

A high frequency microcloning protocol for subsequent cryopreservation in *Kaempferia galanga* L.: An endangered, over exploited medicinal plant in pharmaceuticals

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Abstract: An efficient *in vitro* propagation protocol was standardized in *K. galanga*, wherein shoot cultures were raised from rhizome with axillary bud explants in Murashige and Skoog (MS) medium supplemented with different hormonal regimes. Maximum 10.6 ± 0.83 multiple shoots per explants were obtained in MS medium supplemented with 4.0 mg l^{-1} 6-benzyl adenine (BA) along with 1.0 mg l^{-1} each of α -naphthaleneacetic acid (NAA) and kinetin. Subsequent 2-3 subculture passages enhanced the shoot multiplication rate to 3-4 times. The multiple shoots thus produced were having individual root system and elongated substantially when kept in the same medium for 4-6 weeks. There was no need of elongation and root induction phase and this two-step multiplication procedure reduced the period of plantlet production compared to the earlier protocols. The regenerated plants after a short hardening phase got established in the field at 80-90% efficiency and their genetic uniformity was confirmed through ISSR analysis. The protocol thus standardized is an efficient method for rapid propagation of this high value medicinal species by which at least 30 shoots per explants can be produced within 12 weeks duration. It offers the possibility of raising physiologically uniform plants for easy dissection of shoot tip meristems which can be efficiently utilized for subsequent long-term conservation through cryopreservation.

Key words: endangered medicinal plant, *in vitro* clonal propagation, *Kaempferia galanga*

I. INTRODUCTION

Kaempferia galanga (Family Zingiberaceae) is an endangered medicinal plant [1] possessing several therapeutic applications [2,3]. There is no seed formation naturally and conventional propagation is *via* rhizomes which is dormant during drought and sprouting in spring. Always there is scarcity of propagules as they constitute the commercial product of medicinal importance. Due to the demand for the drugs obtained from rhizomes, the plants are over exploited, thereby threatening its survival. To meet the market demand and considering the propagation problem, developing feasible protocols for rapid multiplication and effective conservation are essential. *In vitro* preservation by cryopreservation ensures the long-term maintenance of plant germplasm for which a reliable micropropagation system is necessary to obtain as many physiologically uniform *in vitro* grown plantlets. Though *in vitro* propagation of *K. galanga* has been reported by several authors [4,5,6,7,8,9,10,11,12] such established procedures does not reveal the production of a large number of relatively homogenous shoot tips of uniform size, physiological state and growth response. The present study provides a high frequency *in vitro* propagation system with the production of genetically uniform plants as evident by ISSR banding pattern which can be subsequently utilized for cryopreservation of shoot tips thereby increasing the chances of positive and uniform response to subsequent cryogenic treatments.

II. MATERIALS AND METHODS

2.1. Plant material

Kaempferia galanga plants (Herbarium voucher: TBG 60637) collected from the Kulathupuzha forest range under Kerala Forest Department and maintained at the field gene bank of JNTBGRI served as the plant material for the present study.

2.2. Culture establishment

Rhizomes with axillary buds collected from the field-grown plants were thoroughly washed under running tap water, outer scales were removed using scalpel and washed in 3-4 drops of Teepol, dissolved in 100 ml distilled water for 20 minutes followed by washing in running tap water. After several rinses in distilled water, the explants were subjected to sterilization with 0.1 % (w/v) HgCl_2 for 8-10 minutes followed by 4-5 rinses in sterile distilled water. Axillary buds along with basal rhizome part (1 cm) excised aseptically were

implanted on MS medium [13] containing 0.6% (w/v) agar, 3% (w/v) sucrose and supplemented with 0.5 mg^l⁻¹ BA. The cultures were incubated for 4-6 weeks at 25±2 °C and 12 hour photoperiod with a light intensity of 50-60 µEm⁻²s⁻¹ provided by cool, white fluorescent tubes (Philips India, Mumbai) under 50-60% RH.

