

In vitro propagation and microrhizome induction in *Kaempferia galanga* Linn. and *K. rotunda* Linn.

P Chirangini, S K Sinha¹ and G J Sharma*

Department of Life Sciences, Manipur University, Imphal 795 003, India

Received 2 April 2004; revised 11 August 2004; accepted 25 August 2004

Rhizomatous buds of *Kaempferia galanga* and *K. rotunda* induced microshoots when cultured on Murashige and Skoog (MS) medium supplemented with plant growth regulators. Multiple shoots were induced on MS medium containing 5.70 μM IAA alone and a combination of 0.57 μM IAA plus 4.65 μM Kn in case of *K. galanga*. Whereas, the medium supplemented with 2.69 μM NAA plus 2.22 μM BAP was the best for *K. rotunda*. Further, subculture of the microshoots gave more multiple shoots (13) on medium containing 4.44 μM BAP in *K. galanga* and (9) on the 2.69 μM NAA and 2.22 μM BAP in *K. rotunda*. Induction of callus was observed in *K. galanga* on the medium supplemented with 2.85 μM IAA. Microshoots were regenerated from the callus on 2.69 μM NAA and 2.22 μM BAP enriched medium. Microrhizome formation was observed within one month of incubation of microshoot cultures (~4 month old, following 4 passages) in the medium supplemented with 6-9% sucrose with either 22.2 μM BAP or 23.25 μM Kn. These microrhizomes produced shoots when transferred to fresh microshoot induction medium within 2-3 weeks of incubation. The microshoots produced roots irrespective of their method of regeneration. Plantlets transplanted to pots grew to mature plants after 3 months of transfer and showed 80-90% survival.

Keywords: 6-benzylaminopurine (BAP), indole-3-acetic acid (IAA), *Kaempferia*, kinetin (Kn), microrhizome, α -naphthalene acetic acid (NAA)

IPC Code: Int. Cl.⁷ A01H4/00, 5/04

Introduction

The rhizomes of *Kaempferia galanga* Linn. and *K. rotunda* Linn. are used in cosmetics and traditional system of medicine for curing various ailments¹⁻⁴. Garo and Khasi tribes of Meghalaya, India use *K. rotunda* rhizome juice as eye drop in removing cataract and also as remedial measure for night blindness⁵. Besides its medicinal importance, *K. rotunda* is grown sporadically in the northeastern India for their scented flowers using rhizome as propagule. Both the species are annual and 2-4 plants can be obtained in a year from one rhizome. For rapid multiplication of slow propagating species, tissue culture technique remains an indispensable tool. Currently, effort on *in vitro* propagation is directed at rhizome or storage-organ induction for efficient acclimatization and to minimize injury during transportation. Microrhizomes also have got enough potential to be used by commercial growers as

disease-free planting material, produced *in vitro* irrespective of seasonal fluctuations, easily transferable and sown like seeds^{6,7}. *In vitro* induction of rhizome has been reported in ginger⁶ and *Curcuma longa*⁸. Though, propagation of *K. galanga* and *K. rotunda* through multiple shoot induction has been reported in both the species^{9,10} but, so far, there is no report on microrhizome induction.

The present investigation, therefore, is an attempt to develop suitable protocol for microshoot induction, callus induction, plantlet regeneration from callus and microrhizome induction in *K. galanga* and *K. rotunda*, two medicinally important species.

