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Research Article

Cryopreservation of aromatic ginger *Kaempferia galanga* L. by encapsulation-dehydration

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Abstract

Kaempferia galanga L. is an endangered multi-purpose medicinal plant in Family Zingiberaceae, the rhizomes of which are used for several ayurvedic formulations. Encapsulation-dehydration (ED) method was optimized for cryopreservation of shoot tips of *K. galanga*. Shoot tips (*STs*) bearing the apical meristem dissected from the established *in vitro* shoot cultures were preconditioned in MS+0.4 M sucrose prior to encapsulation in calcium alginate and the beads subsequently transferred to MS liquid+0.3 M sucrose for 3 days afterward dehydration inside the laminar airflow for 4 hours upon rapid freezing in LN and rapid thawing produced maximum 62.2% survival and 46.7% regeneration rates. Shoot regeneration was observed from the apical meristems exclusive of intermediary callus phase. The plantlets regenerated from cryopreserved *STs* transferred to the field were phenotypically analogous with the mother plant.

Keywords: cryopreservation; encapsulation-dehydration; *Kaempferia galanga*; monocot; preculture; shoot tips

Abbreviations: BA (6-benzyl adenine); GA_3 (giberellic acid); IAA (Indole 3-acetic acid); LN (liquid nitrogen); MS (Murashige and Skoog); NAA (α-naphthaleneacetic acid); *STs* (Shoot tips)

Introduction

Kaempferia galanga L., a high sought endangered multi-utility medicinal plant of the Family Zingiberaceae (Shirin *et al.*, 2000) is cultivated in Tropical Asia for its aromatic rhizome which is having a wide range of medicinal applications (Sadiman, 1992). Various uses of aromatic ginger (*K. galanga*) for health benefits, food and nutritional purposes are well documented (Preetha *et al.*, 2016). Traditionally the rhizomes have been used to treat fever, amoebiasis, bruice, dandruff, headache, rheumatism, cholera, contusion, constipation, stomach ache, cold and chest pain (Limyati and Juniar, 1998; Kanjanapothi *et al.*, 2004). The anticancer, antihypertensive, larvicidal (Choochote *et al.*, 1999, Othman *et al.*, 2002, Norjit*et al.*, 2007), vasorelaxant (Othman *et al.*, 2006), antioxidant (Sahoo *et al.*, 2014), sedative (Ali *et al.*, 2015) and anti-inflammatory (Channabasavaiah *et al.*, 2016) activities of the rhizome extract have been reported. The

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rhizomes contain a high value volatile oil, several alkaloids, starch, protein, aminoacids, minerals and fatty matter. The chemical components and biological activities of volatile oil have been worked out by Tewtrakul *et al.* (2005). The price of essential oil varies from US$ 600-700 per Kg on the international market and is highly exploited by the local people and pharmaceutical industries (Chithra *et al.*, 2005). More recently aromatic ginger was elevated to the status as a functional food (Srivastava *et al.*, 2019). Conventional propagation of *K. galanga* is through clonal means *via* rhizomes, which remains dormant during drought and sprouting in spring. From the conservation point of view, the genetic resources of clonally propagated plants can be maintained as field collections, in green houses, *in vitro* or cryopreserved at ultralow temperatures of liquid nitrogen (FAO, 2013). During maintenance in the field, the plants were rendered to environmental pressures such as diseases, pests and extreme weather conditions (Rantala *et al.*, 2019). To evade the troubles associated with conventional field maintenance, *in vitro* and cryopreservation techniques guarantee the long-term *ex situ* conservation and sustainable utilization of plant genetic resources. Among the *in vitro* strategies, cryopreservation is safe, cost-effective, long-standing method of plant germplasm preservation since during storage all metabolic activities are suspended thus ensuring high genetic stability together requiring minimum space for maintenance (Matsumoto, 2017). There is no need of regular subculturing in cryopreserved materials and the original germplasm can be stored for prolonged time periods with no change in their qualities (Al-Khayri and Naik, 2017). Though *in vitro* plant regeneration systems are established well in *K. galanga* (Vincent *et al.*, 1992; Shirin *et al.*, 2000; Swapna *et al.*, 2004; Rahman *et al.*, 2005; Preetha *et al.*, 2008, 2014; Mohanty *et al.*, 2010; Senarat *et al.*, 2017), so far there is only a single report on *in vitro* long-term conservation by cryopreservation using the PVS2 aided vitrification in this medicinal plant by us (Preetha *et al.*, 2013) that recorded 30-40% recovery after cryopreservation. Since there is a critical necessity for realistic conservation endeavours to establish long-term germplasm collections of recalcitrant and tropical plants as because of the challenge and threat these species are facing (Mohd Noor *et al.*, 2019), we here report the feasibility of cryopreserving shoot tips of *K. galanga,* using encapsulation-dehydration technique which offers a relatively high recovery rate (46.7%) than the earlier established protocol and can be efficiently applied for producing synthetic seeds as well as ensuring the long-term conservation of the same with much more recovery in other economic Zingiberaceae species.