2.3. Shoot multiplication, elongation and rooting

The initiated shoot buds were subcultured individually after 4 weeks to fresh medium augmented with 1.0, 2.0, 3.0 and 4.0 mg^l⁻¹ BA or kinetin either individually or 2.0, 3.0, 4.0 mg^l⁻¹ BA in combination with 1.0 mg^l⁻¹ kinetin or 0.5 and 1.0 mg^l⁻¹ NAA or IAA to enhance the multiplication, elongation and rooting. Scaling up of shoot multiplication was done by repeated subculture of the shoot clumps after removing the outer scales surrounding the individual shoots in selected hormonal combinations. Observations were recorded at regular intervals. Data were statistically analyzed based on ANOVA and their significance was assessed using Duncan's Multiple Range test at p≤ 0.05 using SPSS software.

2.4. Hardening and field transfer

The plantlets weaned away from the culture vessels were washed thoroughly in running tap water to remove traces of agar and treated with 0.1 % Dithane M-45 for 5 minutes to avoid fungal infection. They were transplanted in polybags filled with potting medium of garden soil and river sand mixture (1:3) 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. The hardened plants were then transferred to a shade net house under 50% sunlight and regular watering for 3-4 weeks. Such established plantlets were transferred to the field during premonsoon and monsoon rains to facilitate their establishment.

2.5. Genetic uniformity analysis using ISSR markers

Genomic DNA from leaf samples of regenerated plants were isolated using modified CTAB method [14]. ISSR assay was carried out in 25 µL reaction mixture containing 0.2 mM dNTP's, 10 mM Tris-HCL, 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 1.0 U Taq DNA polymerase (Finzymes, Helsinki, Finland), 15 pmol primers (IDT, Coralville, USA) and 50 ng of genomic DNA. The amplification was performed in a thermal cycler (Eppendorf ESP-S). After the initial cycle of 2 min at 93°C, 2 min at 50 to 55°C (annealing temperature of the primers ranges from 50 to 55°C for the different primers used in this study) and 2 min at 72°C. A total of 39 cycles of 1 min at 93°C, 1 min at 50 to 55°C and 1 min at 72°C were performed. The last cycle was performed by 10 min extension at 72°C. Reaction mixture wherein template DNA replaced by distilled water was used as negative control. Amplified products were resolved in 1.4% agarose gel (1xTBE) followed by ethidium bromide staining. Amplification with each arbitrary primer was repeated 3 times and those primers that produced reproducible and consistent bands were selected for data generation.

III. RESULTS

3.1. Culture establishment

Maximum of 76.67% infection free cultures were obtained from the rhizome explants which were subjected to surface decontamination as described. Seasonal variation influenced the percentage of response. Explants collected during rainy season (June-July) showed maximum contamination leading to a loss of up to 70% and those collected during summer season (January-April) showed minimum of contamination and loss (30%) of explants.

Rhizomes with axillary buds of *K. galanga* inoculated on MS medium supplemented with 0.5 mg^l⁻¹ BA induced direct shoot proliferation, while those transferred to medium devoid of growth regulators exhibited elongation of existing primordium in limited number of explants. The explant responded after 15 days of culture by breaking the outer thick sheath followed by emergence of shoot primordium (Fig.1a). The explants produced 1-3 shoots within 30 days. All the shoots developed were healthy with well developed leaves and one or two roots.

3.2. Shoot multiplication

3.2.1. Effect of cytokinins on shoot multiplication

Single shoots subcultured on medium supplemented with different concentrations and combinations of BA or kinetin either alone or in combination, or in combination with auxins exhibited differential response of multiple shoot bud induction in 60-96.67% of the cultures. The multiplication rate was increased as the concentration of cytokinins increased in the medium. After 30 days of subculture, MS medium supplemented with 2.0, 3.0 and 4.0 mg^l⁻¹ BA produced an average of 4.89±0.74, 5.00±0.38 and 5.20±0.42 shoots respectively (TABLE 1). Medium supplemented with 1.0-3.0 mg^l⁻¹ kinetin produced only 1-2 shoots within 30 days of

culture while an average of 2.71 ± 0.28 shoots were obtained in 4.0 mg l^{-1} kinetin. No significant change in shoot length was observed as the concentration of BA or kinetin increased.