Materials and Methods

Preparation of Plant Material

Rhizomes of zingiberaceous members have characteristic buds/eyes that sprout under conducive environment and grow to complete plant. Such rhizomatous eye was used as explant. Rhizomes were collected from the Experimental Field, Manipur University, during February-March 2001 and washed under running tap water for ~30 min; roots and outer scale were removed. The rhizomes were then washed

*Author for correspondence:

Tel: 91-385-2435154; Fax: 91-385-2435145

E-mail: gjs1951@rediffmail.com

¹Present address: TERI North-Eastern Regional Centre, Guwahati 781005, India

with Labolene (Qualigens, India) for ~10 min followed by treatment with 0.1% Dhanustin-50 (fungicide) for 20-30 min and 70% alcohol for 30 sec. Surface disinfestation was done either with sodium hypochlorite (1% available chlorine) or HgCl_2 (0.2%) for 15 min followed by 2-4 rinses with sterile water. In order to improve the efficiency, HgCl_2 was enriched with few drops of 1 N HCl and Tween 80 (Hi-Media, India). In addition, treatment with antibiotic (0.1% streptomycin) for 30 min was also tried. Disinfestation was followed by 3-4 rinses with sterile distilled water under laminar hood. The rhizomes were then cut into 5-8 mm sized pieces having at least one eye. The outer sheaths of the eyes were removed with a sterile surgical blade and explants (2-4 mm) were inoculated on various media.

Microshoot and Callus Induction

Murashige and Skoog (MS) medium¹¹ supplemented with indole-3-acetic acid (IAA; 0.57-11.42 μM), α -naphthalene acetic acid (NAA; 2.69-21.48 μM) and 2,4-dichlorophenoxyacetic acid (2,4-D; 2.26-18.10 μM) singly or in combination either with 6-benzylaminopurine (BAP; 2.22-8.88 μM) or kinetin (Kn; 2.32-4.65 μM) were tested. About 30 and 70 ml of the media were dispensed into separate tubes ($32 \times 200 \text{ mm}^2$) and conical flasks (250 ml) respectively, which were sealed with non-absorbent cotton wool plugs.

Microshoot Multiplication

The microshoots induced in the induction media were separated from the base, trimmed and sub-cultured on MS media supplemented with IAA (0.57-2.85 μM) and NAA (0.54-10.74 μM) alone or in combination with BAP (0.44-8.88 μM) or Kn (2.32 μM) for *K. galanga*, and NAA (0.54-10.74 μM) in combination with BAP (0.44-8.88 μM) or Kn (2.32 μM) alone for *K. rotunda*.

Statistical significance was calculated by one-way analysis of variance followed by Fischer's LSD (protected 't') test.

Regeneration of Plantlets from Callus

The excised calli were sub-cultured on MS media supplemented with NAA (2.69-21.48 μM) alone or in combination with BAP (2.22-4.44 μM).

In vitro Induction of Rhizome

Microshoot cultures following 4 passages (~4 months old) were transferred to MS media

supplemented with 6-9% sucrose and BAP (22.20-44.40 μM) or Kn (23.25 μM) alone.

All culture media were gelled with 0.8% (w/v) agar and the pH was adjusted to 5.8 with 1 N NaOH or 1 N HCl prior to autoclaving at 121°C for 20 min and 15 lb pressure. All cultures were incubated at 25°C±2 under 30 $\mu\text{mol m}^{-2}\text{s}^{-1}$ illumination for 16/8 h day/night cycle. Sub-culturing was carried out after every 4 weeks by trimming-off leaves and roots. For each experiment, at least ten replicates were used.

Acclimatization of Plantlets

Six-month-old plantlets of both *K. galanga* and *K. rotunda* were taken out from the flask, washed and treated with Dhanustin (0.1%) for 10 min followed by 3-4 washing with tap water. These plantlets were then transplanted to a mixture of river sand and dried powdered cow dung (1:1) filled in earthen pots and kept under 55% shade at the corridor. Watering with tap water during early morning and late evening were done for the period of 3 months with constant monitoring.

Results

The incidence of very high (~90%) contamination in cultures was reported due to underground source of explants. However, it was reduced substantially following addition of 1 N HCl to HgCl_2 solution. Streptomycin treatment of the explants failed to contain contamination.

