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Shoot tip cryopreservation by vitrification in *Kaempferia galanga* L. An endangered, overexploited medicinal plant in Tropical Asia

T. S. Preetha[1], A. S. HemanthaKumar[2], P. N. Krishnan[3]
[2,3]*Biotechnology and Bioinformatics Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram - 695562, Kerala, India.*[1]*Corresponding author*
[1]*Department of Botany, University College, Thiruvananthapuram, Kerala, India*

Abstract: *Shoot tips from the established in vitro shoot cultures of Kaempferia galanga L., an endangered medicinal plant were successfully cryopreserved using the vitrification procedure. Effect of concentration of sucrose for preculture, vitrification treatment with PVS2(Plant vitrification solution 2) and recovery medium on cryopreservation of shoot tip meristems has been analyzed. Overnight preculturing of the dissected shoot tips in MS medium containing 0.4 M sucrose, osmoprotection with loading solution for 20 minutes, dehydration with PVS2 for 20 minutes at 0 ºC were found to be optimum among the various treatments tested. The cryoprotected shoot tips upon rapid freezing in LN (liquid nitrogen) followed by rapid thawing produced 50-60% survival and 30-40% regeneration rates. Incorporation of GA_3 (giberellic acid) in the post-thaw culture medium was essential to induce shoot emergence in the initial phase. Subculturing of the recovered shoot tips in MS medium supplemented with 1.0 mgl^{-1} BA (6-benzyl adenine) and 0.5 mgl^{-1} NAA (α-naphthaleneacetic acid) resulted plantlet production. The regenerated shoots grew to mature plants were free of any morphogenetic variation upon field transfer.*

Key words: *cryopreservation, endangered medicinal plant, long - term conservation, shoot tips, vitrification.*

I. Introduction

Kaempferia galanga L. (Family Zingiberaceae), an endangered medicinal species in Tropical Asia [1,2] is a clonally propagated plant currently cultivated mainly in South East Asia and China for their aromatic rhizomes which are widely used for wide range of medicinal applications [3]. Its rhizome contains a volatile oil, several alkaloids, starch, protein, aminoacid, minerals and fatty matter. Leaves and flowers contain flavanoids [4]. The methanol extract of *K. galanga* rhizome contains ethyl p-methoxy trans cinnamate, which is highly cytotoxic to *He La* cells [5]. Conventional propagation of the species is through rhizomes, which remains dormant during drought and sprouts only in spring. *In vitro* plant regeneration systems are well established in the species [6,7,8] but so far there is no report on *in vitro* conservation strategies. Germplasm of vegetatively propagated plants is usually maintained in field or as *in vitro* collections, which are time and labour consuming and the risk of losing the material is relatively high. A simple and efficient cryopreservation protocol would facilitate long-term storage and much wider utilization of plant germplasm [9]. As a protocol for cryopreservation is not available in *K. galanga*, the present study is relevant in terms of protocol development as also in other problematic species, where cryopreservation requires novel protective agents and procedures.

II. Materials and methods

Plant material

In vitro shoot cultures of *K. galanga* established from rhizome segments were maintained in MS medium [10] supplemented with 2.0 mgl^{-1} BA and 0.5 mgl^{-1} NAA (proliferation medium). Subculturing was done after 4-6 weeks in culture bottles each with 3-4 shoots to enhance the production of strong, robust shoots. The cultures were incubated under 12 hour photoperiod provided by cool white fluorescent tubes (50-60 $\mu molm^{-2}s^{-1}$) at 26±2 ºC. These conditions were maintained in all experiments unless otherwise stated.

Vitrification of precultured shoot tips

Shoot tips (3 mm) (Fig. 2a) from the established shoot cultures were dissected under a dissection microscope (Kyowa, Tokyo) inside the laminar air flow chamber. The dissected shoot tips were precultured overnight in the dark in MS medium supplemented with 0.3, 0.4, 0.5 or 0.75 M sucrose. They were subjected to treatment with Loading solution (2 M glycerol + 0.4 M sucrose) for 20 minutes and subsequently dehydrated with 1 ml PVS2 (30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) Dimethyl sulfoxide) [11] at 0 ºC for 1 hour at 10 minutes interval. The properly dehydrated shoot tips were plunged in to LN for at least 1 hour storage. LN-non-exposed ones were kept as control and processed in the same way as LN-exposed samples.

