993) had studied the effect of NAA and sucrose levels on the development of cultured embryos of coconut. They observed that when NAA was present throughout the culture period, there was a tendency for reduced shoot growth. However when NAA exposure was limited to four weeks late in growth period (between 20-24 weeks), shoot growth was

Table 2. Effect of different types of explants excised from coconut zygotic embryos in medium supplemented with different growth hormones for *in vitro* shoot length

Media	Type of explants					
	Whole embryo	Upper cotyledon	Half upper cotyledon	Plumule + radicle	Plumule	Mean
1.	1.36±1.51	1.39±1.15	1.27±1.70	2.36±1.24	2.86±3.57	1.84±2.06a
2.	0	0.15±0.32	1.08±1.07	4.23±3.01	1.50±0.62	1.39±2.08ab
3.	1.55±1.99	0.17±0.39	0.42±0.59	0.43±0.45	2.30±1.15	0.97±1.33bc
4.	0.47±0.44	0.39±0.36	0.24±0.43	1.42±0.92	1.57±2.30	0.81±1.24bc
5.	0.98±1.62	1.04±0.72	0.18±0.16	1.28±0.59	0.89±0.86	0.87±0.96bc
6.	0.65±0.48	0.93±0.89	0.34±0.65	1.88±1.50	1.99±1.76	1.15±1.30abc
7.	0.44±1.01	0.63±1.58	0.63±1.79	1.84±3.25	0.74±1.04	0.85±1.90bc
8.	0.92±2.18	0.15±0.47	0.15±0.33	0	1.26±1.08	0.49±1.18c
9.	0.89±0.86	0.61±1.55	2.12±2.65	3.10±3.53	1.07±0.70	1.55±2.26ab
10.	0.64±1.01	0.03±0.09	0.05±0.15	3.35±2.06	0	0.81±1.63bc
11.	0.34±0.66	0.04±0.06	3.42±3.86	1.64±3.08	2.20±2.69	1.52±2.73ab
12.	1.71±1.49	0.05±0.15	0.40±1.19	0.47±0.77	2.64±3.54	1.05±2.01bc
Mean	0.82±1.31b	0.46±0.90b	0.85±1.82b	1.83±2.32a	1.58±2.04a	

Mean values followed with similar alphabets did not vary significantly according to DMRT

stimulated even at the highest concentration (800 µM). Moreover, the sensitivity of *in vitro* coconut plants to NAA increased with decreasing sucrose content in the medium. Our present study is confirmatory with earlier results, since in intact embryos, even though germination was noticed initially, it did not proceed further. On the other hand, in the case of plumular tissues the presence of NAA alone in the medium was positive for shoot initiation as well as elongation. The variable sensitivity of whole embryo and plumule to NAA treatment is evident in the present study.

Further, the timing of NAA treatment in germination and growth medium at various sucrose levels needs further study. From plumular explants, plantlets with fully opened leaves with vigorous roots were formed when glutamine (5 mg L^{-1}) and TDZ (1 mg L^{-1}) were incorporated along with NAA in the medium (Fig. 2b). The effect of BAP alone or in combination with other growth hormones (NAA and TDZ) was not significant for shoot growth either for whole embryo or plumular explant.

Ashburner *et al*. (1996) had earlier reported that supplementation of BAP (up to 316 µM) did not significantly affect growth of coconut embryos in culture. Even though shoot initiation was enhanced in media supplemented with GA_3 (1 mg L^{-1}), it was quite inhibitory for further development. Prolonged subculture into medium containing GA_3 resulted in browning and complete death of explants. Increased germination of coconut zygotic embryos was reported by Karun *et al*. (2001) in medium supplemented low concentration of GA_3 (0.01 to 1.0 µM) in relation to untreated controls, but the differences were not significant. Treatment of coconut zygotic embryos with higher concentration of GA_3 (up to 4.6 µM) for five weeks in semi-solid medium and their transfer to GA_3 free-liquid medium for the remaining 32 week culture favoured its germination and conversion into plants (Ake *et al*., 2007). Hence, it has become evident from the present study that the treatment period with GA_3 is critical for the normal germination and conversion of coconut embryo or plumular tissues to plants that otherwise will become lethal after continuous exposure to GA_3 (Fig. 2c). A simple and effective germination medium for conversion of coconut plumule to plantlet, standardized in the present study, has been utilized as a regeneration medium after plumule cryopreservation (Karun *et al*., 2005; Sajini *et al*., 2011).

