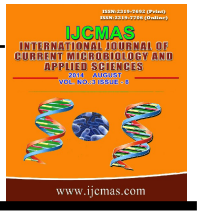
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 9 (2014) pp. 409-414**
http://www.ijcmas.com



Original Research Article

In-vitro plant regeneration in *Kaempferia rotunda* Linn. through somatic embryogenesis - a rare medicinal plant

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Keywords

Bhumichampaka, Micropropagation, Synthetic seeds, Zingiberaceae

A B S T R A C T

The plant Kaempferia rotunda Linn., is an important medicinal herb used in various fields of medicines. In the present study an efficient protocol is developed for the in-vitro plant regeneration in it through somatic embryogenesis. Of various hormonal combinations tried, embryogenic callus were induced on MS solid medium supplemented with 2.5mg/L 2,4-D and 0.5mg/L BAP. This embryonic callus was developed in to embryos on MS medium containing 0.25mg/L 2,4-D and 3.0mg/L BAP. Further plant regeneration was observed on MS medium supplemented with 5.0mg/L BAP. Globoid or torpedo shaped somatic embryos obtained from the callus culture was encapsulated in calcium alginate beads successfully. Plantlets obtained through embryogenesis successfully transferred to the field and they showed 50% establishment in the soil.

Introduction

Kaempferia rotunda Linn. is an important rare medicinal herb with tuberous root stalks of family Zingiberacea used in Ayurveda, Folk, Siddha and Unani. It is called bhumichampaka in Sanskrit, bhuchampa in Hindi, Chengazhi in Malayalam and blackhorm in English. It is used for gastropathy, inflammations, wounds, ulcers, blood clots, tumours, cancerous swellings etc (Udayan and balachandran, 2009). "Indian Crocus" is the popular name of it and its root tubers used as major ingredient in more than twenty ayurvedic preparations especially in "Vata" and "Kapha". Due to unscientific and large scale collection of raw drugs, this plant is being completely root out from the flora and declared as endangered. The conventional propagation is season depended and some soil pathogens affect the quality also. To overcome these problems, *in vitro* propagation tools can be used, through which pathogen free materials can be

produced independent of the seasonal variation to contribute to various fields of research, planting material production, germplasm conservation etc.

There are reports for multiplication via clonal, organogenesis and embryogenesis of different genera of the family *Zingiberaceae* such as *Alpinia* (Chang and Crily, 1993; Agreties et al, 1996 and Anand and Hariharan 1997), Elatteria (Nadgauda et al, 1983), *Zingiber officinale* (Sharma et al., 1994; Babu et al, 1996), *Costus speciosus* (Roy and Pal, 1991), *Kaempferia galangal* (Vincent et al, 1991 and Vincent et al, 1992), *Kaempferia* rotunda Linn. (Anand et al, 1997) etc. The present study has resulted in the development of a protocol for the successful *in vitro* propagation of *Kaempferia rotunda* Linn. via somatic embryogenesis. No report is hither to available on the somatic embryogenesis from rhizome derived callus of *Kaempferia rotunda* Linn.

Materials and Methods

Rhizomes were collected from the Herbal garden, Arya Vaidya Sala Kottakkal and established in the garden pots containing potting mixture (sand, soil and cow dung in the ratio of 2:2:1). The healthy rhizomes collected for *in vitro* studies were washed thoroughly in running tape water followed by 2% solution of Copper oxichloride supplemented with detergent solution extran (5%) and were surface sterilized for 4 minutes with (0.1%) $HgCl_2$ solution. These explants were subsequently washed with double distilled water and cut in to pieces with atleast one viable bud. After thorough washing these explants were taken in to Laminar air flow chamber (LAF). Inside LAF the rhizome pieces were subjected to second $HgCl_2$ (0.1%) treatment for 2 minutes. These were then washed thoroughly with sterile distilled water to remove traces of $HgCl_2$ remains if any and inoculated to MS Medium (Murashige and Skoog 1962.) supplemented with different concentration and combinations of auxin and cytokinin (Table.1). Inoculated cultures were incubated at 25 ± 2^oC and 16-8 photoperiod (1000-2000Lux). Observations were taken at regular intervals of one month. Sufficiently grown plantlets were transferred to a pots containing mixture of soil and sand (1:1) and were kept under room temperature. Humidity was maintained by covering each pot with polythene bags (Das, 1993). The established plantlets were then transplanted to the soil.