Rewarming and recovery of shoot tips

After LN exposure, rewarming was performed by rapidly immersing the cryotubes in a water bath at 40 °C for 30 seconds. PVS2 was replaced with washing solution (MS+1.2 M sucrose) twice at 10 minutes interval, blotted on filter paper layer on MS basal medium for one day and then transferred to recovery medium (MS medium supplemented with 0.5 mgl^{-1} BA and 0.1 mgl^{-1} GA_3; or 1.0 mgl^{-1} BA and 0.5 mgl^{-1} NAA; or 1.0 mgl^{-1} BA along with 0.5 mgl^{-1} NAA and 0.1 mgl^{-1} GA_3). After 7-10 days incubation in the dark, the apices were transferred to standard conditions of illumination. The shoot tips with emerging shoots were transferred after 4-6 weeks to medium supplemented with 1.0 mgl^{-1} BA and 0.5 mgl^{-1} NAA. Observation on survival and regeneration was made after 4 weeks.

Statistical analysis

Ten meristems were used in each experiment with three replicates. Results were analyzed by ANOVA following arc sine transformation with mean separation analysis by LSD multiple't' test.

Genetic stability analysis

Leaf samples from plants regenerated after cryopreservation of shoot apical meristems were selected for the RAPD analysis. Genomic DNA was isolated using modified CTAB method [12]. RAPD assay was carried out in 25 µl reaction mixture containing 2.5 µl 10x amplification buffer (10 mM Tris HCl pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl and 0.01% gelatin from Finnzymes), 0.5 µl dNTP mixture (100 µl each of dATP, dGTP, dCTP and dTTP), 0.5 µl Taq DNA polymerase (Finnzymes), 1.2 µl (15 pM) decamer primer (Operon Technologies Inc, USA) and 50 ng genomic DNA. Amplification was performed in Eppendorf Mastercycler (Hamburg, Germany) at an initial temperature of 94 °C for 5 minutes for complete denaturation. The second step consisted of 42 cycles having three stages of temperature: 92 °C for 1 minute for denaturation of template DNA, 37 °C for 1 minute for primer annealing and 72 °C for 2 minutes for primer extension followed by running the samples at 72 °C for 7 minutes for complete polymerization. The amplified products were resolved by 0.8% agarose gel electrophoresis and stained with ethidium bromide. The gel documentation and analysis system (Alpha Imager™ 2000, Alpha Innotech Corporation, USA) was used to score the bands representing the RAPD markers.

III. Results and Discussion

Effect of overnight preculture with sucrose

Preculturing of shoot tips with different concentrations of sucrose prior to loading treatment has been reported to be effective in improving post-LN survival rates of plant species [13]. The excised *K. galanga* shoot tips precultured overnight in MS medium supplemented with 0.3, 0.4, 0.5 and 0.75 M sucrose produced 66.67% survival in 0.4 M sucrose which was found to be optimum among the various concentrations tested (TABLE 1). Thus, a suitable sucrose pretreatment contributed to improving tolerance to vitrification in agreement with the results reported for several tropical monocots such as banana, taro, orchids and pineapple [14].

Effect of different exposure time to PVS2 on cryopreservation

K. galanga shoot tips exposed to PVS2 at 0 ºC for up to 20 minutes were able to resume 36.67% shoot growth after LN exposure (Fig.1), while those subjected to PVS2 treatment at 25 ºC were very sensitive and very low percentage survival (10-20%) was observed after 10 minutes exposure (data not shown). Application of vitrification solutions at lower temperature (0 ºC) reduced their toxicity to the explants [15]. The process of cell plasmolysis induced by PVS2 dehydration may progress with lower speed at 0 ºC than at 25 ºC and the chemical toxicity of the PVS2 solution may also be reduced. In *K. galanga*, there was a drastic drop in post-LN recovery after 30 minutes PVS2 exposure at 0 ºC and the regrowth rate declined after 40 minutes exposure, which confirms the increased sensitivity of *K. galanga* shoot tips to PVS2 even at 0 ºC. Similarly shoot tips of sweet potato and banana have been reported to be very sensitive to PVS2 treatment, where exposure to PVS2 for 5 minutes at 25 ºC significantly reduced the survival rate of shoot tips [16,17]. In contrast, some monocots like taro and orchids (*Cymbidium*) are more tolerant and 64.5-70% shoot tips resumed growth even after 40 minutes of PVS2 exposure [14,18]. The sensitivity of *K. galanga* shoot tips to PVS2 makes the application of vitrification procedure difficult to achieve enough dehydration before LN exposure. This substantiates the need for additional pretreatments to be investigated to minimize the toxic effects of this solution.