3.2.2. Combined effect of cytokinins on shoot multiplication

Combination of BA and kinetin did not affect multiple shoot bud induction as that of BA alone, but there was significant increase than with kinetin alone (TABLE 1). A linear increase in the production of multiple shoots was noticed with increase in BA concentration in combination with kinetin. Combination of 1.0 mg l^{-1} kinetin and 2.0, 3.0 and 4.0 mg l^{-1} BA produced a mean number of 2.83 ± 0.22 , 3.14 ± 0.26 and 5.75 ± 0.38 shoots respectively (TABLE 1).

3.2.3. Effect of cytokinins in combination with auxins on shoot multiplication

BA in combination with auxins viz. NAA and IAA further increased the shoot multiplication rates and the mean number of shoots varied from 3.8 ± 0.29 to 7.4 ± 0.65 (Fig. 1b) (TABLE 1). BA at a concentration of 2.0 and 3.0 mg l^{-1} in combination with 0.5 mg l^{-1} NAA/ IAA promoted shoot multiplication more or less equally. A significant increase in the number of multiple shoot production was noted when 4.0 mg l^{-1} BA was used in combination with 1.0 mg l^{-1} NAA (7.40 ± 0.65 shoots) in comparison with the addition of 1.0 mg l^{-1} IAA (6.00 ± 0.35 shoots). All the shoots developed were healthy with well developed green leaves. A positive impact on multiple shoot induction was noted when BA, kinetin and NAA were used together. MS medium supplemented with 4.0 mg l^{-1} BA along with 1.0 mg l^{-1} each of kinetin and NAA induced the best shoot multiplication (10.60 ± 0.83 shoots) as well as the highest number of roots per shoot (Fig.1c) (TABLE 1). When the concentration of each hormones was reduced to half (2.0 mg l^{-1} BA along with 0.5 mg l^{-1} each of kinetin/ NAA/ IAA) in the multihormonal combination tested, the rate of multiple shoot production was also reduced to half.

3.3. Scaling up of shoot multiplication

In *K. galanga*, scaling up of shoot multiplication was achieved through repeated subculture of the shoot clumps in those hormonal combinations which exerted the production of more than 5 shoots during the first subculture period. Repeated subculturing for 1-2 cycles each of 4-5 weeks duration induced the production of an increased number of multiple shoots. The shoot cultures responded with differentiation of buds from basal part (resident meristem of the shoots) within 1-2 weeks. Relatively higher rate of multiplication was recorded in the second passage compared to the first (TABLE 2). An average three-fold increase in the number of shoots was observed during two subculture passages in almost all the growth hormone combinations tested. The shoot clumps with maximum multiplication (10.60 ± 0.83 shoots) obtained during first subculture in medium amended with 4.0 mg l^{-1} BA and 1.0 mg l^{-1} each of kinetin and NAA produced 30.2 ± 0.76 shoots after 60 days of culture during the second subculture passage in the same media formulation (Fig. 1d) (TABLE 2). In this combination, beyond two subculture passages (60 days), each subculture passage extended up to 4 weeks, a total of 30.2 shoots were harvested. Therefore, this hormonal combination was selected as the best for multiplication of shoot buds in *K. galanga*. Thus it is possible to produce 30 shoots per explant starting from a single rhizome segment within 60 days of inoculation.

During the third subculture, almost same rate of multiplication was observed after 90 days of culture (TABLE 2). When the isolated shoots obtained in each subculture passage were frequently transferred to the same media for a long period, shoot multiplication rates become reduced.

3.4. Shoot elongation and rooting

Plantlets developed in the multiplication medium elongated substantially within 40 days when left in the same medium without subculture during the first subculture period itself and hence there was no need of including the shoot elongation step. Moreover, root development was parallel with shoot multiplication in all the combinations tested. Small roots were initiated from the basal part of the regenerated shoots after 15-20 days of culture. The roots were green, thick and the number varied from 4-8. This is an advantage over only shoot formation, where additional treatments are required for root induction.