Materials and Methods

Plant material

In vitro shoot cultures of *Kaempferia galanga* L. established from rhizome segments were raised from explants collected from the natural habitat and maintained in the field gene bank of JNTBGRI campus (Herbarium voucher specimen TBG 60637). Shoot cultures were maintained in the proliferation medium, i.e., MS medium (Murashige and Skoog, 1962) fortified with 2.0 mgl^{-1} 6-benzyl adenine (BA) (Sigma) and 0.5 mgl^{-1} α-naphthaleneacetic acid (NAA) (Sigma) (Preetha, 2012). Subculturing was done after 4-6 weeks in culture bottles each with 3 shoots to enhance the production of strong, robust shoots. The cultures were incubated at 26±2 °C under 12-hour photoperiod provided by cool white fluorescent tubes (50-60 $\mu molm^{-2}s^{-1}$) (Philips, India).

Cryopreservation

Preconditioning of STs on different sucrose concentrations at 25°C for ED

STs from the vigorously growing *in vitro* shoot cultures were dissected under a dissection microscope (Kyowa, Tokyo) inside the laminar air flow chamber. The dissected *STs* (3 mm) were cultured overnight at 26±2 °C in semisolid MS medium supplemented with 0.4 M sucrose and incubated in the dark.

Encapsulation of preconditioned STs

The sucrose-precultured *STs* were suspended in liquid MS medium free of calcium supplemented with 0.5 M sucrose and 2.5% (w/v) sodium alginate in 50 ml sterile beakers. This mixture holding the *STs* was dispensed in to liquid MS medium containing 10% (w/v) sucrose and 100 mM $CaCl_2$ (Merck) wherein they were left for 30 minutes to ensure polymerization.

Preculture of encapsulated STs at 4 °C

The beads containing *STs* were then precultured at 4 °C in liquid MS medium containing 0.3 or 0.5 M sucrose (*T1* and *T2*) for 3 and 5 days (*3d* and *5d*) (i.e., the different treatments were denoted as *T1(3d), T1(5d), T2(3d)* and *T2(5d)* respectively).

Dehydration of precultured STs

The precultured beads were dehydrated inside a laminar airflow chamber for 0-8 hrs at 2 hrs interval to determine the optimal dehydration duration. For each dehydration time, half of the beads were transferred to the recovery medium that served as the control and the other halves were placed in 1 ml cryotubes (10 beads to a vial), which were immediately plunged in to LN. The beads of another control group that had received no sucrose preculture or dehydration treatment were similarly dealt with (the absolute control); half being cooled and the other half left uncooled.

Determination of moisture content (MC)

Total fresh weight of 25 beads with three replicates was measured after sucrose preculture and during the dehydration period (0-8 hrs). After each period of dehydration, the beads were oven dried at 130 °C for 2 hrs to determine the dry weight. The MC was determined as:

$$\%MC = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100$$

Rewarming and recovery

Rewarming of the cryopreserved *STs* were performed by rapidly immersing the cryotubes in a water bath at 40 °C for 30 seconds. Encapsulated non-cryopreserved (-LN) and encapsulated cryopreserved (+LN) *STs* were rehydrated for one night in MS+0.3 M sucrose, then transferred to MS medium supplemented with 0.5 mgl^{-1} BA and 0.1 mgl^{-1} GA_3 for 4 weeks and subsequently to 1.0 mgl^{-1} BA and 0.5 mgl^{-1} NAA recovery medium (25 ml) in Petri dishes. The cultures were maintained at optimum culture conditions under dark for initial 10 days and then to standard conditions of illumination. Treated controls were processed in the same way except that they were not stored in LN.