Explant showed swelling 1-2 weeks after inoculation and produced single and multiple shoot in both the species. High frequency (80%) of multiple shoots were observed in treatment with 5.70 μM IAA alone and combination of 0.57 μM IAA plus 4.65 μM Kn for *K. galanga* (Fig. 1, a). Whereas, only low frequency (20%) of multiple shoots were observed with combinations of 2.69 μM NAA plus 2.22 μM BAP and 5.37 μM NAA plus 2.22 μM BAP for *K. rotunda* (Fig. 1, b). In both the species, the appearance of either single shoot or multiple shoots was accompanied by rooting as well. Calli were formed 2 weeks after inoculation. Only low frequency (20%) of callus formation was observed on medium supplemented with 2.85 μM IAA for *K. galanga*; whereas higher frequencies (40-50%) were observed with 2.69 μM NAA and 4.52 μM 2,4 D for *K. rotunda* (Fig. 1, c & d). The detailed data were not presented.

On subculture of microshoots, more multiplication of microshoots (13 in a clump) was observed on the

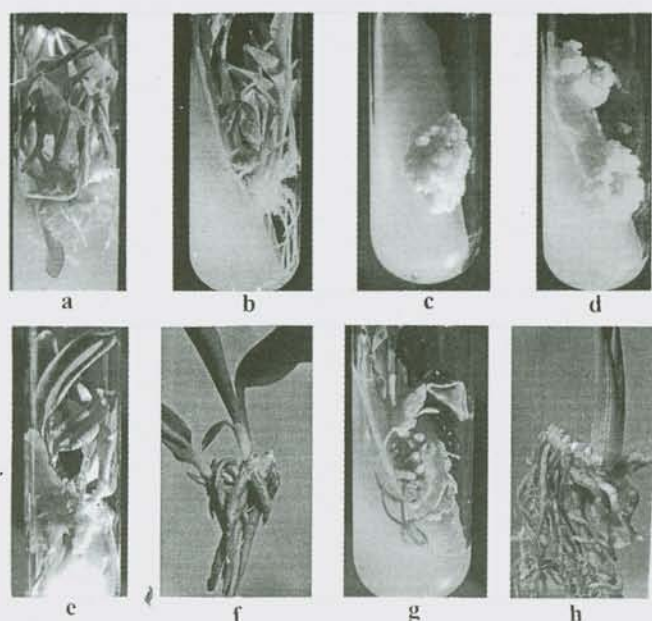


Fig. 1—*In vitro* propagation and microrhizome induction in two species of *Kaempferia*: Induction of multiple shoots with root in *K. galanga* (a) and *K. rotunda* (b); Induction of calli in *K. galanga* (c) and *K. rotunda* (d); Induction of microrhizomes in *K. galanga* (e) and same after cleaning (f), and in *K. rotunda* (g) and same after cleaning (h).

medium containing 4.44 μM BAP in *K. galanga* (Table 1) and (9 in a clump) on the medium containing 2.69 μM NAA and 2.22 μM BAP in *K. rotunda* (Table 2).

In case of *K. galanga*, transfer of callus to MS medium enriched with 2.69 μM NAA plus 2.22 μM BAP resulted in the formation of huge friable calli. However, the best medium for callus multiplication was either NAA (21.48 μM) alone or a combination of NAA (5.37 μM) and BAP (2.22 μM). Callus when sub-cultured on media (either 2.69 μM NAA plus 2.22 μM BAP or 10.74–21.48 μM NAA alone) produced small granular bodies at the initial stage, which later differentiated into shoots along with roots. These shoots when transferred onto microshoot multiplication media produced good number (4–5) of multiple shoots. Interestingly, the roots of these shoots were slender, fibrous and more in number, exceeding 30; whereas, the roots of multiple shoots produced from rhizome bud explants were green, thick and less in number, not exceeding eight.

