INDIRECT ORGANOGENESIS AND EMBRYOGENESIS IN *Kaempferia galanga* L.

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THESIS

Submitted in Partial fulfilment of the
requirement for the degree

Master of Science in Horticulture

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KERALA AGRICULTURAL UNIVERSITY

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1997

DECLARATION

I hereby declare that the thesis entitled "**Indirect organogenesis and embryogenesis in *Kaempferia galanga* L.**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship, associateship or other similar title, of any other university or society.

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Certified that the thesis entitled "**Indirect organogenesis and embryogenesis in *Kaempferia galanga* L.**" is a record of research work done independently by **Mrs.Mini Joseph**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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ACKNOWLEDGEMENT

I express my deep sense of gratitude and sincere thanks to ***Mrs.Lissamma Joseph****, Assistant Professor, Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara and Chairperson of my Advisory Committee, for her keen interest, constant encouragement and inspiration and valuable guidance throughout the course of this investigation. My sincere and heartfelt gratitude ever remains with her.*

I wish to express my profound gratitude to ***Dr.E.V.Nybe****, Professor and Head i/c, Department of Plantation Crops and Spices, College of Horticulture and member of the Advisory Committee for his valuable suggestions and timely help during the course of my research work and preparation of the thesis.*

I am extremely grateful to ***Dr.P.A.Nazeem****, Associate Professor, College of Horticulture and member of the Advisory Committee for her valuable help, constructive criticisms and encouragement which assisted me much in the successful completion of my Masters programme.*

I am grateful to ***Dr.Luckins C. Babu****, Associate Professor, College of Forestry, Vellanikkara and member of the Advisory Committee for his timely help and valuable suggestions during the preparation of the thesis.*

I express my sincere gratitude to Dr.R.Vikraman Nair, Professor, Cadbury-KAU Co-operative Cocoa Research Project for his constant inspiration and for his good will in rendering me all help during the period of investigation.

A special word of thanks is due to Dr.R.Keshavachandran, Associate Professor, Department of Plantation Crops and Spices, College of Horticulture for taking photographs.

The help I received from Dr.N.K.Vijayakumar, Associate Professor, College of Forestry and Dr.A.M. Ranjit, Assistant Professor, Department of Entomology to make use of the stereomicroscope is gratefully acknowledged.

I am grateful to Dr.V.K.Mallika, Associate Professor, CCRP for her valuable suggestions.

I express my sincere thanks to all the staff members of the Department of Plantation Crops and Spices for their sincere help and valuable co-operation which helped me a lot for successful completion of my Masters programme.

I also wish to express my thanks to all the staff members and labourers of the Tissue Culture Laboratory for their help during the time of investigation.

A word of thanks to Sri.Joy for the neat typing and prompt service.

I wish to thank all my friends and fellow students for their immense help and encouragement throughout the period of investigation.

I have no words to express my thanks to my husband, parents and in-laws for their constant prayers, sincere encouragement, moral support and for bearing all the inconvenience caused all along this study.

Above all, I place my heartfelt gratitude and fervent indebtedness to God, the Almighty. for his bountiful blessings.

MINI JOSEPH

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ABBREVIATIONS

BA	- benzyl adenine
BAP	- benzyl amino purine
$^{\circ}C$	- degree celsius
cm	- centimeter
2,4-D	- 2,4-dichlora phenoxy acetic acid
EDTA	- Ethylene diamino tetra acetic acid
GA_3	- gibberellic acid
h	- hour(s)
IAA	- Indole-3-acetic acid
IBA	- Indole-3-butyric acid
2iP	- iso pentenyl adenine
KIN	- Kinetin, N^6-furfuryl adenine
mg l^{-1}	- milligram(s) per litre
μM	- micro molar
mM	- milli molar
MS	- Murashige and Skoog's (1962) medium
½ MS	- Murashige and Skoog's (1962) medium with half the salt concentration
NAA	- α-Napththalene acetic acid
pH	- hydrogen ion concentration
ppm	- parts per million
psi	- pounds per square inch
rm	- revolutions per minute
uv	- ultra violet
w/v	- weight in volume

Introduction

INTRODUCTION

Kaempferia galanga L., popularly known as 'Kacholam' is a zingiberaceous medicinal plant indigenous to India (Sivarajan and Balachandran, 1994) distributed in the tropics and subtropics of Asia, Africa and in Philippine Islands. Of the 55 species reported in the genus, only 10 are known in India, among which *K. galanga* L. and *K. rotunda* L. are the economically important ones. It is a rhizomatous herb with stem and leaves, spreading horizontally, lying flat on the surface of ground. The inflorescence, with 2 to 12 fragrant flowers, arise directly from the rhizome. Eventhough flowers are well adapted for pollination, no seed set has so far been reported (Rekha and Viswanathan, 1996).

In India *K. galanga* is cultivated through out the plains for its aromatic rhizome. The methanol extract of the rhizome contains ethyl P-methoxy trans-cinnamate which is highly cytotoxic to He La cells (Kosuge *et al.*, 1985). Recently, larvicidal and anticancer principles have been obtained from its rhizomes (Kosuge *et al.*, 1985; Kiuchi *et al.*, 1988). The hot water extract of *Kaempferia* rhizomes showed strong larvicidal activity against the larvae of dog round worm, *Toxocara canis* (Kiuchi *et al.*, 1988). The constituents of the rhizome include cineol, borneol, 3-carene, camphene, kaempferol, kaempferide, cinnamaldehyde, P-methoxy cinnamic acid and an ethyl cinnamate.

Rhizomes of *K. galanga* are used in bulk quantities in ayurvedic medicine and agarbathi industries. It finds an important place in indigenous medicine as a stimulant, expectorant, diuretic and carminative. Because of these multivarious attributes, the export value of this crop has increased in recent years. Dried rhizomes

fetch a market price of Rs.40 per kg in the year. The suitability for intercropping in coconut gardens has attracted a good number of farmers in a state like Kerala for undertaking the cultivation of Kacholam.

The conventional method of propagation of *K. galanga* is through rhizomes. Tissue culture/micropropagation has been proved to be a viable method of propagule multiplication in this crop (Vincent *et al.*, 1991). Improvement of this crop by conventional methods of breeding is not possible for want of seed production. Seedlessness also contributes to the limited variability available with the crop. In this context the advantage of callus mediated organogenesis and embryogenesis can be exploited to complement the existing natural variability. Thus, an investigation was undertaken to develop a suitable protocol for indirect organogenesis and embryogenesis in *Kaempferia* with a view to increase/induce the spectrum of variability.