Viability and plant growth

Stereomicroscopic (Nikon Eclipse) examination of the samples was performed regularly after plating on recovery medium. The *STs* were considered viable when they initiated growth and expanded. The survival levels were recorded based on the number of viable shoots out of the total number of thawed shoots. Shoot formation (recovery) was expressed as a percentage of the total number of shoots that proliferate normally after 4 weeks of transfer to recovery/ regrowth conditions.

The plants were maintained and multiplied under *in vitro* for up to two generations. The plantlets with 3-5 leaves and 2-3 roots weaned from the vessels were washed thoroughly in running tap water and transplanted in garden soil and river sand mixture (1:1) filled in polybags and hardened in a mist house under constant irrigation at 28±2 °C and 80±5 % RH (M/s Indo-American Exports Ltd.) for 1-2 weeks and then in a shade net house under 50% sunlight and regular watering for 3-4 weeks. After hardening, the plantlets were transferred to the field and monitored.

Experimental design and statistical analysis

Treatments were arranged in a completely randomized design (CRD). Each experiment consisted of ten meristems per treatment and was repeated thrice. Results were analysed by ANOVA and t-test. The means were separated according to the least significant difference (LSD) at $p \leq 0.05$.

Results

The shoot meristems dissected out from *in vitro* shoot cultures were preconditioned in MS medium supplemented with 0.4 M sucrose for one day and then used for cryopreservation experiments. A preliminary experiment conducted to see the effect of encapsulation without preculture on the regeneration of *STs* exhibited 95-100% survival rate and subsequent shoot regeneration after 3 days. Results on shoot tip cryopreservation in *K. galanga* through Encapsulation-dehydration indicated significant effects of three significant parameters such as (i) sucrose concentration during preculture, (ii) duration of preculture, (iii) duration of dehydration and their interaction.

Effect of sucrose preculture on recovery of encapsulated and cryopreserved STs

The effect of preculture in two different concentrations of sucrose (0.3 and 0.5 M) on shoot recovery after cryopreservation was studied. The best results in terms of shoot recovery (around 50%) were obtained by preculturing with 0.3 M sucrose for 3 days [*T1(3d)*] together with 4 hrs of dehydration (Table 1). Maximum 62.2% survival and 46.7% regeneration were observed in cryopreserved *STs* in T1(3d) (Table 1). However, preculture in 0.3 M sucrose for 5 days [*T1(5d)*] reduced the survival and regeneration frequencies (Table 2). Also, an increase in the concentration of sucrose in the preculture medium from 0.3 to 0.5 M sucrose significantly reduced the survival and regeneration of cryopreserved *STs* to around 20-30% and 10-20% correspondingly and it recorded maximum 25.6% survival and 16.7% regeneration frequencies (Tables 3 and 4).

Table 1. Recovery of *K. galanga STs* subjected to cryopreservation using encapsulation-dehydration after preculture of beads in MS+0.3 M sucrose for 3 days [T1(3d)]

Dehydration period (hrs)	% Moisture content	% Survival		% Regeneration	
		-LN	+LN	-LN	+LN
0	86.15	100.0±0.0^{a}	0.0±0.0	96.7±1.8^{a}	0.0±0.0
2	74.21	96.7±1.8^{a}	0.0±0.0	90.0±3.1^{a}	0.0±0.0
4	29.25	76.7±1.8^{c}	56.7±1.8^{a}	60.0±0.0^{b}	33.3±1.8^{a}
6	20.09	50.0±3.1^{d}	46.7±1.8^{c}	40.0±3.2^{c}	36.7±1.8^{b}
8	15.17	43.3±1.8^{e}	33.3±1.8^{d}	30.0±3.1^{d}	16.7±1.8^{c}

*Values are mean ± SE. Ten shoot tips were tested for each of three replications. Means followed by the same letter in the superscript in a column do not differ significantly based on ANOVA and LSD multiple 't' test at p≤0.05.