The concentration of NAA and IAA affected the number of roots developing on subcultured plantlets in *K. galanga*. The mean number of roots produced per shoot slightly increased as the concentration of NAA and IAA increased in the medium. MS medium supplemented with 4.0 mg l^{-1} BA along with 0.5 mg l^{-1} NAA produced 3.5 roots per shoot which was increased to 4.8 up on enhancement of the concentration of NAA to 1.0 mg l^{-1} . Increase in the rate of root induction was also observed in MS medium supplemented with 4.0 mg l^{-1} BA along with 0.5 mg l^{-1} IAA, where 4.6 roots per shoot were produced; while supplementation of 4.0 mg l^{-1} BA along with 1.0 mg l^{-1} IAA induced the formation of maximum 5.2 roots per shoot (TABLE 1). Root induction was also observed even in MS medium supplemented with cytokinins alone.

3.5. Hardening and field transfer

The plantlets were successfully acclimatized in the mist house for a period of 1-2 weeks and then to a shade net house for 3-4 weeks before transplanting into selected areas inside the JNTBGRI campus. Plantlets with 7-8 cm height showed better establishment after hardening. The regenerated plantlets transplanted in polybags showed 90-95% survival (Fig.1e) and got established in the field at 80-90% rate. They grew to mature plants after 3-4 months of transfer, were free of any morphological variations and showed normal growth as field grown plants in their yield, rhizome formation, etc. They were genetically uniform in ISSR analysis (Fig. 1f). Based on the results obtained, a schematic representation for *in vitro clonal* propagation of *K. galanga* is presented in Fig.2.

IV. DISCUSSION

The rhizome explants were subjected to vigorous decontamination due to the underground, soiled nature of the explants. Underground plant parts harbor different microorganisms and in such cases, multistep disinfection process [15] is usually followed *viz.* initial wash with a detergent, followed by immersion in commercial bleach, sodium or calcium hypochlorite and thereafter in 0.1% (w/v) HgCl₂ for 5-10 minutes depending on the nature of the tissue. The surface decontamination initially with teepol followed by aseptic treatment with 0.1% HgCl₂ for 8-10 minutes offered a reasonable decontamination protocol in *K. galanga*. For successful decontamination, the explants had to be collected during summer months (January-March). The infection-free axillary buds together with the basal rhizomatous part cultured on MS basal medium responded with the elongation of the existing shoot tip with no additional proliferation of shoot buds. Exogenous supply of cytokinins was essential to keep the apical bud alive and active.

In vitro-derived single shoots subcultured on medium supplemented with different concentrations and combinations of growth regulators exhibited multiple shoot bud induction. BA played a significant role in multiple shoot formation and gave better results compared to kinetin. MS medium supplemented with 4.0 mg l⁻¹ BA produced an average of 5.20±0.42 shoots, where as 2.71±0.28 shoots were obtained in 4.0 mg l⁻¹ kinetin. The high performance of BA over other cytokinins for stimulation and proliferation of lateral bud growth was reported earlier in *K. galanga* [7]. The effectiveness of BA on clonal multiplication of medicinal plants of Zingiberaceae has been reported in *Curcuma* and *Zingiber* [16], *Alpinia calcarata* [17] and *A. galanga* [18]. However, an increase in the production of multiple shoots was noticed when kinetin was used in combination with BA and a mean number of 2.83±0.22, 3.14±0.26 and 5.75±0.38 shoots were obtained in a combination of 1.0 mg l⁻¹ kinetin along with 2.0, 3.0 and 4.0 mg l⁻¹ BA. Thus combination of the two cytokinins increased multiple shoot bud induction and this is in agreement with earlier findings [19]. Such a synergistic effect has been reported by many workers in different plant species [20,21].