Review of Literature

REVIEW OF LITERATURE

Kaempferia galanga L. is a zingiberaceous medicinal plant. The plant is valued for its aromatic underground rhizomes which possesses stimulatory, expectorant, carminative and diuretic properties.

The conventional method of propagation of *Kaempferia galanga* is through rhizomes. Improvement of this crop by conventional method of breeding is not possible for want of seed production. Seedlessness also contributes to the limited variability available with the crop. Sathyabhama (1988) reported that presence of spines on stigmatic surface and excessive length of style prevented seedset in ginger. Similarly spiny stigma and lengthy style contributed towards seedlessness in *K. galanga* also (Rekha and Viswanathan, 1996).

Plant tissue culture enables faster rate of propagule production and also provides exciting possibilities for creation of variability in crop plants. This is of utmost importance in plants like *Kaempferia galanga* in which the natural variability is less.

2.1 General aspects of tissue culture

The cell theory postulated by Schleiden (1838) and Schwann (1839) which reveals the totipotent nature of plant cells is the basis of plant tissue culture. Haberlandt (1902) reported that isolated cells were capable of resuming uninterrupted growth. Skoog and Miller (1957) put forth the concept of hormonal control of organ formation and showed that the differentiation of roots and shoots was a function of auxin cytokinin ratio and that organ differentiation could be

regulated by changing the relative concentration of these substances in the medium. The early development of plant tissue culture was based on the efforts of many pioneering investigators including White (1934), Gautheret (1939), Nobecourt (1939), Miller *et al.* (1956), Reinert (1958), Steward *et al.* (1958), Bergmann (1960) and Vasil and Hildebrandt (1965). It was Murashige and Skoog (1962), who developed a completely defined nutrient medium for plant tissue culture.

Murashige (1974; 1977) had devised three possible routes for *in vitro* propagule multiplication.

1. Enhanced release of axillary buds
2. Production of adventitious buds through organogenesis
3. Somatic embryogenesis

2.1.1 Enhanced release of axillary buds

Morel (1960) reported application of shoot apex culture for rapid clonal multiplication of plants for the first time. The greatest success using this technique has been achieved in herbaceous horticultural plants. The success may be due to the weak apical dominance and strong root regenerating capacity of the herbaceous plants (Hu and Wang, 1983). In this route, primary meristems like shoot tips and axillary buds are cultured which assure genetic uniformity of the progeny to a great extent (Rao and Lee, 1986). Successful release of axillary buds has been reported in many medicinal and aromatic plants like *Glycirrhiza glabra* (Shah and Dalal, 1980), *Catharanthus roseus, Thymus vulgaris* (Bajaj *et al.*, 1988), *Adhatoda vasica* (Jaiswal *et al.*, 1989), *Tylophora indica* (Sharma and Chandel, 1992), *Adhatoda beddomei* (Sudha and Seeni, 1994) and *Aristolochia indica* (Kavitha and Raju, 1995).

2.1.2 Somatic organogenesis

Levels of plant growth regulating substances in the culture medium, particularly high auxins and low cytokinins, often lead to callus formation. On the other hand, if the auxin level is reduced in the medium, it may lead to adventitious shoot formation (Skoog and Miller, 1957). Though callus may be obtained from virtually any species, only in some plants it can be regenerated. The reason for this inability may be due to the higher proportion of polyploid or aneuploid cells in those callus (Smith and Street, 1974). Somatic organogenesis may be direct or callus mediated. Callus mediated somatic organogenesis is ideal for recovering useful mutant lines to complement the existing natural variability (Hussey, 1986).

Plant regeneration through direct or indirect organogenesis has been reported in many medicinal and aromatic plants. In aromatic grasses like lemon-grass, citronella and palmarosa callus regeneration has been reported by Mathur *et al.* (1989). They could obtain high yielding somaclonal variants through callus regeneration of leaf sheath explants. Similar report on organogenesis is in *Gloriosa superba* (Somani *et al.*, 1989).

Plant regeneration from callus cultures of *Piper longum* was achieved through organogenesis. IAA in combination with BA in MS medium was necessary for organogenesis as reported by Bhat *et al.* (1992). Shoot regeneration could be obtained from callus derived from leaf and stem segments of *Gomphrena officinalis* in MS media supplemented with BAP and α-NAA. MS media containing IBA resulted in rooting of the regenerated shoots as reported by Mercier *et al.* (1992).

2.1.3 Somatic embryogenesis

Somatic embryogenesis is the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes. The first report of somatic embryogenesis was given by Reinert (1959) in carrot cultures. General pattern of *in vitro* embryogenesis includes direct initiation from differentiated tissue and indirect initiation via callus intermediary. Direct embryogenesis proceeds from embryogenic determined cells (Kato and Kateuchi, 1963). Indirect embryogenesis requires differentiation of the cells, callus proliferation and differentiation of embryogenic cells (Sharp *et al.*, 1980). The positive results are limited to a few species, but is a more rapid mode of plant regeneration (Evans *et al.*, 1981). It requires auxin medium for the induction of embryos and a medium devoid of growth regulators for its maturation (Ammirato, 1983). Hussey (1986) also reported that the level of growth regulators in the culture medium, particularly when the auxin level was lowered, there was the chance for the embryo formation.

Reports on somatic embryogenesis had been published by Murashige (1978), Sharp *et al.* (1979), Vasil and Vasil (1980) in various crops. Cheng and Raghavan (1985) could obtain somatic embryos where pattern of development was similar to that of zygotic embryos. The somatic embryos were formed from friable calli produced from petiole and ovary explants in medium containing 2,4-D. Embryogenesis was initiated when the friable calli were cultured in a medium lacking auxin. In *Nardostachys jatamansi* it was found that embryogenesis could be initiated from callus upon subculture to a medium containing lesser auxin (NAA) and more cytokinin (kinetin) while the concentrations of NAA was to be more and

kinetin less for callus initiation as reported by Mathur (1993). Induction of somatic embryogenesis by different growth regulators was observed in leaf disc cultures of *Nicotiana tabaccum* L. by Gill and Saxena (1993). Successful somatic embryogenesis has also been reported in many crops like *Coffea arabica* (Sondahl *et al.*, 1979), *Carica papaya* (Litz and Connover, 1982), *Malus pumila* (James *et al.*, 1984), *Oryza sativa* (Raghava Ram and Nabors, 1984), *Citrus limon* (Carini *et al.*, 1994) and *Elaeis guineensis* (Teixeira *et al.*, 1994).