Table 2. Recovery of *K. galanga STs* subjected to cryopreservation using encapsulation-dehydration after preculture of beads in MS+0.3 M sucrose for 5 days [T1(5d)]

Dehydration period (hrs)	% Moisture content	% Survival		% Regeneration	
		-LN	+LN	-LN	+LN
0	85.14	80.0±3.1^{a}	0.0±0.0	63.3±1.82^{a}	0.0±0.0
2	75.17	73.3±1.8^{a}	0.0±0.0	60.0±3.16^{a}	0.0±0.0
4	51.61	66.7±1.8^{b}	36.7±1.8^{b}	60.0±0.0^{a}	30.0±3.1^{b}
6	20.00	56.7±1.8^{c}	43.3±1.8^{a}	43.3±1.82^{b}	26.7±1.8^{a}
8	17.15	36.7±1.8^{d}	33.3±1.8^{b}	30.0±0.00^{c}	20.0±3.2^{b}

*Values are mean ± SE. Ten shoot tips were tested for each of three replications. Means followed by the same letter in the superscript in a column do not differ significantly based on ANOVA and LSD multiple 't' test at p≤0.05.

Table 3. Recovery of *K. galanga STs* subjected to cryopreservation using encapsulation-dehydration after preculture of beads in MS+0.5 M sucrose for 3 days [T2(3d)]

Dehydration period (hrs)	% Moisture content	% Survival		% Regeneration	
		-LN	+LN	-LN	+LN
0	84.39	80.0±3.1[a]	0.0±0.0	66.7±3.7[a]	0.0±0.0
2	77.97	66.7±1.8[b]	0.0±0.0	46.7±1.8[b]	0.0±0.0
4	54.30	50.0±3.1[b]	33.3±1.8[a]	43.3±1.8[b]	26.7±3.6[a]
6	28.15	53.3±1.8[b]	33.3±1.8[a]	40.0±0.0[b]	16.7±3.6[a]
8	18.66	40.0±3.1[c]	26.7±1.8[b]	33.3±1.8[c]	10.0±3.1[b]

*Values are mean ± SE, ten shoot tips were tested for each of three replications. Means followed by the same letter in the superscript in a column do not differ significantly based on ANOVA and LSD multiple 't' test at p≤0.05.

Table 4. Recovery of *K. galanga STs* subjected to cryopreservation using encapsulation-dehydration after preculture of beads in MS + 0.5 M sucrose for 5 days [T2(5d)]

Dehydration period (hrs)	% Moisture content	% Survival		% Regeneration	
		-LN	+LN	-LN	+LN
0	77.22	76.7±1.8[a]	0.0±0.0	56.7±1.8[a]	0.0±0.0
2	57.54	63.3±1.8[b]	0.0±0.0	43.3±1.8[b]	0.0±0.0
4	21.62	50.0±3.1[c]	20.0±3.1[a]	36.7±3.6[c]	16.7±1.8[b]
6	16.43	46.7±3.6[d]	26.7±1.8[a]	36.7±3.6[c]	16.7±1.8[a]
8	12.65	43.3±1.8[d]	20.0±0.0[a]	30.0±0.0[c]	10.0±1.8[b]