BA in combination with auxins further increased the shoot multiplication rates and the number of shoots varied from 3.80±0.29 to 7.40±0.65 in MS medium supplemented with 2.0 mg l⁻¹ BA in combination with 1.0 mg l⁻¹ IAA and NAA respectively. Shirin *et al.* (2000) [7] showed that BA (2.7 mg l⁻¹) and NAA (0.5 mg l⁻¹) was effective for the multiplication of *K. galanga*. The highest frequency of multiple shoot induction required a specific ratio of auxin and cytokinin which varied with species and physiological status of the explants as evident from earlier works on *Curcuma zedaoria*, *K. galanga* and *K. rotunda* [9,19,22,23]. Strong synergistic effect of BA-NAA interactions on enhanced shoot production is also reported in banana also [24].

Scaling up of shoot multiplication was achieved through at least two subculture passages of 4 to 6 weeks each in media supplemented with 2.0 and 3.0 mg l⁻¹ BA or 2.0-4.0 mg l⁻¹ BA along with 1.0 mg l⁻¹ NAA/IAA. Maximum multiple shoots (10.60±0.83 shoots) obtained in MS medium supplemented with 4.0 mg l⁻¹ BA along with 1.0 mg l⁻¹ each of kinetin and NAA after the first subculture period (30 days) increased to three times (30.2±0.76 shoots) after 90 days of culture in fresh medium of the same combination indicating the additive effect of this multihormonal combination in effecting multiple shoot induction. Once aseptic shoots are induced, the successive multiplication rate gets maximized under *in vitro* conditions in most of the Zingibers [25]. The plantlets were sufficiently elongated when left in the same medium during the first subculture period.

In *K. galanga*, irrespective of the growth regulator treatment healthy roots were induced with the multiplication of shoots simultaneously. Hence there is no need of including separate root induction step, which is an advantage in micropropagation of this species. Root induction in shoot inducing medium was reported in other Zingibers. This may be due to intrinsic root inducing factors in the rhizome; and such a phenomenon has been reported in rhizomatous plants [5]. Root induction was observed even in MS medium supplemented with cytokinins alone which may be due to the increased endogenous auxin levels in the plant. There was no need of media alternation for shoot elongation and root induction which enables easy propagation of the crop within a short duration of time. Rooted plants hardened in the mist house for initial 4-6 weeks got established at

significant frequencies (90-95%) in pure sand and garden soil medium under irrigated conditions indicating the feasibility of obtaining reasonable frequencies of establishment of *K. galanga* under less sophisticated conditions.

The *in vitro* clonal plants established in the mist house when transferred to open field showed conspicuous growth and emergence of new leaves. The introduction of the plants during the premonsoon showers in late May or monsoon rains in early June facilitated easy establishment. The plantlets transferred to selected areas inside JNTBGRI campus were free from morphological and growth abnormalities with high establishment rate (80-90%) and showed uniform growth. This confirmed the utility of the methods for conservation and revegetation purposes. The genetic stability assessed using ISSR markers established the genetic fidelity of the regenerants.