2.2 *In vitro* studies in *Kaempferia galanga*

Vincent *et al.* (1991) reported successful establishment of callus cultures from vegetative buds of *Kaempferia galanga* on MS medium supplemented with 2,4-Dichlorophenoxy acetic acid (2,4-D) and 6-benzyl amino purine (BAP). Maximum regeneration capacity was exhibited with 1.5 mg l^{-1} BAP and 1 mg l^{-1} NAA. They have also reported successful field establishment of *in vitro* grown plantlets with normal growth as the field grown plants. Vincent *et al.* (1992a) reported micro-propagation of *K. galanga* from axillary buds and the subsequent field establishment of plantlets. Maximum number of plantlets were produced in MS medium containing BAP alone or BAP and Kinetin.

Embryogenesis from callus cultures of *K. galanga* had also been reported by Vincent *et al.* (1992b). Rhizome explants with vegetative buds developed highly embryogenic callus when cultured in MS medium supplemented with BAP and 2,4-D (0.5 mg l^{-1} and 1.0 mg l^{-1} respectively). Callus subcultured on MS medium supplemented with BAP and NAA (0.1 mg l^{-1} and 1.0 mg l^{-1}) produced small globular embryoids, on the peripheral layers, which further developed into plants when transferred to hormone free medium.

Ajithkumar and Seeni (1995) obtained multiple shoot formation in *K. galanga* through axillary bud proliferation, indirect organogenesis and somatic embryogenesis in MS medium supplemented with appropriate growth regulators. They also observed variations in the leaf shape and size to varying degrees in two month old transplanted plants obtained through different routes.

2.3 *In vitro* studies in other Zingiberaceous members

2.3.1 Enhanced release of axillary buds

Clonal propagation of ginger under *in vitro* condition through enhanced release of axillary bud was first reported by Hosoki and Sagawa (1977). Buds from rhizome in storage were cultured on a medium consisting of MS major elements, Ringe-Nitsch minor elements and vitamins, two per cent sucrose and BA 1 mg l^{-1}. Similar results were also obtained by Nadgauda *et al.* (1980). Pillai and Kumar (1982) found that the sprouted buds of ginger rhizome grew and established well when they were cultured on SH medium also. De Lange *et al.* (1987) developed a method for elimination of nematodes from ginger by tissue culture. Later on a number of reports on successful micropropagation of ginger were published (Bhagyalakshmi and Singh, 1988; Ikeda and Tanabe, 1989; Inden *et al.*, 1988 and Choi, 1991a). Choi (1991b) found that rooting of shoots were enhanced by the addition of two per cent activated charcoal to the medium. Dogra *et al.* (1994) reported that when ginger rhizome buds were cultured on MS medium supplemented with 2.5 mg BA + 0.5 mg NAA l^{-1} produced greatest number of multiple shoots.

In turmeric (*Curcuma longa* L.), Nadgauda *et al.* (1978) reported that the young sprouting buds of turmeric rhizome when excised and cultured in

modified MS medium on Smith's medium supplemented with coconut water (10 per cent), Kinetin (0.1 ppm) and BA (0.2 ppm) gave good growth and plantlet formation. Plantlets were obtained in turmeric when bud tissues were cultured on MS medium supplemented with Kinetin 1 mg l^{-1}, BA 1 mg l^{-1} and sucrose 50 g l^{-1} (Keshavachandran and Khader, 1989). Similar results were also obtained by Balachandran *et al* in 1990.

The first report on the application of shoot tip culture for rapid clonal propagation in cardamom was published by Nagauda *et al.* (1983). Reghunath (1989) also obtained *in vitro* clonal propagation in cardamom. Rapid shoot proliferation was reported in *Costus specious* when shoot tips excised from rhizomes were cultured in modified SH medium containing BA (0.5 ppm), IAA (1.0 ppm) and adenine sulphate (15.0 ppm) (Chaturvedi *et al.*, 1984). Ferworda (1994) obtained *in vitro* clonal propagation from rhizome buds of Languas *Alpinia galanga* in a medium with MS salts, vitamins, two per cent sucrose and BA 2 mg l^{-1}. Illg and Faria (1995) reported that inflorescence buds inoculated on Murashige and Skoog medium (MS) containing 10 μM 6-benzyladenine with 5 μM naphthalene acetic acid gave rise to multiple shoot formation in *Alpinia purpurata* K. Schum.

2.3.2 Callus mediated organogenesis

Ilahi and Jabeen (1987) have successfully produced ginger plantlets through callus mediated organogenesis. They reported that half strength of MS inorganic was more favourable for micropropagation of ginger than full strength. Choi (1991c) also reported callus mediated organogenesis in ginger from explants of pseudostem containing one leaf blade. Callus was produced in MS medium containing 0.5 ppm NAA. shoot and root regeneration was obtained in a medium

containing 0.1 to 1.0 ppm NAA and 1.0 ppm BA. Malamug *et al.* (1991) reported plantlet regeneration and propagation from ginger callus. The highest degree of callus formation was obtained from the shoot tips cultured on a medium consisting of Murashige and Skoog major elements, Ringe and Nitsch minor elements and organic additives, 2% sucrose, 0.8% agar with 2,4-D 0.5 mg l^{-1} and BA 1 mg l^{-1}. The highest regeneration was obtained on the medium with BA concentration of 1 and 3 mg l^{-1}. Babu *et al.* (1992b) have also reported callus mediated regeneration in ginger. The presence of 2,4-D in the medium (9 to 22.6 μM) resulted in callus growth. Organogenesis and plantlet formation occurred when the concentration of 2,4-D was reduced to 0.9 μM and with the addition of 44.4 μM BA into the medium.

In turmeric, Shetty *et al.* (1980) reported that when sprouting rhizome buds were cultured on a modified MS medium supplemented with kinetin (0.2 ppm), BA (0.4 ppm) and GA_3 (0.01 ppm), their bases swelled up leading either to the formation of a translucent soft friable callus or a bunch of adventitious roots. When regeneration was through callus, on an average 10-12 plantlets per culture tube could be obtained during a culture period of 6-8 weeks.

Rao *et al.* (1982) observed callus production when five-week-old *in vitro* grown cardamom seedlings were cultured on MS medium containing 2.0 ppm of either 2,4-D, IAA or NAA and 2 ppm BA and 18.0 per cent coconut water. On subculturing to a medium devoid of 2,4-D but containing IAA (2.0 ppm) or NAA (1.0 ppm), green nodular structures could be developed in the callus indicating the initiation of organised growth structures. Four to six shoot buds could be obtained from each callus subculture of uniform size. Reghunath (1989) reported that the

induction, growth, and differentiation of callus in cardamom was the best when shoot meristem were grown in MS medium containing NAA 4 ppm + BA 1.0 ppm or NAA 2 ppm + IAA 1.0 ppm and BA 1.0 ppm. The calli obtained from the treatment combination involving NAA and BA or Kinetin were compact and semi-hard in nature which when subcultured to an auxin free medium containing BA 3.0 ppm + Kinetin 0.5 ppm or BA 2.0 ppm and Kinetin 1.0 ppm induced shoot organogenesis.