Results and Discussion

In the family Zingiberaceae, somatic embryogenesis has been reported in *Hedychium* (Verma and Bansal, 2012), *Kaempferia galanga* by Vincent et al (1992) and Lakshmi and Mythili (2003).

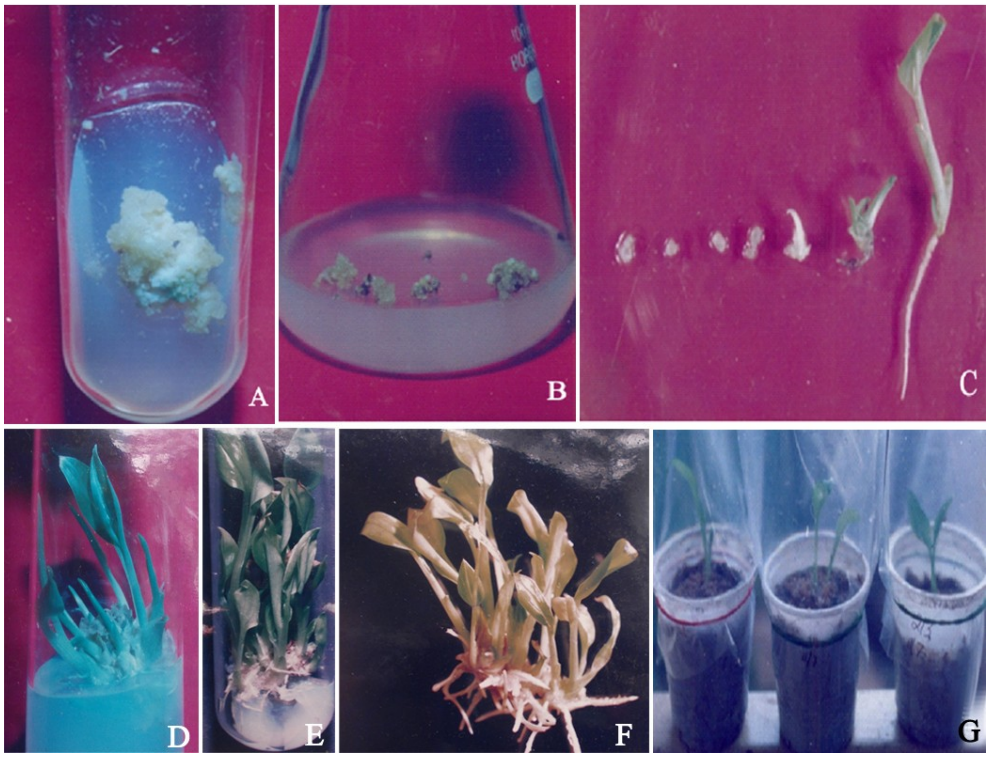
Embryonic callus were proliferated from the explants of *Kaempferia rotunda* Linn. after 14 days of inoculation on MS medium supplemented with high auxin/cytokinin (2.5mg/L. 2,4-D + 0.5mg/L BAP). This combination was found to be more effective for embryonic callus induction (Table.1; Fig. 1A & B). This callus upon transfer to the medium containing low auxin and high cytokinin produced friable embryos (Fig. 1C) .Up on keeping the callus, the yellowish green globular embryos start separating from the embryonic clumps. It was found that the initial embryonic competence induced because of unorganized growth of callus in the presence of 2,4-D. It is necessary to remove 2,4-D from the medium or reduce its concentration auxin to a considerable extend, when the callus achieved embryonic competency. Same was opined by Vincent et al (1992) while working with *Kaempferia galangal.* This was in corroboration with the findings of

Babu et al (1992, 1996). Moreover, the combined effect 2,4-D and BAP for the induction callus was very effective as explained by Agritius et al (1996) in *Alpinia calcarata*.

Rahman et al (2004) reported the plant regeneration technique through somatic embryogenesis from leaf base derived callus of *Kaempferia galanga*. Highest percentage of callus regeneration was obtained on MS medium supplemented with 2,4-D and BA. The regenerated plants showed 85% of establishment in the nursery. Saensouk (2011) reported somatic embryogenesis and plant regeneration in this same hormone combination in *Cornukaempferia aurantiflora*. Verma and Bansil (2012) developed an efficient protocol for somatic embryogenesis and plant regeneration of *Hedychium coronarium* J. Koenig on different concentration of auxin and *ex vitro* establishment of regenerated plants was 95%.

