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In vitro regeneration of pathogen free *Kaempferia galanga* L. - a rare medicinal plant

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***Kaempferia galanga* L. is an aromatic and medicinal herb of the family Zingeberaceae. The plant is propagated through rhizomes for which various pathogens are transmitted through it. Conventional breeding methods are ineffective for producing disease free crop but in vitro regeneration can serve the purpose. Direct *in vitro* regeneration using shoot buds in Murashige and Skoog media supplemented with various concentrations and combinations of cytokinin were used for regeneration. The sprouts responded within 3-4 weeks. Maximum number of plantlets were observed in the media supplemented with 4 mg/L BAP (4.33 shoots/explant) when different cytokinins were used alone. Combinations of BAP and kinetin produced better results. Maximum regeneration was observed in media supplemented with 3 mg/l BAP + 4 mg/l kinetin (6.52). Decline in the rate of regeneration were observed after 1st or 2nd subcultures depending upon the plant growth regulator/s. Rooting followed shooting in all the hormone combinations. High survival percentage was observed on hardening. The regenerated plantlets were detected to be pathogen free.**

Key words: *Kaempferia,* pathogen free, cytokinins, shoot sprouts, regeneration

Kaempferia galanga L. (black thorn) is an aromatic and medicinal herb with tuberous rootstocks belonging to the family Zingeberaceae. The stem less plant has broad flat leaves spreading on the ground. Black thorn is facing threat of extinction owing to indiscriminate and unsustainable harvesting in the wild. Conventional breeding is difficult in this plant, and in vitro multiplication is important to conservation and propagation (Swapna *et al.*, 2004). Though native in India, it is cultivated in South East Asia and China (Kirtikar *et al.*, 1996). The rhizome extract of the plant are used as expectorant, stimulant, diuretic, carminative, and stomachic (Rahman *et al.*, 2004 & Kumar *et al.*, 1997). The extract is also used to cure nasal block, asthma and hypertension. The leaf extracts are applied to sore eyes, sore throat, rheumatism, swellings and fevers. The aroma and flavour of the plant is used in flavouring foodstuffs, beverages, perfumes and cosmetics industries (Rahman *et al.*, 2004).

Black thorn is propagated through rhizomes like all other members of the family. Various pathogens of bacterial (*Pseudomonas* sp) and fungal (*Pythium* sp) origin get transmitted through the seed materials and affect the cultivation of this crop. The conventional breeding methods are inapplicable in improvement of the members of Zingiberaceae due to low seed set. *In vitro* culture offers a method for producing variations and exploring the resultant variations for crop improvement. Regeneration of *Kaempferia galanga* L. by

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callusing and somatic embryogenesis (Vincent et al., 1991 and Vincent *et al.,* 1992) and regeneration of from the leaf base-derived callus cultures (Rahman *et al.,* 2004) were reported. The present study was conducted to find an efficient method for direct *in vitro* regeneration of pathogen free *Kaempferia galanga* from shoot sprout explants.

Materials and methods

Collection of plant materials and culture conditions

The rhizomes of *Kaempferia galanga* were collected from the field and were washed under running tap water to remove the soil from the surface. They were treated with Diathane M 45 (0.1% in water) and were kept on trays filled with sand for germination. The rhizomes sprouted within 2-4 weeks and the sprouts were used as explants. The young sprouts were washed in running water to remove the soil of the germinating tray and dipped in 1% Extran for 10 minutes. They were washed several times with double distilled water. The explants were taken to laminar air flow cabinet and were dipped in mercuric chloride solution of 0.5% (w/v) for 10 minutes**.** The treated explants were washed several times with sterile double distilled water and dipped in 70% Ethyl alcohol for 1 minute. The traces of Ethyl alcohol were removed by washing them with sterile double distilled water for 5 times. The open ends of the explants were cut off and were inoculated on the culture media.