Jain and Chaturvedi (1985) obtained callusing in costus (*Costus speciosus* Sm) by culturing rhizome bud sprouts on a modified SH medium containing adenine sulphate (10 ppm), BA (0.25 ppm) and IAA (0.5 ppm). Rhizogenesis followed by shoot formation occurred when they were subcultured on the same medium. Normal shoots were obtained when the regenerated shoots were subcultured in a medium containing IAA (2.0 ppm), adenine sulphate (15.0 ppm) and BA (0.25 ppm).

2.3.3 Somatic embryogenesis

Kacker *et al.* (1993) reported somatic embryogenesis in ginger. The leaf segments in MS medium with eight per cent sucrose and 2.7 μM Dicamba resulted embryogenesis. IAA and NAA were not effective in producing embryogenic cultures. Plant regeneration was achieved when embryogenic cultures were transferred to MS medium containing 8.9 μM BA. Histological studies revealed various stages of somatic embryogenesis characteristic of monocot system. The *in vitro* raised plants were successfully established in soil.

Reghunath (1989) obtained somatic embryogenesis in cardamom in MS medium containing NAA 4 ppm + BAA 1.0 ppm or NAA 2.0 ppm + IAA 1.0 ppm + BA 1.0 ppm. Treatments involving 2,4-D though produced profuse callusing, they failed to initiate somatic embryogenesis. For induction of somatic embryogenesis, the callus from shoot meristem (from culture) was better than others.

2.4 Somatic organogenesis or embryogenesis in other monocotyledons

Vander Valk *et al.* (1992) obtained high frequency somatic embryogenesis and plant regeneration from zygotic embryo-derived callus cultures of *Allium cepa, Allium fistulosum* and *Allium porvum*. Compact embryogenic callus was induced on Murashige and Skooge (MS) medium supplemented with 2,4-D. The plants could be regenerated at a high frequency from this compact callus through somatic embryogenesis, when using Kinetin supplemented MS medium. Addition of abscisic acid to the regeneration medium stimulated the formation both somatic embryos and shoots. Ruiz *et al.* (1992) reported that there was a significant genotype x explant interaction in the initiation of embryogenic callus in barley. Some phenotypic variants were detected among the regenerated plants and most were originated by epigenetic changes. Nair and Seo (1993) observed that BDS medium with 4.5 μM 2,4-D and 13.3 μM BA to be the most suitable for shoot multiplication from callus initiated from flower buds of *Allium senescens* var. minor.

Callus induction was obtained from basal parts of *Vetiveria zizanoids* Stapf. leaves cultured on Murashige and Skoog (MS) medium supplemented with 9.0 μM 2,4-D, 5.7 μ IAA and 4.6 μM Kinetin. Shoot formation was obtained from fast growing 14-day-old callus on the same basal medium supplemented with 0.9 μM

2,4-D and 9.3 μM Kinetin (Mucciarelli *et al.*, 1993). Teixeira *et al.* (1993) obtained somatic embryogenesis from immature zygotic embryos of oil palm on modified Y3 medium containing cystein 500 mg l^{-1}, PVP-40 0.5% (w/v), 2,4-D 500 μM and charcoal 0.3% (w/v). Normal embryo and plantlet development was observed only from friable embryogenic tissue.

Denchev and Conger (1995) reported that callus could be initiated from mature caryopsis of switch grass (*Panicum virgatum* L.) on a medium containing 11.3 to 45 μM 2,4-D in combination with 15 or 45 μ 6-benzyladenine (BA). Addition of low concentration (0.1-0.3 mg l^{-1}) of BA to Murashige and Skoog (MS) callus induction medium containing 1 or 2 mg l^{-1} 2,4-D stimulated somatic embryogenesis and strongly increased the percentage of seeds producing shoot forming callus in *Poa pratensis* (Vander Valk *et al.*, 1995).

Bregitzer *et al.* (1995) reported that there was a positive relationship between 2,4-D concentration and normal karyotype in plants regenerated from barley callus. The percentage of diploid callus in 16-week-old callus peaked at 20.4 μM 2,4-D.

Hong and Debergh (1995) obtained high frequency plant regeneration via somatic embryogenesis from *in vitro* shoot base cultures of seedlings of gardenleek (*Allium porum* L.). They obtained embryogenic callus from nodular callus in BDS medium with 9 mM 2,4-D + 7.6 mM abscisic acid. Plant regeneration was maximum with 9.8 mM N^6 (2-isopentenyl) adenine. The presence of 2,4-D in the medium and light condition were shown to be essential for nodular callus induction and somatic embryogenesis.

Rout and Lucas (1996) produced embryogenically competent cells and optimized the embryogenic responses for *in vitro* cultured immature inflorescence of rice *indica* cultivar Ptb-31, Pallavi and V 20 A and *japonica* cultivar Calmochi 101, fujisaka 5 and Taipeizoa. Histological and morphological analysis revealed that the parenchymatous ground tissue present in the second whorl of sterile bracts and base of fertile bracts was involved in the embryogenic response.

Materials and Methods

MATERIALS AND METHODS

The present study pertaining to indirect organogenesis and embryogenesis in *Kaempferia galanga* L. was carried out in the Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara, Thrissur, during the period from 1994 to 1996. The materials used and methodology adopted for the study are described in this chapter.

3.1 Production of axenic plants as explant source

In vitro plants were raised following the methodology already standardised in the laboratory (KAU, 1994). From this axenic plants, parts like leaf bit, pseudostem bits and pseudostem base were taken as explants for callus induction.

3.1.1 Collection and preparation of explants

Rhizomes were collected from the medicinal plant garden attached to the All India Co-ordinated Research Project on Medicinal and Aromatic Plants at Vellanikkara. Sprouts arising from mature rhizomes were used as explants for raising *in vitro* plants. The sprouting buds were excised from the rhizomes, washed thoroughly in tap water, removed the scale leaves and dipped in teepol solution for 10 minutes. These sprouts were then washed with distilled water and taken to the laminar flow chamber for further surface sterilization.

3.1.2 Surface sterilization

Surface sterilization of the explants was carried out under perfect aseptic

condition maintained in a laminar air flow cabinet which was made contamination free using uv lamp. The working table and sides of the laminar flow were thoroughly wiped with absolute alcohol.