The 4-month-old microshoots after 4 passages when sub-cultured to higher concentration of sucrose (6–9%) showed growth retardation with swollen base after 1 month. However, on subsequent sub-culture

Table 1—Multiplication of microshoots in *K. galanga* on MS medium supplemented with growth regulators

| Growth regulator (μM) | | | | Number of microshoots/subculture (Mean* \pm S.E.) | Highest number of microshoots |
|------------------------------------|-------|------|------|---|-------------------------------|
| IAA | NAA | BAP | Kn | | |
| 2.85 | - | - | - | 2.00 \pm 0.41 ^b | 3 |
| - | 2.69 | - | - | 1.50 \pm 0.33 ^b | 2 |
| - | 5.37 | - | - | 1.50 \pm 0.50 ^b | 3 |
| - | - | 4.44 | - | 8.75 \pm 2.18 ^a | 13 |
| - | - | 2.22 | 2.32 | 1.75 \pm 0.25 ^b | 2 |
| 0.57 | - | 4.44 | - | 3.67 \pm 0.42 ^b | 8 |
| - | 0.54 | 0.44 | - | 1.50 \pm 0.50 ^b | 3 |
| - | 0.54 | 4.44 | - | 1.25 \pm 0.25 ^b | 2 |
| - | 5.37 | 0.44 | - | 1.50 \pm 0.29 ^b | 2 |
| - | 2.69 | 2.22 | - | 3.33 \pm 0.40 ^b | 6 |
| - | 2.69 | 4.44 | - | 2.22 \pm 0.58 ^b | 4 |
| - | 5.37 | 4.44 | - | 2.00 \pm 0.20 ^b | 3 |
| - | 10.74 | 8.88 | - | 1.20 \pm 0.20 ^c | 2 |

* Means followed by the same letter do not differ significantly at $p=0.01$ (protected 't' test)

Table 2—Multiplication of microshoots in *K. rotunda* on MS medium supplemented with growth regulators

| Growth regulator (μM) | | | Number of microshoots/subculture (Mean* \pm S.E.) | Highest number of microshoots |
|------------------------------------|------|------|---|-------------------------------|
| NAA | BAP | Kn | | |
| 0.54 | 0.44 | - | 1.50 \pm 0.27 ^b | 5 |
| 0.54 | 4.44 | - | 1.17 \pm 0.12 ^c | 2 |
| 2.69 | 2.22 | - | 6.10 \pm 0.34 ^a | 9 |
| 2.69 | 4.44 | - | 1.67 \pm 0.89 ^b | 3 |
| 2.69 | 8.88 | - | 5.33 \pm 1.33 ^a | 8 |
| 5.37 | 0.44 | - | 1.22 \pm 0.44 ^c | 2 |
| 5.37 | 2.22 | - | 2.50 \pm 0.36 ^b | 5 |
| 5.37 | 4.44 | - | 1.22 \pm 0.22 ^c | 2 |
| 5.37 | 8.88 | - | 2.50 \pm 0.87 ^b | 4 |
| 10.74 | 8.88 | - | 3.00 \pm 0.34 ^b | 5 |
| - | - | 2.32 | 3.00 \pm 0.58 ^b | 8 |

* Means followed by the same letter do not differ significantly at $p=0.01$ (protected 't' test)

most of the shoots died. *K. galanga* microshoots cultured in 6% sucrose with either BAP (22.20 μM) or Kn (23.25 μM), showed pear-shaped microrhizome formation (Fig. 1, e & f). Whereas in *K. rotunda*, microrhizome formation was observed in both 6 and 9% sucrose concentrated media supplemented with either (BAP, 22.20–44.40 μM) or Kn (23.25 μM) (Fig. 1, g & h). Irrespective of concentration of sucrose or the type of growth regulator, *K. rotunda* shoots also produced microrhizome in cultures when kept undisturbed for 6 months. A visual observation of the microrhizome of *K. rotunda* formed in higher sucrose and cytokinin concentration supplemented

media were found to be larger in size (data not shown) with 8-10 buds/eyes than those obtained in basal medium with lower growth regulator concentration supplemented culture, which were smaller in size (4-6 buds/eyes). These microrhizomes when transferred onto fresh microshoot induction media produced microshoots within 2-3 weeks of incubation.