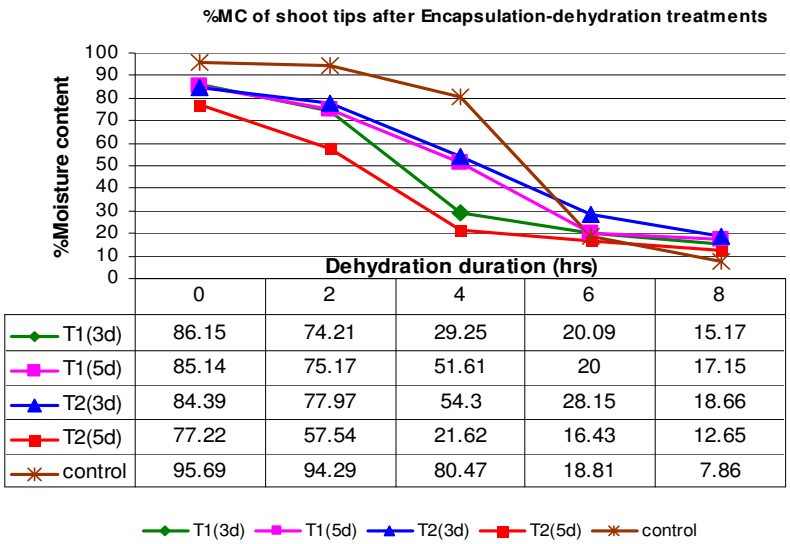
*Values are mean ± SE, ten shoot tips were tested for each of three replications. Means followed by the same letter in the superscript in a column do not differ significantly based on ANOVA and LSD multiple 't' test at p≤0.05.

Effect of preculture duration on recovery of encapsulated and cryopreserved STs

To study the effect of preculture period on shoot tip survival after cryopreservation, preculture with 0.3 and 0.5 M sucrose for 3 and 5 days followed by dehydration for 0-8 hrs was assessed. Period of preculture greatly influenced the survival and regeneration of both desiccated (control) and frozen (treated) shoot tips. Higher survival rates (60-70%) after cryopreservation were obtained in preculturing with 0.3 M sucrose for 3 days [*T1(3d)*] (Table 1). Preculture with 0.3 M sucrose for 5 days [*T1(5d)*] (Table 2) and 0.5 M sucrose for 3 and 5 days [*T2(3d)* and *T2(5d)* respectively] (Tables 3 and 4) notably reduced the survival of the *STs* to around 30% after LN exposure.

Effect of desiccation period on recovery of encapsulated and cryopreserved STs

The survival and regeneration percentage of cryopreserved *K. galanga STs* also depended on the duration of dehydration. Encapsulated *STs* precultured in culture medium with 0.3 and 0.5 M sucrose concentrations for 3 and 5 days and dehydrated afterwards at 0-8 hrs exhibited faster dehydration of the beads that have been precultured in lower concentrations of sucrose even for lesser dehydration duration. Longer dehydration period (6 hrs) was required to reduce the bead moisture content to optimum levels during preculture in higher concentrations of sucrose (0.5 M). However, devoid of dehydration, no survival was obtained after exposure to LN. Preculture of the encapsulated *STs* in high sucrose medium reduced the moisture content of the beads from 95.69% (in absolute control) to 86.15% during the initial 3 days preculture in 0.3 M sucrose [*T1(3d)*] (Figure 1) which continued to decrease during dehydration reaching 29.25%, 15.17% and 12.25% after 4, 6 and 8 hrs of dehydration respectively. Desiccation beyond 6 hrs resulted in drastic decline in the survival and regeneration of LN-exposed *STs* and desiccation controls in all the treatments. Highest regeneration percentages were recorded when the precultured encapsulated *STs* were dehydrated for 4-6 hrs in laminar airflow, resulting in a bead moisture content of 20-30%.



	0	2	4	6	8
T1(3d)	86.15	74.21	29.25	20.09	15.17
T1(5d)	85.14	75.17	51.61	20	17.15
T2(3d)	84.39	77.97	54.3	28.15	18.66
T2(5d)	77.22	57.54	21.62	16.43	12.65
control	95.69	94.29	80.47	18.81	7.86

Figure 1 Moisture content (%) of encapsulated *K. galanga* shoot tips subjected to 0-8 hrs dehydration

Effect of bead moisture content on recovery of encapsulated and cryopreserved STs