The explants were dipped in 50 per cent alcohol for two minutes. They were then treated with mercuric chloride for seven minutes with frequent agitation. After surface sterilization, the mercuric chloride solution was drained off and the explants were washed thrice with sterile water to make it free of the chemical. The explants were then drained on a blotting paper before inoculation.

3.1.2 Culture media

The basal media suggested by Murashige and Skoog (MS medium) 1962, modified MS medium with half the concentration of inorganic nutrients (½ MS), Gamborg *et al.*, 1968 (B5), Schenk and Hildebrandt, 1972 (SH) were used for the study. The composition of different basal media tried are given in Annexure I. The best basal medium was identified and used for further studies.

3.2 **Media preparation**

The various chemicals used for preparation of media were of analytical grade supplied by SISCO Research Laboratories, SRL, British Drug House (BDH), Merck and Sigma. Borosilicate glass wares of Corning/Borosil brand were used for the study. They were cleared by initially soaking in potassium dichromate solution in sulphuric acid for half an hour, followed by thorough washing with jets of tap water in order to completely remove all traces of dichromate solution. They were then soaked in detergent solution (Teepol 0.1%) over night, thoroughly washed with tap water and rinsed twice with double distilled water. The glass wares were then dried

in a hot air oven at 100°C for 24 hours. They were then stored in cupboards away from contaminants and dust until used.

Standard procedure (Gamborg and Shyluk, 1981) was followed for the preparation of media. Stock solutions of major and minor elements were prepared first and were stored under refrigerated condition in amber coloured bottles. The amino acids and vitamin stocks were also prepared separately. Fresh stock of amino acids and vitamins, were prepared at every six weeks interval. The stock solutions for various phytohormones were prepared as per Sigma, 1991 and stored in refrigerated conditions.

An aliquot of different stock solutions was pipetted out into a clean vessel, which was rinsed with distilled water. Sucrose and inositol were added fresh and dissolved. Required quantities of growth regulators were also added and the solution was made upto the required volume. The pH of the solution was adjusted between 5.5 to 5.8 using in NaOH or HCl.

Inorder to prepare the semisolid media, good quality agar (BDH) was added (0.7% w/v) and the solution was heated for melting the agar. The bulk media was poured into culture vessels and plugged with cotton plugs. Different types of culture vessels like test tubes (15 x 25 cm) jam bottles (3 cm diameter, 250 ml volume) and conical flask (100 ml) were used for the study. Sterilization of the media was done in an autoclave by applying 15 psi pressure for 20 minutes. After sterilization, the culture vessels were stored in an air conditioned culture room for further use.

3.3 Inoculation procedure

Inoculation was carried out under strict aseptic condition in a laminar air flow cabinet. Sterilized forceps, petridishes, surgical blades and blotting paper were used. The surface sterilized explants were carefully inoculated into the media.

3.4 Culture conditions provided

The cultures were incubated in a culture room provided with fluorescent lamps to give a light intensity of 3,000 lux for 16 hours light period. The temperature was maintained at $26^{\circ} \pm 2^{\circ}$C. Humidity in the culture room varied between 60 and 80 per cent according to the climate prevailed.

3.5 Callus induction

From the axenic plants, leaf bits, pseudostem bits and pseudostem base were taken as explants.

3.5.1 Basal media

MS medium, ½ MS medium, B5 medium and SH medium were tried for callus induction.

3.5.2 Growth regulators

Different combinations of auxins like 2,4-D (0.1-10.0 mg l^{-1}), NAA (0.1-5.0 mg l^{-1}) and cytokinins like kinetin (0.1-5.0 mg l^{-1}), 2ip (0.25-10.0 mg l^{-1}), BA (0.25-10.0 mg $^{-1}$) were incorporated in the basal media for inducing callus from the different explants. The details of combinations tried are given in Annexure-II.

3.5.3 Carbon source

Sucrose at three per cent concentration was used as source of carbon.

The cultures were incubated in a culture room under dark condition. A culture period of sixty days was allowed for each treatment and observations were taken at fortnightly interval. Observations were recorded on number of days taken for inducing calli, growth rate of calli and callus morphology. Callus index was worked out as follows.

$$CI = P \times G$$

where P is the percentage of callus initiation and G is the growth score.

Scoring was done based on the spread of the calli and a maximum score of four was given for those that have occupied the whole surface of the media within sixty days of culture period in culture tubes.

3.6 Indirect organogenesis

The calli maintained in half strength MS medium supplemented with 2,4-D 1 mg l^{-1} were used as starting material for obtaining indirect organogenesis.

3.6.1 Growth regulators

Different combinations of auxins like 2,4-D (0.1-10.0 mg l^{-1}), NAA (0.1-5.0 mg l^{-1}), cytokinins like kinetin (0.25-6.0 mg l^{-1}), 2ip (0.25-6.0 mg l^{-1}) and BA (0.25-10.0 mg l^{-1}) and GA_3 (0.5-2.0 mg l^{-1}) were incorporated in the media. The details of combinations tried are given in Annexure III.

3.6.2 Other media additives

Other media additives like silver nitrate and activated charcoal were incorporated in the media to evaluate the effects on organogenesis. The details of different concentrations used are given in Annexure-IV.

3.6.3 Carbon source

Sucrose at three per cent concentration was used as source of carbon.

3.6.4 Culture conditions provided

The cultures were incubated in the culture room both under light and dark conditions.

Suspension cultures were maintained in conical flask and shaken at 100 rpm on an incubator shaker. The details of combinations tried for suspension culture are given in Annexure-V.

The response of the calli was observed and recorded at fortnightly intervals. Observations were taken on the percentage of cultures responded, nature of response and the average number of shoots/roots produced per culture per treatment. The plantlets produced were subcultured into the same medium at monthly interval.

3.7 Somatic embryogenesis

3.7.1 Induction of somatic embryogenesis

The calli maintained in ½ MS medium supplemented with 2,4-D 1 mg l^{-1} were used as starting material.

3.7.1.1 Growth regulator

Different combinations of NAA (1.0-5.0 mg l^{-1}) and cytokinins like BA (0.25-10.0 mg l^{-1}) and Kinetin (0.25-6.0 mg l^{-1}) were tried. The details of combinations tried are given in Annexure-VI.

3.7.1.2 Carbon source

Sucrose at three per cent concentration was used as carbon source.

The cultures were maintained both under light and dark conditions. The cultures were viewed under stereomicroscope and observations were taken at weekly interval on the nature of calli produced and percentage of cultures showing embryogenesis.