Murashige and Skoog (M.S.) basal medium (Murashige & Skoog, 1962) was used for the present study. Sucrose (Hi Media-1.11925.1000) was used as the carbon source at the rate of 30 mg/l in all the experiments. The medium was supplemented with cytokines like benzyl amino purine (BAP), kinetin and zeatin at different concentrations and combinations. For solidifying the culture media, agar (E Merk-RM1158-500G) was used at a concentration of 8 mg/l. The pH was adjusted to 5.7 ± 0.1. The media was sterilized at 121^{o}C for 20 minutes at 1.08 Kg/cm^{2} pressure. Filter sterilized vitamins and growth regulators were added to the media after autoclaving. The culture vessels after inoculation were incubated at 25 ± 2^{o}C and were given a photoperiod of 16 hours with a light intensity of 2000-2500 Lux provided by cool white fluorescent tubular lamps. For secondary cultures, the plantlets regenerated from primary culture were taken out of the culture tubes. The shoots were separated from each other and were reinoculated in the media containing the same concentration and combinations of plant growth regulator.

Hardening of plantlets

Healthy, *in vitro* regenerated plants with good number of roots were selected for hardening. The plantlets were taken out and washed carefully to remove all traces of agar sticking to the roots. They were then transplanted into polycups containing a mixture of garden soil and sand (1:1). They were kept covered with plastic bags with holes to provide 60-70% relative humidity. Subsequently they were transferred to the garden and planted in the field after one month.

Detection of pathogen in regenerated plants

Two diagnostic tests were performed to detect the presence or absence of the pathogen. 1. Rhizome pieces of these clones were transferred to potato dextrose agar (PDA) and observed for 8 – 10 days for fungal growth on the medium. 2. Rhizomes harvested from the tissue culture-derived plants were stored in river sand, and the number of rotted and healthy rhizomes was recorded after 6 months of storage.

Results

The explants responded within 2-3 weeks. In the primary culture 26 and 47 days were required respectively for shoot and root

initiation. Surface sterilization with 0.5% mercuric chloride for 10 minutes followed by 70% ethanol for 1 minute showed extremely low rate of contamination in the primary culture. Fungal and bacterial contaminations were 12% and 04% respectively in the primary culture. The number of days required for shoot and root initiation increased with the increase in the concentration of mercuric chloride.

Plant regeneration

Significant variations were observed in the results obtained by supplementing the media with different cytokinins (Table-1).

Table 1. Effect of cytokinin on regeneration of *Kaempferia galanga*

BAP	Kinetin	Zeatin	Shoot/explants	Height(Cm)/shoot
1			1.91 ± 0.12	2.67 ± 0.61
2			2.03 ± 0.10	3.05 ± 0.07
3			3.17 ± 0.11	4.31 ± 0.16
4			4.33 ± 0.11*	3.97 ± 0.19
5			3.00 ± 0.12*	3.40 ± 0.11
	1		2.15 ± 0.20	1.81 ± 0.04
	2		3.21 ± 0.31	2.26 ± 0.18
	3		3.44 ± 0.08	2.56 ± 0.12
	4		2.13 ± 0.21	2.74 ± 0.08
	5		1.94 ± 0.16	2.82 ± 0.21
		1	2.25 ± 0.10	1.99 ± 0.23
		2	3.58 ± 0.41	2.21 ± 0.06
		3	2.28 ± 0.31	2.42 ± 0.11
		4	2.27 ± 0.38	2.48 ± 0.11
		5	1.63 ± 0.34	2.44 ± 0.22
1	1		2.56 ± 0.11	0.71 ± 0.12
1	2		2.73 ± 0.12	1.62 ± 0.36
1	3		3.38 ± 0.41	2.21 ± 0.16
1	4		3.93 ± 0.30	2.74 ± 0.12
2	1		3.13 ± 0.35	4.31 ± 0.24
2	2		4.33 ± 0.27	5.41 ± 0.12
2	3		4.51 ± 0.15	6.81 ± 0.16
2	4		6.06 ± 0.24	8.23 ± 0.20
3	1		4.76 ± 0.11	6.28 ± 0.12
3	2		5.75 ± 0.72	7.14 ± 0.38
3	3		6.00 ± 0.29	8.15 ± 0.16
3	4		6.52 ± 0.22	7.41 ± 0.21
4	1		5.48 ± 0.39	5.86 ± 0.16
4	2		5.76 ± 0.20	6.39 ± 0.28
4	3		5.53 ± 0.26	7.09 ± 0.09
4	4		5.43 ± 0.45	6.26 ± 0.26

Data ± standard deviation was taken 12 weeks following inoculation. All treatments had 5 replicates and were repeated thrice.