Difference in the shoot growth was not significant between plumule and plumule along with a portion of radicle. Extraction of plumule alone was easier as compared to the explant preparation of plumule with portion of radicle. Hence, plumule alone was chosen for the cryopreservation studies.

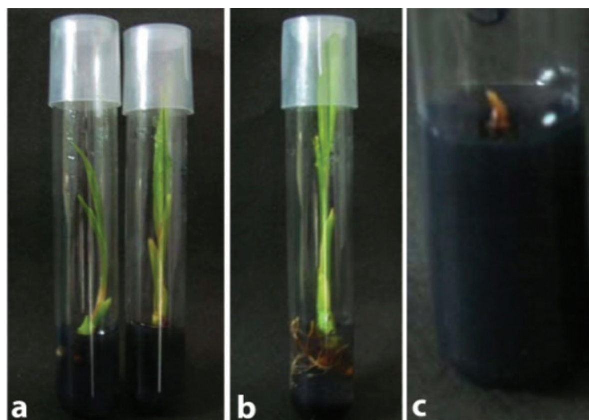

Fig. 2. Growth and development of plantlets from plumular explants along with a portion of radicle of coconut. (a) Plantlet development in NAA (18 mg L^{-1}) medium; (b) Plantlet development in NAA (18 mg L^{-1}), glutamine (5 mg L^{-1}) and TDZ (1 mg L^{-1}) and (c) Browning and death of shoot of embryos in medium supplemented with GA_3 throughout the culture period

Effect of sucrose concentration in pre-culture medium and duration of PVS_3 treatment during cryopreservation of coconut plumule

The desiccated plumules started swelling and enlargement within 7-10 days after inoculation to the regeneration medium (Y3 with 18 mg L^{-1} NAA). But for cryopreserved plumules, a further delay of 5-7 days occurred for resumption of growth. The non-viable plumules were white in colour without any sign of growth or enlargement. The survival ranged from 60 to 90 per cent in plumule pre-grown in media containing 0.5 M sucrose after dehydration with PVS_3 for various durations, whereas it was 14 to 75 per cent in cryopreserved ones (Fig. 3). The survival obtained from the cryopreserved plumule envisages the possibility of using droplet

vitrification for long term storage of coconut germplasm. The droplet vitrification was initially developed from the droplet freezing method using 10 per cent dimethyl sulphoxide (DMSO) as cryoprotectant. It was originally implemented for cryopreservation of shoot tips of potato (Schafer-Menuher *et al.*, 1997) and asparagus (Mix-Wagner *et al.*, 2000). Later, Leunufia and Keller (2003) and Panis *et al.* (2005) substituted DMSO by PVS_2. The main advantage of this method is the fast cooling rate (130 ºC s^{-1}) compared (Towill and Bonnart, 2003) to vitrification (6 ºC s^{-1}) that facilitates accomplishing vitrified state rapidly during freezing (Fahy *et al.*, 1984). Consequently, high survival and regeneration rates have also been achieved in crops like yam (Leunufna and Keller, 2003) banana (Panis *et al.*, 2005), garlic (Kim *et al.*, 2007) and taro (Sant *et al.*, 2008).