3.7.2 Embryo maturation

The embryogenic calli at early stages of embryogenesis were transferred to maturation media. The different media combinations tried were ½ MS medium with three per cent sucrose, MS medium with three per cent sucrose, MS medium with five per cent sucrose and MS medium with five per cent sucrose supplemented with BA at 0.5, 1.0, 1.5 and 2.0 mg l^{-1} concentrations. Observations were taken on the average number of mature embryoids produced per culture vessel, the percentage of cultures producing mature embryoids and the number of plantlets produced per culture.

3.8 Hardening and plant out

Plantlets with well developed leaves and roots were taken out of the culture tubes, medium was washed off and planted in small polythene bags (11 cm x

8 cm) filled with potting mixtures. The polybags were kept in the nethouse and watering was done daily. After four weeks the plantlets were transferred to pots of size seven inch and kept under full sunlight.

Field evaluation

The plantlets obtained through indirect organogenesis, embryogenesis, direct organogenesis and conventional method of propagation were planted in pots. Ten pots were maintained in each type. The observations were taken for three months at fortnightly interval. Observations were recorded on percentage survival of plants in the field, number of tillers produced, leaf area and leaf orientation. Leaf area was calculated using the formula

$$\text{Leaf area} = 3.99 + 0.617 \times l \times b$$

where 'l' is the length of leaf and 'b' is the breadth.

The data were compared for evaluating the performance of tissue culture derived plantlets with that of conventionally propagated plants.

Results

RESULTS

The results of the investigations on indirect organogenesis and embryogenesis in *Kaempferia galanga* conducted during 1994-'96 in the Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara are presented in this chapter.

4.1 Production of axenic plants as explant source

The results of the trial for standardisation of basal media are presented in Table 1. Among the various basal media tried, MS and half strength MS media gave the highest sprouting percentage and maximum tiller production at 30 DAI. Sprouting percentage was the highest in half strength MS medium (70.0) followed by full MS medium (68.9). The average number of tillers produced per treatment was same (3) in both the cases. The plants rooted in the same medium and these cultures were used as explant source for callus induction (Plate 1).

4.2 Callus induction

4.2.1 Standardisation of basal media and explants

The data on the response of different explants for callus induction in different basal media, when supplemented with 2,4-D are presented in Table 2. Among the explants tried, pseudostem base recorded the highest callusing percentage (27.8) at 30 DAI (Plate 2). But at 45 and 65 DAI callusing percentage (72.7 and 81.8) was the highest for pseudostem bit explants (Plate 3). The response of leaf segments to callusing was slow and poor (Plate 4). Callus induction was initiated only 45 DAI of leaf explants. Eventhough callusing was observed in full MS media,

Plate 1. Axenic plants produced from sprouts arising from mature rhizome explant source

Plate 2. Callus induced from pseudostem base explants in medium supplemented with 2,4-D 4 mg l^{-1}

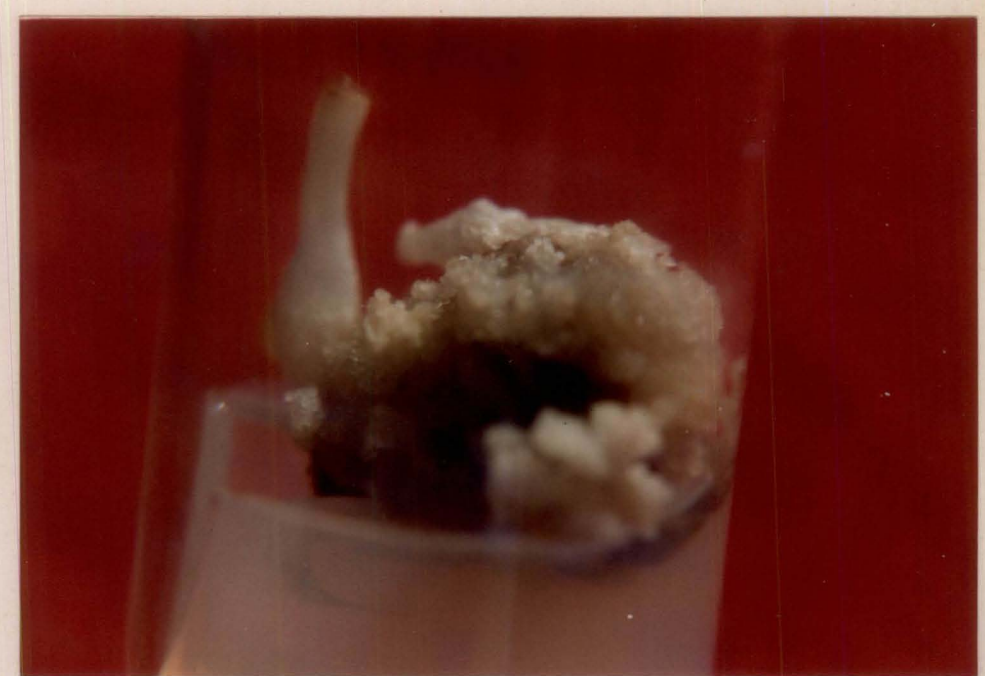


Plate 3. Callus induced from pseudostem bits in medium supplemented with 2,4-D 4 mg l^{-1}

Plate 4. Callus induced from leaf segments in medium supplemented with 2,4-D 4 mg l^{-1}

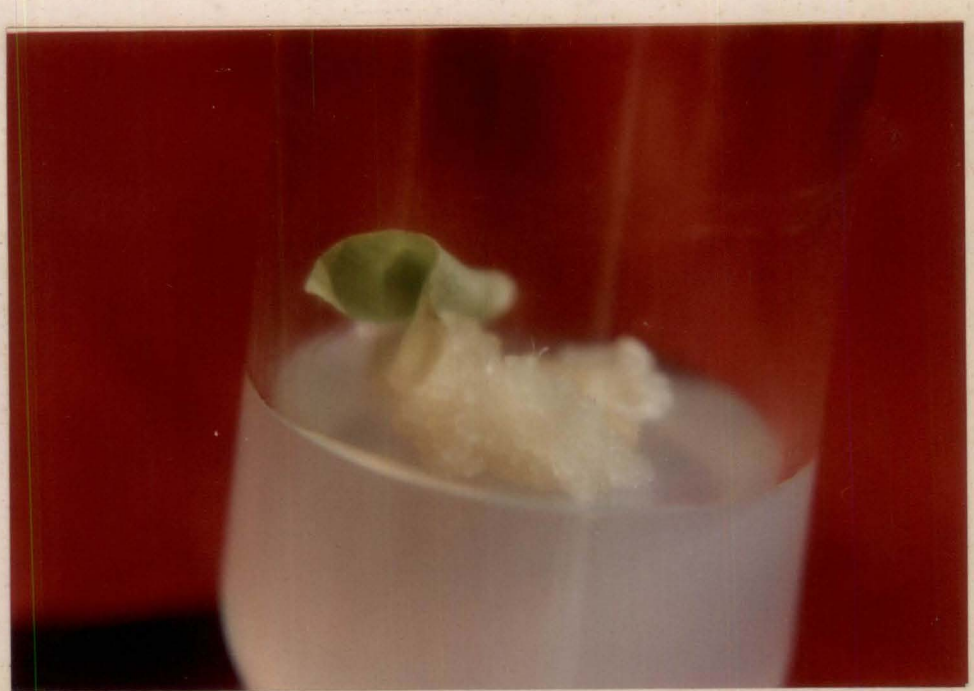


Table 1. Response of different basal media for production of *in vitro* plants from sprouts arising from mature rhizomes of *K. galanga*