BAP gave better results compared to kinetin and Zea. The maximum numbers of plantlets/explant (4.33) were observed in the media supplemented with BAP at a concentration of 4 mg/l while the maximum height (4.31cm) was found with BAP 3 mg/l. In the media supplemented with kinetin the maximum numbers of plantlets/explant (3.44) were observed at 3 mg/l and the maximum height (2.82cm) at 5mg/l. The highest numbers of plantlets/explant (3.58) regenerated with zeatin were at 2 mg/l whereas the maximum height (2.48cm) was at zeatin 4 mg/l. Rooting were observed in all the cultures. Lower concentrations of cytokinins produced less number of shoots from the explants while it declined above a critical level. Callusing of the explants was observed in the media supplemented with 4 and 5 mg/l BAP. The rates of callusing were more in the secondary culture than the primary culture.

The regeneration of plantlets varied considerably with different combinations of BAP and kinetin. The maximum numbers of plantlets were obtained in the media supplemented with 3 mg/l BAP + 4 mg/l kinetin followed by 2 mg/l BAP +4 mg/l kinetin, while the maximum plantlet height was obtained in the media supplemented with 2 mg/l BAP + 4 mg/l kinetin. Regeneration of plantlets gradually increased with increase in hormone concentrations while it declined beyond 3 mg/l BAP + 4 mg/l kinetin. Combinations of cytokinins were more potential in regeneration rather than when they were used alone.

Responses in primary and secondary cultures

The effects of plant growth regulators on regeneration were not the same in primary and secondary cultures (Figure 1). In all the cases the least number of plantlets were produced in the primary culture and it declined after the 1st or 2nd subcultures. In the cultures supplemented with kinetin 3 mg/l, BAP 2 mg/l + kinetin 4 mg/l, BAP 3 mg/l + kinetin 3 mg/l and BAP 3 mg/l + kinetin 4 mg/l the maximum regeneration were found in the 1st subculture while in the culture supplemented with BAP 4 mg/l maximum regeneration was in the 2nd subculture.