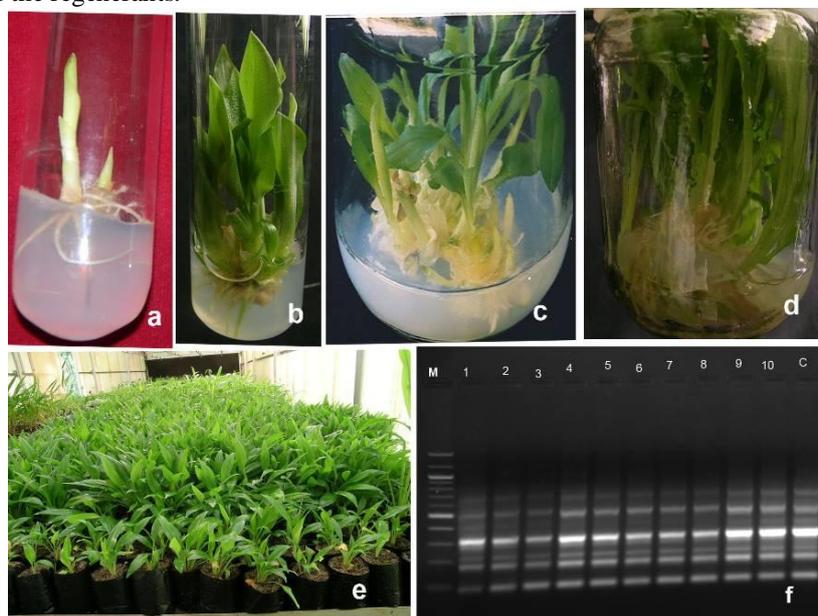
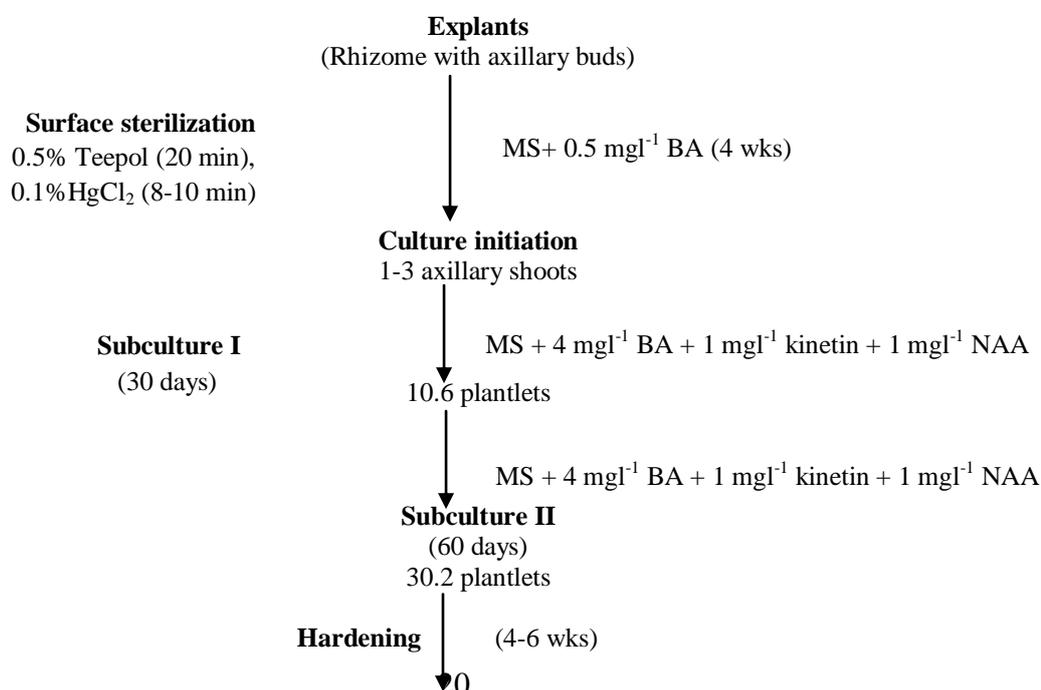


Figure 1: Microcloning of *K. galanga* L. a) Shoot bud initiation in MS+0.5 mg l⁻¹ BA; b) Multiple shoots in MS+0.5 mg l⁻¹ NAA+2.0 mg l⁻¹ BA; c) Shoot multiplication in MS+4.0 mg l⁻¹ BA+1.0 mg l⁻¹ each of kinetin and NAA; d) Multiple shoots in MS+4.0 mg l⁻¹ BA+1.0 mg l⁻¹ each of kinetin and NAA after 2 subculture passages; e) Hardening of the plantlets inside the mist chamber; f) Monomorphic bands in ISSR analysis (M: Marker, 1-10 samples, C: mother plant)

Figure 2: Protocol for *in vitro* clonal propagation of *K. galanga* L.