Treatments	Sprouting percentage 30 DAI	Average number of tillers/ explant 30 DAI
MS	68.9	3.0
½ MS	70.0	3.0
B5	6.0	1.0
SH	9.0	1.0

Table 2. Response of different explants in different basal media supplemented with 2,4-D 4 mg l^{-1} for callus induction in *K. galanga*

Explants	½ MS			MS			B5			SH		
	Callusing percentage DAI			Callusing percentage DAI			Callusing percentage DAI			Callusing percentage DAI		
	30	45	60	30	45	60	30	45	60	30	45	60
Leaf segment	-	22.2	55.6	-	-	11.1	-	-	-	-	-	-
Pseudostem bits	26.7	72.7	81.8	-	16.7	22.2	-	-	-	-	-	-
Pseudostem base	27.8	38.9	38.9	5.5	5.5	5.5	-	-	-	-	-	-

DAI - Days after inoculation

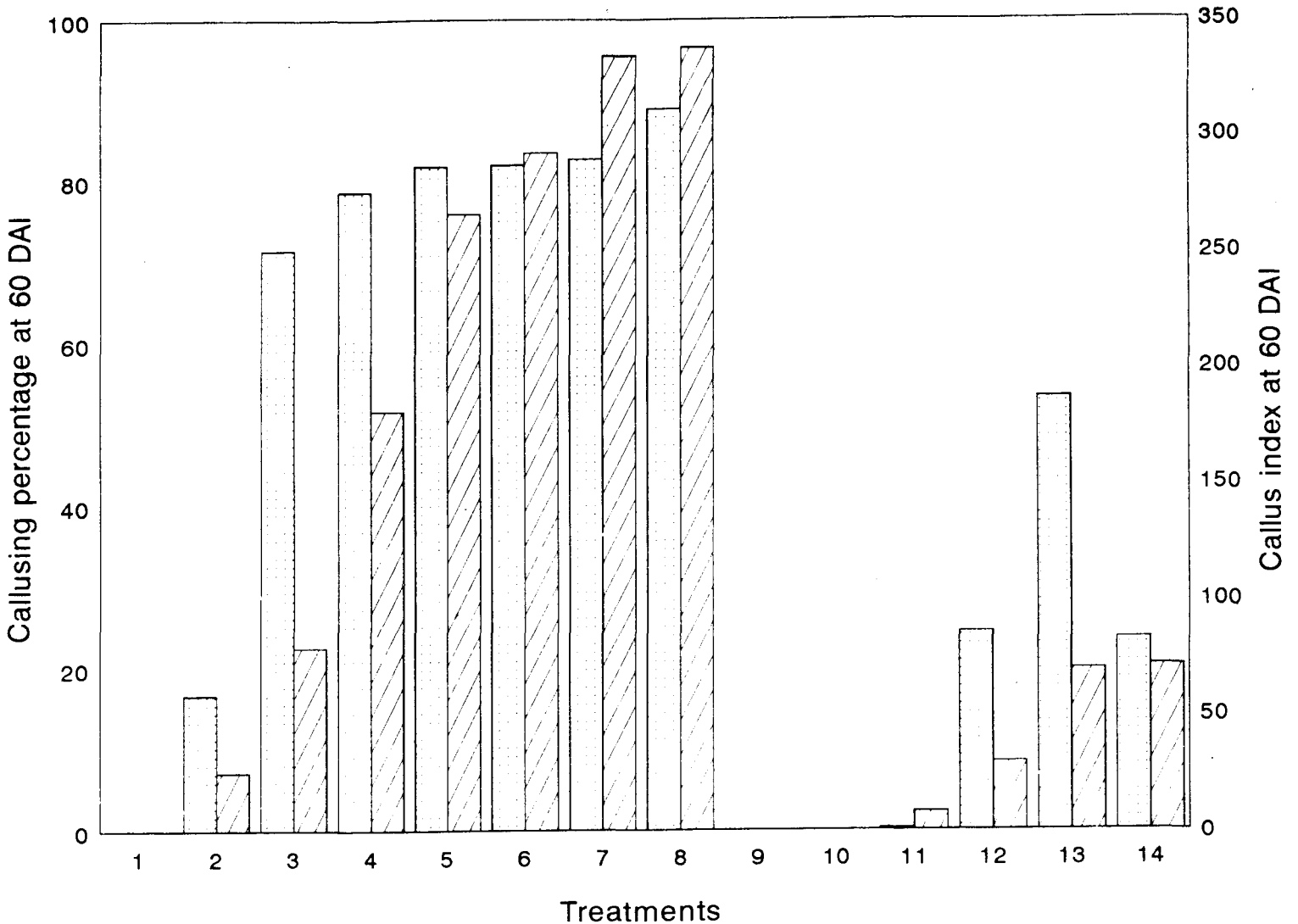


Fig.1. Effect of auxins on callus induction and later proliferation in pseudostem bit explants kept under dark condition

callusing percentage was less. The calli produced from all the explants proved to have regeneration potential in later experiments. Callusing was not initiated in B_5 and SH medium even 60 DAI.

4.2.2 Effect of growth regulators

4.2.2.1 Response to auxins

The data on the response to various combinations of auxins on induction of calli from pseudostem bit explant are presented in Table 3 and Fig.1. 2,4-D was found to be the best compared to NAA for inducing calli and for its further proliferation. It was observed that percentage of callusing increased with increasing concentration of 2,4-D. Maximum callusing percentage and callus index were recorded with 10 mg l^{-1} 2,4-D. At 0.1 mg l^{-1} concentration of 2,4-D, callus was not induced even 60 DAI. Whereas, a callusing percentage of 71.4 and callus index of 78.6 were obtained with 1.0 mg l^{-1} 2,4-D at 60 DAI. At all the levels of 2,4-D used, the calli produced were creamy and friable with good morphogenic potential.