The regrowth of both non-cryopreserved (-LN) and cryopreserved (+LN) shoots was greatly influenced by the water content of the precultured beads. For shoot tips desiccated to different MC ranging from 80-20% (control), very low percentage of survival was observed after cryopreservation. While in the absolute control, where no cryopreservation step was used, 95-100% *STs* survived and regenerated. The initial water content of the control beads was high (95.69%), which reduced to 86.15% by 3 days of preculture on 0.3 M sucrose enriched medium, and to 15-20% by subsequent dehydration in the airflow chamber. Around 50% shoot recovery was obtained in this preculture treatment, together with 4-6 hours of dehydration, where moisture content was 29.25-20.09% (Figure 1). In *K. galanga*, highest regeneration percentage (46.7%) was noticed when the precultured encapsulated *STs* were dehydrated for 4 hrs, resulting in a bead moisture content of 29.25%. Similarly, bead moisture content decreased to 84.39% after 3-day preculture and to 77.22% after 5-day preculture in 0.5 M sucrose. Subsequent 6 hrs desiccation period further reduced the moisture content to 28.15% and 16.43% respectively, thereby effecting 26.67-33.33% survival rates after cryopreservation (Figure 1).

Shoot recovery

Shoot recovery after LN exposure in *K. galanga* was proceeded through apical meristem regrowth (Figure 2b) with subsequent formation of leaf primordia (Figure 2c) followed by leaf expansion (Figure 2d). Shoot emergence occurred directly from the apical dome without callus development. Meristems which did not recover became dark brown or remained white when observed after 3-4 weeks of transfer to recovery medium. The shoots transferred to multiplication medium (MS+0.5 mgl^{-1} NAA+1.0 mgl^{-1} BA), produced multiple shoots along with roots after 30 days (Figure 2e). The plantlets (5-8 cm) transferred to the field after short hardening phase were morphologically identical with the mother plants (Figure 2f).

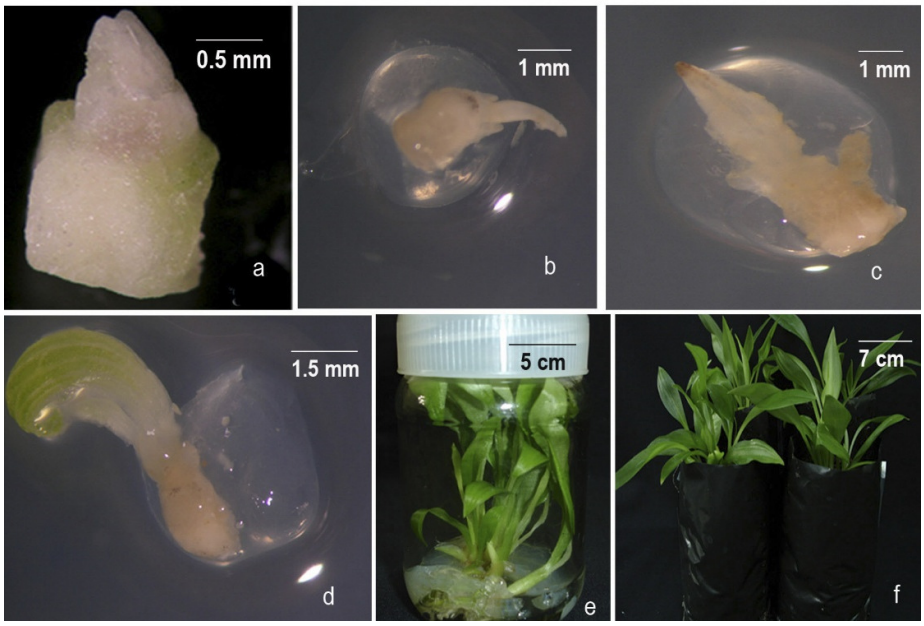


Figure 2. Shoot tip cryopreservation of *K. galanga* by encapsulation-dehydration
a Shoot tip (3 mm) selected for encapsulation; **b& c** Stereomicroscopic view of shoot apical growth by breaking the encapsulation matrix in LN-exposed shoot tips after 4 weeks of rewarming; **d** Initiation of shoot primordial growth after 8 weeks of rewarming; **e** Plantlet development in MS+0.5 mgl^{-1} NAA+2.0 mgl^{-1} BA; **f** Hardened LN-recovered plantlets ready for field transfer.