Plantlets transplanted to pots showed 80-90% survival and grew to mature plants after 3 months of transfer. The characteristic black smear at the lower side of leaf appeared in *K. rotunda* after 2 months. When microrhizomes were transferred to river sand and cow dung mixture (1:1) filled earthen pots, they sprouted into 4-6 shoots after 2 weeks of transplantation and grew into complete plants within 4 weeks.

Discussion

Establishment of aseptic culture from underground explants was found to be difficult due to higher contamination¹². In the present case, additions of one drop of 1 N HCl and Tween 80 in the HgCl₂ solution (0.2%) produced 80% aseptic culture initiation. It appeared that acidification of the sterilant in extreme cases of contamination was beneficial. The organogenic route of *in vitro* cultured dormant rhizomatous bud/eye started responding by breaking the outer thick sheath followed by emergence of shoot primordium. The highest frequency of multiple shoot initiation for *K. galanga* and *K. rotunda* as shown in the results suggest that multiple shoot induction required a specific ratio of auxin and cytokinin that varied with species and physiological status of the explant. This was evident in earlier works on *C. zedoaria*¹³ and *K. galanga* and *K. rotunda*^{9,10}. Multiplication of microshoots in *K. galanga*¹⁰ at the rate of 1:10 and in *K. rotunda*^{9,10} at 1:6 and 1:7 has been reported. In the present case, the highest number of microshoots induced in *K. galanga* (13) and in *K. rotunda* (9) showed much higher response. Nadgauda *et al*¹⁴ reported that root initiation and development declined with increasing levels of BAP in turmeric tissue culture. Whereas in our experiment, irrespective of the growth regulator treatment, these microshoots produced roots as well, which is an advantage over only shoot formation where additional treatment is required for root induction. Similar findings have been reported in *C. longa*⁸. Callus induction in *K. galanga* does not pose any difficulty as compared to *K. rotunda*. Medium containing either

NAA alone or in combination with BAP supports faster callus multiplication than 2,4-D supplements. Literature survey reveals that our findings on callus induction in *K. galanga* and *K. rotunda* are the first reports. In the present work, NAA either alone or in combination with BAP supplemented in the medium was found useful for calli multiplication as well as plantlet differentiation, which is in agreement with the earlier report in *Curcuma* spp¹³.

Assimilate partitioning from source to sink is essential for the harvestable component of economically important plants¹⁵⁻¹⁷. The harvestable yield is the result of carbon dioxide fixation and the subsequent allocation of fixed carbon and assimilates into economically yield component. For Zingiberales in general, and *Kaempferia* in particular, rhizome is the harvestable organ of economic importance. Rhizome serves as sink where assimilates are unloaded. In *in vitro* culture condition, assimilate provided as sucrose may have been transported to the stem for rhizome initiation, as plantlets cultured in sucrose rich medium show restricted leaf and shoot growth with swollen basal part. *In vitro* ginger plantlets cultured in MS medium with 3% sucrose incubated in higher light intensities at 180 $\mu\text{mol m}^{-2}\text{s}^{-1}$ of continuous illumination produce optimal rhizome formation¹⁸. The carbon source when supplied as sucrose (9 or 12%) to ginger plantlets produce rhizomes irrespective of variations in photoperiod⁶. Even in lower photoperiod of 8 hr illumination, turmeric plantlets cultured in 8% sucrose with lower concentration of growth regulator produced rhizomes⁸. In the present investigation, higher concentration of sucrose (6 or 9%) with higher cytokinin supplementation has been found to induce microrhizomes in normal photoperiod of 16 hr illumination. Therefore, it is possible that higher concentration of sucrose is needed as a source of energy in the process of *in vitro* rhizome formation under normal photoperiod.