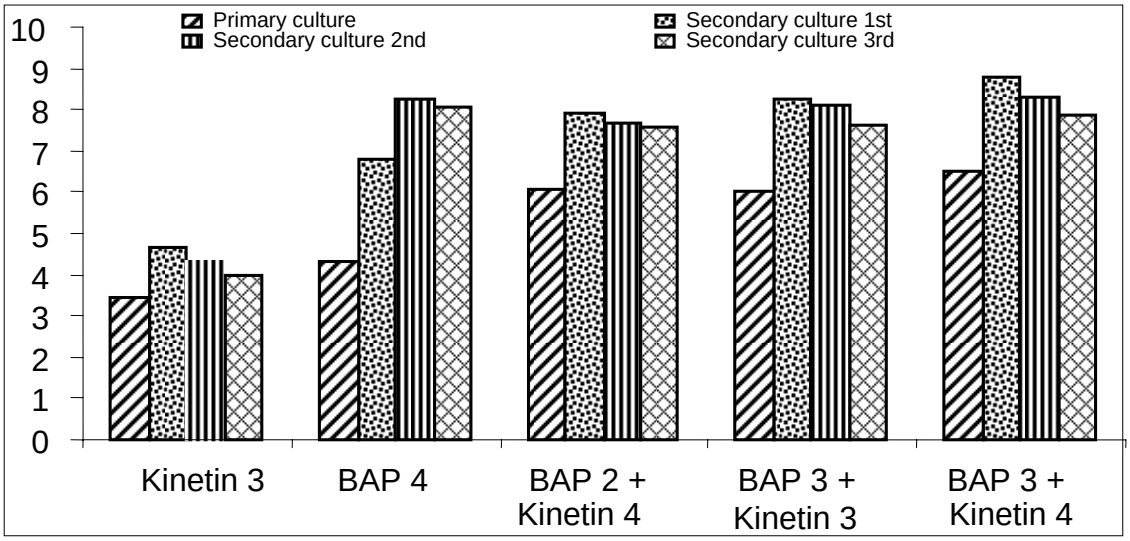

Figure: 1. effect of subculture on the rate of plantlet regeneration. The results are the average of 10 replicates and were repeated twice.

Figure: 2. *In vitro* sprouting of the explants, b. Regeneration in medium supplemented with BAP 3, c. Regeneration in medium supplemented with BAP & Kinetin 2+2, d. Regeneration in medium supplemented with BAP & Kinetin 3+4, e. Showing in vitro rooting and f. Hardening of plantlets.

Hardening of plantlets

The ultimate success of *in vitro* propagation lies in the successful establishment of plant in the soil. The regenerated plantlets of *Kaempferia* after hardening were transferred to garden after one month. The regenerated plants showed a survival percentage of 94% in poly cups (green house) and 100% in field (environment).

Detection of pathogen in regenerated plant

Rhizome pieces of micropropagated plants were transferred onto PDA to examine the infection, if any. Mycelia growths were not observed on medium after 8 – 10 days of incubation while 58 % of the pieces of conventional rhizomes showed mycelia growth. Rhizomes obtained from the tissue culture-derived plants stored in sand for 6 months also did not show any rotting of rhizomes 53% of the rhizomes of mother-derived plants rotted during storage.

Discussions

In all the cases the number of days required for shoot and root initiation increased with the increase in the concentration and duration of mercuric chloride treatment. This may be due to cell death of explants during surface sterilization with higher concentrations of mercuric chloride.

Combinations and concentrations had significant effect on the regeneration of plantlets. Among the cytokinins tried, BAP gave better results compared to kinetin and Zea. BAP was found to be very much effective in the generation of ginger tissue culture (Sit *et al.* 2005 and Bhattacharya & Sen, 2006). Moderate rooting were observed in all the cultures which indicates that plant growth regulators does not have any influence on rooting. Lower concentrations of cytokinins produced less number of shoots from the explants while it declined above a critical level. This decline in the rate of shooting may be due to some inhibitory effect produced by higher concentrations of cytokinins. Similar results showing gradual increase and decline of the number of shoots after a certain level of cytokinins was observed in ginger tissue culture (Balachandran *et al.,* 1990 and Sit *et al.,* 2005). Regeneration of plantlets gradually increased with increase in hormone concentrations while it declined beyond a critical level. Combinations of cytokinins were more potential in regeneration rather than when they were used alone. Regeneration of plantlets were more in the media supplemented with low BAP + high kinetin ratio, but BAP was more effective than kinetin when used alone. This indicates that high concentrations of kinetin are effective only in combinations. Adequate numbers of roots/ plantlets were generated in the same media.

In all the cases the least number of plantlets were produced in the primary culture and it declined after the 1st or 2nd subcultures. Similar results were observed by Sit et al. 2005, in ginger tissue culture. Maximum regeneration was in the 2nd subculture. The increase in the number of plantlets in the secondary cultures can be due to the adaptations of the explants to the culture media and diminished effect of sterilant. The gradual decline in the rate of regeneration can be due to ageing of the explants and decline in their totipotency. The absence of pathogen in the rhizomes of regenerates plantlets may be due to *in vitro* selection of explants.

The high regeneration rate, shooting and rooting in the same media along with very high survival percentage indicates that this method could easily be adopted for *in vitro* regeneration of *Kaempferia galanga.* This protocol has the ability of detecting the presence of pathogen in the regenerated plantlets.

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