A delay in PVS2 toxicity between controls and cryopreserved samples was also noticed. The PVS2 controls depicted toxicity after 50 minutes exposure, while the cryopreserved samples became sensitive after 30 minutes exposure. Such delay in PVS2 toxicity between controls and cryopreserved samples was also observed in cryopreserved shoot tips of wasabi, apple and taro [13,14], where controls had 100% recovery for up to 20 minutes PVS2 exposure; where as cryopreserved samples had a significant drop after 10 minutes. It is assumed that the viability of the controls depends solely on toxicity of PVS2 while that of frozen samples depends on both toxicity and crystallization. Successfully recovered shoot tips remained green and resumed growth within

2-3 weeks. The shoot growth was evident by axillary meristem development and apical meristem regrowth (Fig.2b-d). The shoots upon transfer to multiplication medium induced the emergence of shoots directly from the apical dome without callus formation (Fig.2e). Rooting occurred in the same medium and plantlets (5-8 cm height) were obtained after 30 days. The plantlets transferred to the soil after a short hardening period were free from morphological variations (Fig.2f).

Effect of recovery medium

Since only small tissue areas survive cryopreservation, it is necessary to apply phytohormones in to the regeneration medium to stimulate cell division and growth. MS basal medium did not show regeneration of post-thaw shoot tips indicating the requirement of plant growth regulators for regrowth. Medium supplemented with 0.5 mgl^{-1} BA and 0.1 mgl^{-1} GA_3 as well as 1 mgl^{-1} BA, 0.5 mgl^{-1} NAA and 0.1 mgl^{-1} GA_3 produced significantly higher levels of regeneration in comparison to medium supplemented with BA-NAA combination (TABLE 2). Combinations of cytokinins and GA_3 in the recovery medium appear to be more beneficial for plant growth and development after LN immersion. Cytokinins are a vital component of recovery media and they stimulate cell division and control morphogenesis. Following cryogenic treatments, it is necessary to induce active cell growth to produce organized tissues rather than undifferentiated callus. GA_3 inhibits callus formation under some conditions. Subculturing of the regenerating shoots in medium supplemented with 1.0 mgl^{-1} BA and 0.5 mgl^{-1} NAA was necessary for plantlet production, substantiating the fact that the plantlets after recovery needed to be returned to their normal stock culture medium. Thus the study indicates the importance of altering growth regulator levels in the culture medium in different stages of shoot recovery to improve survival thereby allowing direct regeneration of plantlets as in *Dioscorea* [19].

On the basis of the above investigations, the optimized protocol for the cryopreservation of shoot tips of *K. galanga* can be summarized as follows: (1) choice of shoot tips with 1-2 leaf primordia from 1 month old *in vitro* plants; (2) Overnight preculture with 0.4 M sucrose; (3) loading treatment for 20 minutes; (4) dehydration with PVS2 for 20 minutes at 0 ºC; (5) Rapid freezing in LN; (6) Rapid thawing at 40 ºC; (7) Unloading in washing solution; (8) Culture on MS + BA (0.5 mgl^{-1})+ GA_3 (0.1 mgl^{-1}) for recovery; (9) Transfer to MS + BA (1.0 mgl^{-1})+ NAA (0.5 mgl^{-1}) for plantlet production.

Genetic uniformity analysis by RAPD

Assessment of true-to-type regenerants from cryopreserved materials is important while using cryogenic protocol for the long-term conservation. Significant differences in morphological or DNA based markers have not been found in most studies [20]. In the present study, randomly selected cryopreserved samples fingerprinted using 10 RAPD primers revealed a monomorphic banding pattern (Fig.3). Similarly stability in the RAPD patterns was found in the potato plants derived from meristems cryopreserved by encapsulation/ vitrification [21]. These results further confirm that long-term conservation through cryopreservation of shoot apical meristems using vitrification is feasible for conservation and sustainable utilization of *K. galanga.*

IV. Table

Table 1. Effect of sucrose preculture on the regeneration of *K. galanga* shoot tips subjected to cryopreservation using vitrification

Sucrose concentration (M)	% survival*	% regeneration*
0.0	43.33±1.82^{b}	13.33±1.82^{b}
0.3	46.67±1.82^{b}	33.33±1.82^{b}
0.4	66.67±1.82^{a}	36.67±1.82^{a}
0.5	13.33±1.82^{c}	23.33±1.82^{c}
0.75	0.00±0.00	0.00±0.00

*Mean ± SE, Values followed by the same letter in the superscript do not differ significantly based on ANOVA and t-test at $p \leq 0.05$

Table 2. Effect of plant growth regulators in recovery medium on *K. galanga* shoot tip cryopreservation using vitrification

PGR concentration (mgl^{-1})			% survival *	% regeneration*
BA	**NAA**	**GA_3**	**(Mean±SE)**	**(Mean±SE)**
0.0	0.0	0.0	46.67 ± 1.82^{c}	6.67 ± 1.82^{c}
0.5	-	0.1	66.67 ± 1.82^{a}	46.67 ± 1.82^{b}
1.0	0.5	-	50.00 ± 3.16^{b}	43.33 ± 1.82^{b}
1.0	0.5	0.1	56.67 ± 1.82^{b}	50.00 ± 3.16^{a}