The schematic representation of the protocol developed for shoot tip cryopreservation of *K. galanga* using Encapsulation-dehydration is:

Shoot tips (~3 mm) with 1-2 leaf primordia dissected from 1 month old plants

↓

Overnight preculture with 0.4 M sucrose

↓

Encapsulation
(2.5% Sodium alginate in 0.5 M sucrose + 100 mM $CaCl_2$ in 10% sucrose)

↓

Preculture of beads in 0.3 M sucrose for 3 days

↓

Desiccation inside the laminar airflow (4 hrs)

↓

Rapid freezing in LN

↓

Thawing at 40 °C

↓

Culture on regrowth medium for recovery

i. MS + 0.3 M sucrose medium (overnight)
i. MS + 0.5 mgl^{-1} BA + 0.1 mgl^{-1} GA_3 (3 weeks)
ii. MS + 1.0 mgl^{-1} BA + 0.5 mgl^{-1} NAA (3-4 weeks)

↓

Plantlets

Discussion

Plant tissues have more than 70% water content in their cells and when these specimens were exposed to -196 °C, the ultra low temperature of LN, there is the chance of intra cellular ice crystal formation inside the cells, the greatest challenge in cryopreservation technique. These ice crystals formed during cooling in LN cause 'freezing injuries' such as rupture of cell membranes resulting in the loss of semi-permeability, cellular compartmentation and structural integrity leading to cell death (Oliveira *et al.* 2019). Hence the cellular water content needs to be optimized to prevent the intracellular crystallization, which can be achieved by inducing osmotic dehydration with the help of different cryoprotection treatments such as PVS2/ droplet vitrification, Encapsulation-dehydration, Encapsulation-vitrification, step-wise freezing, etc. Here, an efficient method was achieved using a procedure of encapsulation-dehydration for cryopreserving the shoot tip of *K. galanga*. Recently this method was suggested for the eradication of viruses from apple root stock (Bettoni *et al.*, 2018). The technique was developed initially by Dereuddre *et al.* (1990) for cryopreservation of shoot tips and somatic embryos is based on the technology for producing synthetic seeds (Redenbaugh *et al.*, 1986) and it combines two cryoprotective treatments, preculture with sucrose and air drying for inducing cytoplasmic vitrification; thereby avoiding the formation of intracellular ice crystals during subsequent rapid cooling in LN. In encapsulation-dehydration, the extraction of water from the shoot tips results from the progressive osmotic dehydration with the sucrose enriched medium followed by additional loss of water through evaporation from the beads (Fabre and Dereuddre, 1990). In the present study also, resistance to air dehydration and rapid cooling in LN was induced by overnight pretreatment of the excised shoot tips in MS medium supplemented with 0.4 M sucrose and subsequent preculture of encapsulated shoot tips in high sucrose enriched medium. Preconditioning of the explant materials in medium enriched with osmotic agents like sucrose, mannitol/ sorbitol has increased the efficiency of cryopreservation in many taxa (San Jose *et al.*, 2014; Zhang *et al.*, 2017) as well as in the cryopreservation approach by vitrification in *K. galanga* itself (Preetha *et al.*, 2013).

Experiments on cryopreservation by encapsulation-dehydration generally use sucrose as the cryoprotectant, which induces osmotic dehydration as well as contributing to the increased concentrations of sugar, starch and proline in the shoot tips, thus enhancing the stability of membranes under conditions of severe dehydration (Matsumoto and Sakai, 2003; Kaczmarczyk*et al.*, 2011). In *K. galanga*, the dissected *STs* were encapsulated using Sodium alginate solution having high sucrose concentration (0.5 M) which was dropped in to liquid culture medium containing high concentration of $CaCl_2$ (100 mM) and sucrose (10%). Accordingly, the sucrose is absorbed by the explants, thereby increasing the concentration of internal solutes.