Pre-conditioning of explants before exposure to ultra-low temperature proved to be very essential for its survival and regrowth. This is generally achieved by culturing the explants in a sucrose-enriched medium. In the vitrification method followed for coconut zygotic embryos, three days of pre-culture in 0.6 M sucrose proved to be optimal for its survival (Sajini *et al.*, 2011). In the present study, no significant difference was observed for survival and regrowth of cryopreserved plumules after pre-culture in Y3 medium supplemented with 0.4 or 0.5 M sucrose. But significant interaction was noticed for pre-culture media and liquid nitrogen exposure. After three days pre-culture in medium containing 0.5 M sucrose and subsequent dehydration for 20 min to 100 min with PVS_3, maximum shoot production noticed was 39 per cent in the case of dehydrated plumules. After cryopreservation, it was 20 per cent in plumule pre-cultured for three days in medium containing 0.4 M sucrose and subsequent dehydration for 20 to 100 minutes with PVS_3. Here the plumular explants showed flaccidity and callus formation after incubation in the regeneration medium (10 to 30%). But among the treatments undertaken, three days pre-culture in medium containing 0.5 M sucrose and subsequent dehydration for 20 min in PVS_3 resulted in maximum shoot growth in dehydrated (60%) or cryopreserved plumules (35%) (Fig. 6.). Abnormality was also very less in this treatment (5-10%). The mode and duration of pre-culture in sucrose-enriched medium before cryopreservation varies depending on the plant species studied. In droplet vitrification method developed for yam, Leunufna and Keller (2003) reported that three day pre-culture in medium containing 10 per cent or 20 per cent sucrose was effective for improving survival and regrowth rates. Preconditioning of lily meristems in media supplemented with 0.3 M sucrose for two days was very effective for improving post-thaw survival (Chen *et al.*, 2011). In chrysanthemum, step wise pre-culture with 0.3, 0.5 and 0.7 M sucrose significantly improved survival and regrowth of axillary shoot tips after cryopreservation (Lee *et al.*, 2011). Various biochemical changes like accumulation of endogenous sugars and starch grains (Bachiri *et al.*, 2000), changes in proteins and fatty acids (Zhu *et al.*, 2006) and other structural changes (Helliot *et al.*, 2003) were found to occur after pre-culture of explants in sucrose medium. Lee *et al.* (2011) suggested that sucrose pre-culture lower free water activity in cells and trigger the metabolic pathways which lead to the acquisition of tolerance to subsequent dehydration and freezing. The accumulation of endogenous sucrose in cells will strengthen membrane stability during severe dehydration and support survival after cryopreservation (Crowe *et al.*, 1989). Previously, encapsulation dehydration method resulted in only 20 per cent survival of plumule after cryopreservation (Alla-N'Nan *et al.*, 2008). Cryopreserved plumular explants of Brazilian Green Dwarf coconut following droplet vitrification technique yielded better survivability (~96%) and embryogenic callus production (Ledo *et al.*, 2019).

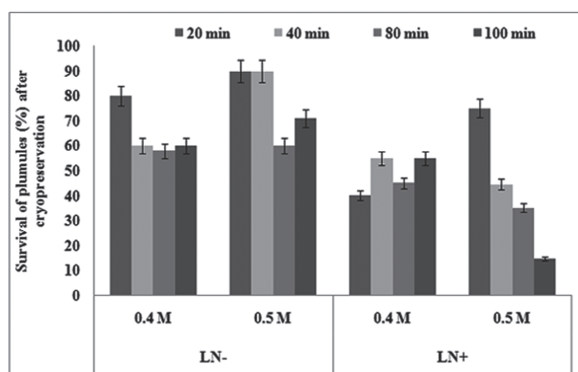

Fig. 3. The effect of pre-growth media and dehydration duration in PVS_3 for the survival of plumules before and after cryopreservation

Table 3. The effect of pre-growth media and dehydration duration in PVS_3 for the regeneration of plumules after cryopreservation

PVS_3 dehydration (minutes)	Pregrowth treatment with sucrose (M)					
	-LN			+ LN		
	0.4 M	0.5 M	Mean	0.4 M	0.5 M	Mean
20	35.0 ± 9	60.0 ± 9	47.5 ± 6.4a	15.0 ± 9	35.0 ± 9	5.0 ± 6.4 a
40	25.0 ± 9	46.5 ± 9	35.7 ± 6.4ab	15.0 ± 9	20.0 ± 9	17.5 ± 6.4 ab
80	8.5 ± 9	15.0 ± 9	11.7 ± 6.4b	20.0 ± 9	10.0 ± 9	15.0 ± 6.4 b
100	10.0 ± 9	35.5 ± 9	22.7 ± 6.4b	30.0 ± 9	5.0 ± 9	17.5 ± 6.4 ab
Mean	19.6 ± 4.5	39.2 ± 4.5	29.4 ± 3.2	20.0 ± 4.5	17.5 ± 4.5	18.7 ± 3.2

Mean values followed with similar alphabets did not vary significantly according to DMRT