*Mean ± SE, Ten meristems were tested for each of three replications. Observations were made after 4 weeks. Values followed by the same letter in the superscript do not differ significantly based on ANOVA and t-test at $p \leq 0.05$

V. Figures

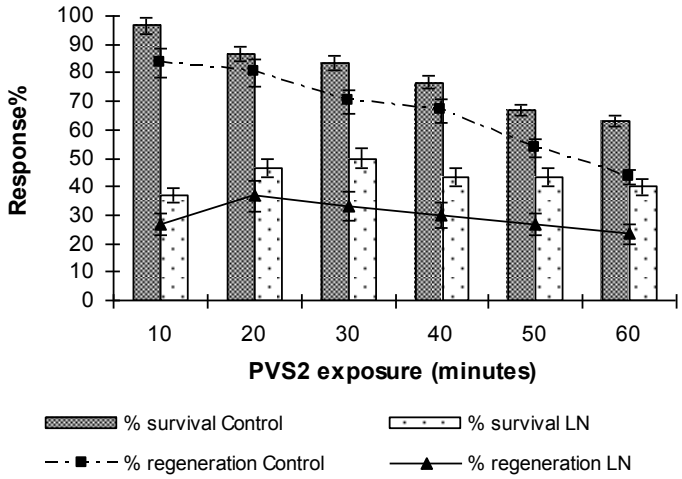

Fig. 1: Recovery of *K. galanga* shoot tips subjected to cryopreservation using PVS2 vitrification. Note: Ten meristems were tested for each of three replications. Observations were made after 4 weeks.

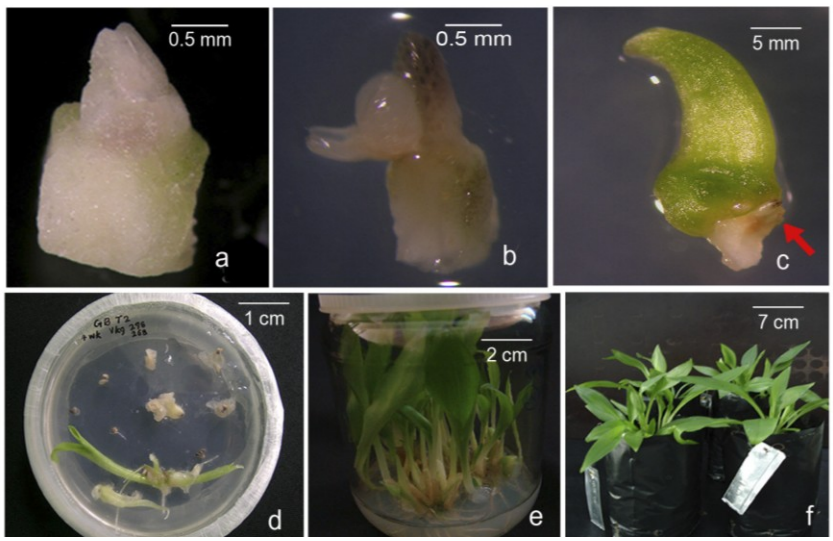

Fig. 2: Shoot recovery after cryopreservation. a) Shoot tip explant (3 mm). b,c&d) Shoot regeneration after 3 weeks of LN exposure in MS+ 0.1 mgl^{-1} GA_3 + 0.5 mgl^{-1} BA; (a,b&c Stereomicroscopic view). e) Plantlet production in MS + 0.5 mgl^{-1} NAA + 2.0 mgl^{-1} BA. f) LN-recovered plantlets.

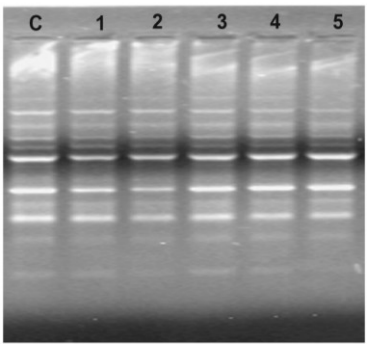

Fig. 3: RAPD banding pattern of LN-recovered plants (Lane 1-5) (Lane C- control)

VI. Conclusion

The efficient vitrification procedure demonstrated here with reasonable recovery rates for cryopreservation of *K. galanga*, an endangered medicinal plant in the tropical region is the first report of the successful cryopreservation of shoot tips and it holds promise for the long-term conservation and sustainable utilization of this highly sought, over exploited medicinal species.

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