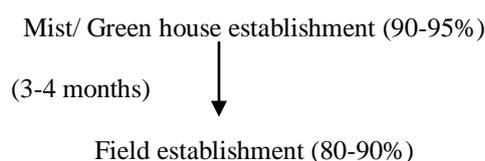


TABLE 1. Effect of plant growth regulators on shoot multiplication in *K. galanga*

Growth regulators (mg l ⁻¹)				% response	Mean no. of shoots ± SE	Mean length of shoots ± SE	Mean number of roots ± SE
BA	KIN	NAA	IAA				
1.0	-	-	-	73.33 ^c	2.10±0.22 ^g	3.19±0.15 ^c	2.9±0.22 ^e
2.0	-	-	-	76.67 ^c	4.89±0.74 ^e	3.57±0.26 ^c	3.8±0.25 ^d
3.0	-	-	-	70.00 ^c	5.00±0.38 ^d	3.07±0.19 ^c	3.3±0.26 ^d
4.0	-	-	-	80.00 ^b	5.20±0.42 ^d	3.41±0.24 ^c	4.1±0.24 ^c
-	1.0	-	-	66.67 ^d	1.40±0.15 ^h	3.72±0.24 ^c	2.0±0.21 ^e
-	2.0	-	-	63.33 ^d	1.70±0.14 ^h	3.10±0.16 ^c	2.9±0.23 ^e
-	3.0	-	-	60.00 ^d	1.91±0.21 ^h	3.18±0.12 ^c	2.8±0.20 ^e
-	4.0	-	-	63.33 ^d	2.71±0.28 ^g	3.68±0.13 ^c	3.0±0.26 ^d
2.0	1.0	-	-	76.67 ^c	2.83±0.22 ^g	3.53±0.17 ^c	3.3±0.21 ^d
3.0	1.0	-	-	73.33 ^c	3.14±0.26 ^f	3.54±0.12 ^c	3.4±0.16 ^d
4.0	1.0	-	-	66.67 ^d	5.75±0.38 ^d	3.75±0.19 ^c	3.5±0.17 ^d
2.0	-	0.5	-	86.67 ^b	4.10±0.18 ^e	3.69±0.12 ^c	4.6±0.22 ^c
3.0	-	0.5	-	76.67 ^c	5.14±0.31 ^d	3.58±0.12 ^c	4.7±0.39 ^c
4.0	-	0.5	-	76.67 ^c	6.14±0.26 ^c	3.79±0.12 ^c	4.6±0.26 ^c
2.0	-	1.0	-	86.67 ^b	5.57±0.41 ^d	3.74±0.13 ^c	4.8±0.29 ^c
3.0	-	1.0	-	83.33 ^b	5.62±0.47 ^d	3.43±0.10 ^c	4.9±0.31 ^c
4.0	-	1.0	-	86.67 ^b	7.40±0.65 ^b	3.66±0.07 ^c	5.2±0.29 ^b
2.0	-	-	0.5	86.67 ^b	3.80±0.29 ^f	3.64±0.10 ^c	3.8±0.25 ^d
3.0	-	-	0.5	93.33 ^a	4.14±0.39 ^e	3.82±0.17 ^c	3.3±0.26 ^d
4.0	-	-	0.5	90.00 ^a	4.80±0.52 ^e	3.83±0.07 ^c	3.5±0.22 ^d
2.0	-	-	1.0	96.67 ^a	4.87±0.29 ^e	4.01±0.13 ^b	4.6±0.30 ^c
3.0	-	-	1.0	83.33 ^b	5.71±0.44 ^d	4.17±0.20 ^b	4.8±0.24 ^c
4.0	-	-	1.0	80.00 ^b	6.00±0.35 ^c	4.10±0.15 ^b	4.8±0.29 ^c
4.0	1.0	1.0	-	86.67 ^b	10.60±0.83 ^a	5.13±0.12 ^a	5.6±0.37 ^b
2.0	0.5	0.5	-	83.33 ^b	5.40±0.84 ^d	4.16±0.49 ^b	4.1±0.85 ^c
4.0	1.0	-	1.0	76.67 ^c	7.90±0.54 ^b	5.58±0.30 ^a	5.0±0.30 ^b
2.0	0.5	-	0.5	73.33 ^c	4.40±0.31 ^e	3.78±0.19 ^c	6.1±0.41 ^a

Data represents mean ± SE of 10 replicates repeated thrice; observations were recorded after 30 days of culture. Means followed by the same letter in superscript in a column do not differ significantly based on ANOVA and Duncan's multiple range test ($p \leq 0.05$).