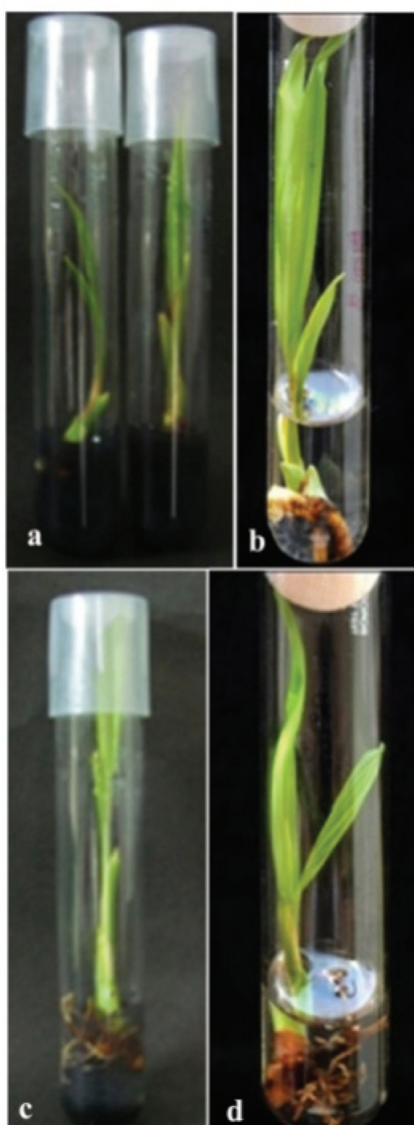

Fig. 4. Performance of retrieved plumular explants after cryopreservation in NAA (18 mg L^{-1}) medium (a & b) and in NAA (18 mg L^{-1}), glutamine (5 mg L^{-1}) and TDZ (1 mg L^{-1}) medium (c & d)

In the present study, shoot regeneration depended on duration of exposure to PVS_3. The maximum shoot growth recorded was 47.5 per cent and 25 per cent, respectively in the case of 20 minutes dehydrated or cryopreserved plumules. It decreased progressively in line with increasing treatment durations (Table 3) for a minimum of 11.7 per cent (-LN) and 15 per cent (+LN) after 80 min exposure to PVS_3. In previous studies, using coconut zygotic embryos, treatment with PVS_3 for 16 hours was found to be very effective for regeneration of cryopreserved embryos using vitrification method (Sajini *et al.*, 2011). In the present study too, PVS_3 was utilized as cryoprotectant. PVS_3 was found to be good cryoprotectant in other crops like garlic (Kim *et al.*, 2009) and sugarcane (Barraco *et al.*, 2011) using droplet vitrification method. PVS_3 contains non-penetrating cryoprotectants like sucrose (50%) and glycerol (50%) that may also be harmful due to high osmotic pressure it exerts on plant cells (Kim *et al.*, 2009). So the period of exposure to vitrification solution determines the extent of cell dehydration or the amount of cryoprotectants permeated into cells. A short exposure period of 20 minutes in PVS_3 after 20 minutes of loading treatment was found to be highly beneficial for the post survival (57%) and regeneration (25%) of cryopreserved coconut plumules. In the droplet vitrification method followed in the present study, high survival observed at critical exposure period of 20 minutes in PVS_3 proposes osmotic tolerance for plumular tissue in coconut. Tolerance to osmotic stress in coconut zygotic embryos have already been reported while applying vitrification (Sajini *et al.*, 2011) and pre-growth desiccation method (Assy Bah *et al.*, 1992;

Sajini *et al*., 2006). The optimal length of PVS_3 treatment for different plant species varies considerably. In the case of sugarcane, an exposure time of 20-40 minutes after 20 minutes of loading treatment using 2 M glycerol and 0.4 M sucrose resulted in recovery percentage of 33 per cent after cryopreservation. The axillary shoot tips of chrysanthemum showed 96 per cent survival and 71 per cent regeneration after exposure to PVS_3 for 60 minutes at room temperature (Lee *et al*., 2011). The recovery rate achieved in the case of 56 accessions of banana shoot tips (Panis *et al*., 2005) using droplet vitrification method ranged between 50 and 95 per cent. It was 89 per cent in 18 taro cultivars (Sant *et al*., 2008) and 95 per cent in garlic bulbil primordial (Kim *et al*., 2009).

Conclusions

Coconut plumule with a portion of radicle was an ideal explant for regeneration into complete plantlet. Eeuwen's Y3 solid medium, supplemented with NAA (18 mg L^{-1}), was found to be better regeneration medium for plumule. Supplementation with glutamine (5 mg L^{-1}) and TDZ (1 mg L^{-1}) improved the growth. Plumule cryopreservation by droplet vitrification method is feasible in coconut and achieved high survival (75%) and re-growth (35%) after cryopreservation. Further improvement of the procedure including the incorporation of cryoplate and cryomesh is necessary to find the right combination of factors so as to enhance complete recovery of plants without much injury to plumules during cooling and rewarming.

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