Acclimatization of plantlets is the crucial phase where plantlets are in transition from *in vitro* phase to *in vivo* phase. Generally, higher sophistication (with controlled high humidity, temperature and accurate potting mixture) is required for higher plantlet survival. In the present case, acclimatization of *Kaempferia* plantlets has been achieved easily with 80-90% survival by way of simply keeping the plant in 55% shade and watering twice a day. Microrhizomes when transferred to pots containing

mixture of river sand and cow dung (1:1) sprouted into 4-5 plants each and showed normal growth.

Acknowledgements

The authors are grateful to the Department of Biotechnology, Government of India, New Delhi for providing financial support (*vide* Grant No. BT/PR/1158/ Agr/08/76/98).

References

1. Watt G, *Dictionary of the economic products of India, vol. IV: Gossypium to Linociera* (Periodical Experts, Delhi, India) 1972.
2. Prakash V & Mehrotra B N, Zingiberaceae of India: Biological screening and ethnobotanical diversity, in *Proc 2nd Symp Family Zingiberaceae* (South China Institute of Botany, China) 1996, 229-237.
3. Sinha S C, *Medicinal plants of Manipur* (MASS and Sinha, Imphal, India) 1996.
4. Siriruga P, Thai Zingiberaceae: Species diversity and their uses, *Pure Appl Chem*, 70 (1998) 2111-2118.
5. Rao R R, Ethnobotanical studies on the flora of Meghalaya: Some interesting reports of herbal medicines, in *Glimpses of Indian ethnobotany*, edited by S K Jain (Oxford and IBH Publishing Co, New Delhi, India) 1981, 137-148.
6. Bhat S R, Chandel K P S & Kacker A, *In vitro* induction of rhizomes in ginger, *Zingiber officinale* Rosc., *Indian J Exp Biol*, 32 (1994) 340-344.
7. Sharma T J & Singh B M, *In vitro* microrhizome production in *Zingiber officinale* Rosc., *Plant Cell Rep*, 15 (1995) 274-277.
8. Sunitibala H, Damayanti M & Sharma G J, *In vitro* propagation and rhizome formation in *Curcuma longa* Linn., *Cytobios*, 105 (2001) 71-82.
9. Anand P H M, Harikrishan R N, Martin K P & Hariharan M, *In vitro* propagation of *Kaempferia rotunda* Linn., 'Indian Crocus—A medicinal plant', *Phytomorphology*, 47 (1997) 281-286.
10. Geetha S P, Manjula C, John C Z, Minoo D, Nirmal Babu K *et al*, Micropropagation of *Kaempferia* spp (*K. galanga* L. and *K. rotunda* L.), *J Spices Aromatic Crops*, 6 (1997) 129-135.
11. Murashige T & Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol Planta*, 15 (1962) 473-497.
12. Balachandran S M, Bhatt S R & Chandel K P S, Clonal multiplication of turmeric (*Curcuma* spp) and ginger (*Zingiber officinale* Rosc.), *Plant Cell Rep*, 8 (1990) 521-524.
13. Yasuda K, Tsuda T, Shimizu H & Sugaya A, Multiplication of *Curcuma* species by tissue culture, *Planta Med*, 1 (1988) 75-79.
14. Nadgauda R S, Mascarenhas A F, Hendre R R & Jagannathan V, Rapid multiplication of turmeric (*Curcuma longa* Linn.) plants by tissue culture, *Indian J Exp Biol*, 16 (1978) 120-122.
15. Gifford R M & Evans L T, Photosynthesis, carbon partitioning and yield, *Annu Rev Plant Physiol*, 32 (1981) 485-509.
16. Wardlaw I F, The control of carbon partitioning in plants, *New Phytol*, 116 (1990) 341-381.
17. Farrar J F, The whole plant: Carbon partitioning during development, in *Carbon partitioning within and between organisms*, edited by C J Pollock *et al* (BIOS Scientific Publishers, Oxford) 1992, 163-179.
18. Sakamura F, Ogohara K, Suga T, Taniguchi K & Tanaka R, Volatile constituents of *Zingiber officinale* rhizome produced *in vitro* shoot-tip culture, *Phytochemistry*, 25 (1986) 1333-1336.