Transfer of fully developed embryos in to MS Medium containing only BAP (6.0mg/L) resulted maturation of embryonic plantlets. This pattern of embryonic callus was noticed by Samsudeen (1996) while working with ginger callus. The efficiency of embryonic callus was comparatively less (Table. 1). The maturation of embryos should be controlled by the auxin - cytokinin balance in the medium. As revealed by the present experiment, lesser frequency of embryogenesis may be due to the hormonal imbalance and lesser amount protein in the medium which may be cause the decreased mitotic division during the embryonic development.

Figure.1 Somatic embryogenesis and plant regeneration in *Kaempferia rotunda* Linn



A & B- Callus formation; C- Stages of plant regeneration; D & E- Plant multiplication; F- Plantlets ready for planting; G- Hardening

Table.1 Effect of 2, 4-D, BAP and Kinetin on somatic embryogenesis and plantlets formation from rhizome disc derived callus cultures of *Kaempferia rotunda* Linn in MS medium

Growth regulators	Concentration Mg/L	Morphogenetic Responses	Perc. of Responses	Average Number of Embryos/Culture ± SD	Average Number of Shoots/Culture ± SD
2,4-D+BAP	0.5+0.5	No response.	0.0	0.0	0.0
2,4-D+BAP	1.0+0.5	Slow callus formation	30%	0.0	0.0
2,4-D+BAP	2.0+0.5	Moderate Callus Induction	50%	3.5 ± 1.0	0.0
2,4-D+BAP	2.5+0.5	Embryogenic rounded callus induction	60%	7.8 ± 0.9	0.0
2,4-D+BAP	3.0+0.5	Callus induction reduced	40%	4.1 ± 0.8	0.0
2,4-D+BAP	0.25+1.0	Size of the Embryos increased	50%	3.5 ± 1.0	3.5 ± 1.0
2,4-D+BAP	0.25+2.0	Embryos and shoots growth noticed	50%	5.9 ± 0.8	4.1 ± 0.8
2,4-D+BAP	0.25+3.0	More globular embryos along with shoots	60%	5.9 ± 0.8	7.8 ± 0.9
BAP only	5.0	More growth of embryos noticed along with shoots	50%	5.4 ± 0.9	9.0 ± 0.7
BAP only	6.0	Embryos showed more growth along with shoots	50%	5.9 ± 0.8	9.0 ± 0.7
2,4-D+KIN	2.5+0.5	White callus induced	40%	0.0	0.0
2,4-D+KIN	0.25+3.0	Shoots and roots developed	60%	0.0	4.1 ± 0.8
KIN only	5.0	Only shoots and roots	40%	0.0	4.1 ± 0.8
KIN only	6.0	Only shoots and roots	50%	0.0	3.1 ± 1.0

MS=MS+30g/L Sucrose+0.8g/L Agar,

(All measurements after two months of *in vitro* incubation)

Encapsulation of somatic embryos

For encapsulation of somatic embryos, 1-5% solution of sodium alginate was prepared, containing the ingredients of MS Medium (devoid of calcium chloride) and with 1.0mg/L to 2.0mg/L BAP. The medium was autoclaved after adjusting the pH to 5.8 and cooled, and the somatic embryos were mixed with above preparation. The above mixture dropped in to the calcium chloride solution (25-150 µm) using a glass column fitted on a stand under Lamina air flow chamber. The various concentrations of sodium alginate tried and 3% was found to be most suitable for the medium textured bead production. The drops set as small transparent beads when left in the hydrated $CaCl_2$ solution for 30 minutes of curing. In *Elattaria cardamomum* the encapsulation of shoot tips were successfully done by Ganapath et al (1994). Sharma et al (1994) found that 4% sodium alginate was most suitable for the production of disease free encapsulated buds of *Zingiber officianale* Rosc. The beads were removed and washed with sterile double distilled water.

All the synthetic seeds were put in petriplates and stored at 3-4^{o}C.

The plantlets developed through somatic embryogenesis were transferred after three months of total growth directly to pots containing sterile sand and soil in the ratio of 1:1 (Fig. 1D, E& F). During hardening the pots were kept under room temperature by maintaining the humidity by covering it with polythene cover. The coverings were removed after two weeks and plantlets were established in the soil with 50% survival. Thus the present study emphasizes the somatic embryogenesis from the rhizome derived callus of *Kaempferia rotunda,* a precious medicinal plant.

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