Preculture of the encapsulated *STs* again in high sucrose medium resulted in the accumulation of sugars in the alginate bead and tissues (Gonzales Arnao and Engelmann, 2006) which retained the viability of the tissue during dehydration treatment. Also, it reduced the MC of the explants through an osmotic effect. In contrast, Panta *et al.* (2014) found that sucrose pretreatment had no positive effect on survival rate in encapsulation dehydration technique of potato shoot tips. After preculturing with 0.3 M sucrose for 3 days, 100% survival was obtained with encapsulated *K. galanga STs* that were not subjected to dehydration or LN freezing. Period of preculture greatly influenced the survival and regeneration of both -LN (control) and +LN (treated) *STs*. Very little of the LN-exposed shoot tips were survived and regenerated while the sucrose concentration as well as the pre-culture duration was increased. Better responses with respect to survival percentage and recovery rates were obtained by preculturing with 0.3 M sucrose for 3 days [*T1(3d)*] together with 4 hours of dehydration. Preculture for two more days in the same medium or high sucrose concentration (0.5 M) caused a considerable decrease in shoot recovery, which might be due to the toxicity created during exposure to high sucrose concentrations.

Dehydration of the precultured beads to optimum moisture levels is extremely important to achieve survival and regeneration of explants after cryopreservation as the desiccation step is removing a significant amount of crystallizable water from the shoot tissue facilitating their survival after cryopreservation. In *K. galanga*, no survival was observed when non-desiccated, precultured explants were frozen in LN. The survival

and regeneration frequency of LN-non-exposed shoot tips decreased with the increasing dehydration duration which completely agrees with the findings of Shatnawi (2011) in *Capparis spinosa*, where the shoot tips were exposed to similar treatments prior to cryopreservation. Samples need to withstand a moisture reduction to an optimum level to ensure no detrimental ice crystallization during LN treatment (Bachiri *et al.*, 2001). The optimal duration of dehydration in this case was 4-6 hours by which time the MC in beads was 20-30%. Bead MCs between 15-30% are required for optimal survival after cryopreservation (Engelmann 1997), which was also the case in *K. galanga*. Highest regeneration percentage (46.7%) was recorded when the precultured encapsulated *STs* were dehydrated for 4 hrs in laminar airflow, resulting in a bead moisture content of 29.25%. In agreement with this, the encapsulated beads of *Clitoria ternatea* dehydrated for 4 hrs had 60% survival after freezing in LN, of which 65% regrown after cryostorage (Nair *et al.*, 2019). The results indicate that preculture duration, concentration of sucrose in the medium and optimal MC in the beads are critical factors for cryopreserving *K. galanga STs*.

Since only small tissue areas endure cryopreservation, it is necessary to apply plant growth regulators (PGRs) in to the regeneration medium to stimulate cell division and growth. Combinations of cytokinins and GA_3 in the recovery medium appear to be more beneficial for plant growth and development after LN immersion in many species (Lee *et al.*, 2011; Preetha *et al.*, 2013) and here, shoot recovery was obtained in medium supplemented with 0.5 mgl^{-1} BA and 0.1 mgl^{-1} GA_3. Transfer of the shoots to medium supplemented with 1.0 mgl^{-1} BA and 0.5 mgl^{-1} NAA promoted the production of plantlets indicating that altering PGR levels in the recovery medium has improved the survival and thereby stimulated direct regeneration of whole plantlets by meristematic dome development as observed in *Dioscorea* (Mandal and Ahuja-Ghosh, 2007). The LN-recovered plantlets after the short hardening phase got established in the field at high rate (80%) and were free from morphological deformities.

Conclusions

The results projected here demonstrate the feasibility and utility of cryopreservation in long-term conservation approaches in *K. galanga.* The novel method of *K. galanga* shoot meristem cryopreservation by encapsulation-dehydration that recorded a much-improved recovery than the already established vitrification method holds promise for the long-term cryo-banking of this species. The morpho genetically uniform cryo-regenerants also revealed consistent utilization of this economically important plant genetic resource. Furthermore, this procedure can be considered as a footstep for seed production of the same or allied species of zingiberaceaeous group according to its demand.

Authors' Contributions

TSP has designed, conducted the experiments and wrote the manuscript, ASH helped in the analysis of the results and checking the draft and PNK provided overall supervision of the work. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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