TABLE 2. Effect of repeated subculture on shoot multiplication in *K. galanga*

Growth regulators (mg l ⁻¹)				Mean no. of shoots ± SE			
BA	KIN	NAA	IAA	Subculture I (30 days)	Subculture II (60 days)	Subculture III (90 days)	Subculture IV (120 days)
3.0	-	-	-	5.20 ± 0.42 ^d	13.0 ± 0.62 ^e	11.6 ± 0.52 ^g	9.8 ± 0.39 ^h
4.0	-	-	-	5.75 ± 0.38 ^d	19.8 ± 0.92 ^c	16.1 ± 0.48 ^d	14.3 ± 0.71 ^d
4.0	1.0	-	-	5.00 ± 0.38 ^d	12.0 ± 0.47 ^f	10.3 ± 0.58 ^h	9.3 ± 0.30 ^h
2.0	-	0.5	-	5.57 ± 0.41 ^d	11.7 ± 0.49 ^f	10.1 ± 0.56 ^h	8.9 ± 0.27 ⁱ
3.0	-	0.5	-	5.62 ± 0.47 ^d	15.1 ± 0.48 ^d	13.1 ± 0.48 ^e	10.6 ± 0.31 ^g
4.0	-	0.5	-	6.14 ± 0.26 ^c	18.3 ± 0.70 ^c	15.9 ± 0.52 ^d	14.5 ± 0.34 ^d

2.0	-	1.0	-	4.87 ± 0.29 ^e	13.0 ± 0.71 ^e	11.5 ± 0.50 ^g	11.0 ± 0.39 ^f
3.0	-	1.0	-	5.71 ± 0.44 ^d	17.5 ± 0.62 ^c	16.2 ± 0.36 ^d	14.9 ± 0.67 ^d
4.0	-	1.0	-	7.40 ± 0.65 ^b	20.7 ± 0.73 ^b	18.4 ± 0.65 ^c	17.1 ± 0.31 ^c
2.0	-	-	0.5	4.10 ± 0.18 ^e	12.8 ± 0.66 ^e	12.0 ± 0.65 ^f	10.1 ± 0.46 ^g
3.0	-	-	0.5	5.14 ± 0.31 ^d	16.3 ± 0.58 ^d	13.9 ± 0.52 ^e	12.4 ± 0.37 ^e
4.0	-	-	0.5	6.00 ± 0.35 ^c	18.0 ± 0.61 ^c	16.4 ± 0.54 ^d	14.5 ± 0.40 ^d
4.0	1.0	1.0	-	10.60 ± 0.83 ^a	30.2 ± 0.76 ^a	27.5 ± 0.52 ^a	24.8 ± 0.39 ^a
4.0	1.0	-	1.0	7.90 ± 0.54 ^b	22.7 ± 0.80 ^b	21.5 ± 0.73 ^b	18.1 ± 0.40 ^b

Data represents mean ± SE of 10 replicates repeated thrice; observations were recorded after 30 days of culture. Means followed by the same letter in the superscript in a column do not differ significantly based on ANOVA and Duncan's multiple range test ($p \leq 0.05$).

V. CONCLUSION

K. galanga is a clonally propagated medicinal plant having no seed set and a shoot tip cryopreservation protocol is essential for its long-term conservation. Shoot tips are widely used for cryopreservation, because they are totipotent, contain organized tissue and are genetically stable. For successful cryopreservation of plant species, a reliable micropropagation system is necessary to obtain many *in vitro* grown plantlets. The *in vitro* propagation system established here ensures the production of a large number of relatively homogenous shoot tips in terms of size, physiological state and growth response as it is possible to raise at least 30 shoots per rhizome bud, thereby increasing the feasibility of the procedure to subsequent cryotreatments which has also been successfully accomplished [26]. The *in vitro* preservation and micropropagation thus ensures the conservation and sustainable utilization of this endangered pharmaceutically important plant species.

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