With NAA at concentrations of 0.1 and 0.5 mg l^{-1}, no callus induction was observed even after 60 days culture period. Increasing the concentration of NAA to 3 mg l^{-1}, resulted in callus induction after 30 days of inoculation. Further, proliferation and growth of calli initiated at 3 mg l^{-1} concentration of NAA was good. Higher concentration of NAA (5 mg l^{-1}) was found to delay callus production. The calli induced in the presence of NAA showed morphogenetic potential. Production of hairy structures was observed in the calli initiated in the presence of NAA.

Table 3. Effect of auxins on callus induction and later proliferation in pseudostem bit explants kept under dark condition in *K. galanga*

Basal media ½ MS

Treatment No.	Treatments (mg l^{-1})	Callusing percentage			Callus index 60 DAI	Callus morphology
		30 DAI	45 DAI	60 DAI		
T_1	2,4-D 0.1	-	-	-	-	--
T_2	2,4-D 0.5	-	16.7	16.7	25.0	Cream, friable with morphogenic potential
T_3	2,4-D 1.0	14.2	71.4	71.4	78.6	,,
T_4	2,4-D 2.0	22.8	71.1	78.6	180.7	,,
T_5	2,4-D 4.0	26.7	72.7	81.8	265.9	,,
T_6	2,4-D 6.0	42.2	75.6	82.0	292.2	,,
T_7	2,4-D 8.0	50.0	80.7	82.7	334.3	,,
T_8	2,4-D 10.0	44.4	88.9	88.9	337.8	,,
T_9	NAA 0.1	-	-	-	-	--
T_{10}	NAA 0.5	-	-	-	-	--
T_{11}	NAA 1.0	-	-	0.2	8.0	Cream, friable, hairy with morphogenic potential
T_{12}	NAA 2.0	-	4.0	24.4	29.2	,,
T_3	NAA 3.0	5.5	33.3	53.3	69.3	,,
T_{14}	NAA 5.0	-	-	23.6	71.0	Cream, compact callus with hairy structure

4.2.2.2 Combined effect of auxins with cytokinins

The effect of different growth regulator combinations for callus induction from pseudostem bit explants is presented in Table 4. Maximum response to callusing and high callus index were observed with combinations of 2,4-D (1.0 mg l^{-1}) and BA 0.25, 0.5, 0.75 and 1 mg l^{-1}. The highest callusing percentage (92.3) and callus index (193.9) were recorded with 2,4-D 1.0 mg l^{-1} and BA 0.25 mg l^{-1} concentration. The effectiveness of 2,4-D in callus initiation and proliferation was found to be enhanced with the addition of lower concentrations of BA (0.25 to 1.0 mg l^{-1}). However, higher concentrations of BA (1.0 to 10.0 mg l^{-1}) with 2,4-D expressed inhibitory effect on callus initiation and growth. Combination of low concentration of BA (0.25 to 2.0 mg l^{-1}) with 2,4-D produced cream coloured, friable and hairy callus with good morphogenic potential whereas higher levels of BA (4.0 to 10.0 mg l^{-1}) with 2,4-D resulted in browning of callus with poor morphogenic potential.

The callus inducing property of 2,4-D was found to be nullified with the addition of kinetin. The explants turned yellowish and dried after 60 days of inoculation. Combination of 2ip 0.25 to 8.0 mg l^{-1} with 2,4-D 0.5 and 1.0 mg l^{-1} showed positive response for callusing. But addition of 2ip reduced the effectiveness of 2,4-D for callus induction. 2,4-D 0.5 and 1.0 mg l^{-1} with all the levels of 2ip resulted in brown coloured callus except in combination of 2,4-D (0.5 mg l^{-1}) with 2ip at 0.25 and 0.5 mg l^{-1} in which cream coloured friable and hairy calli were produced.

Table 4. Effect of different growth regulator combinations on callus induction and later proliferation in pseudostem bit explants in *K. galanga*

Basal media: ½ MS

Treatments (mg l^{-1})	Callusing percentage			Callus index	Remarks
	30 DAI	45 DAI	60 DAI	60 DAI	
1	2	3	4	5	6
2,4-D 0.5 + BA 0.25	11.1	55.6	66.7	29.6	Cream, friable and hairy callus with regeneration potential
2,4-D 0.5 + BA 0.5	22.2	44.4	44.4	33.3	,,
2,4-D 0.5 + BA 0.75	20.0	30.0	60.0	35.0	,,
2,4-D 0.5 + BA 1.0	10.0	20.0	50.0	50.0	,,
2,4-D 0.5 + BA 2.0	21.4	42.3	42.3	21.4	,,
2,4-D 0.5 + BA 4.0	25.0	31.3	50.0	18.6	Pale brown, friable callus
2,4-D 0.5 + BA 6.0	21.4	50.0	50.0	21.4	,,
2,4-D 0.5 + BA 8.0	-	12.5	18.8	3.1	,,
2,4-D 0.5 + BA 10.0	-	5.9	5.9	2.9	Brown, compact callus
2,4-D 1.0 + BA 0.25	30.8	84.6	92.3	193.9	Cream, friable and hairy calli with regeneration potential
2,4-D 1.0 + BA 0.5	50.0	82.9	82.9	167.1	,,
2,4-D 1.0 + BA 0.75	22.2	77.8	77.8	124.4	,,
2,4-D 1.0 + BA 1.0	16.7	58.3	75.0	82.5	,,
2,4-D 1.0 + BA 2.0	14.3	57.1	57.1	36.5	,,
2,4-D 1.0 + BA 4.0	8.3	25.0	25.0	16.6	Brownish, hairy callus
2,4-D 1.0 + BA 6.0	12.5	25.0	43.8	22.8	,,
2,4-D 1.0 + BA 8.0	5.5	11.1	33.3	22.2	,,
2,4-D 1.0 + BA 10.0	11.1	16.7	16.7	11.1	Brownish, compact and hairy callus

Contd.

Table 4. Continued

1	2	3	4	5	6
2,4-D 0.5 + Kin 0.1	-	-	-	-	Explant turned yellowish and dried
2,4-D 0.5 + Kin 0.2	-	-	-	-	,,
2,4-D 0.5 + Kin 0.5	-	-	-	-	,,
2,4-D 0.5 + Kin 1.0	-	-	-	-	,,
2,4-D 0.5 + Kin 2.0	-	-	-	-	,,
2,4-D 0.5 + Kin 3.0	-	-	-	-	,,
2,4-D 0.5 + Kin 4.0	-	-	-	-	,,
2,4-D 0.5 + Kin 5.0	-	-	-	-	,,
2,4-D 1.0 + Kin 0.1	-	-	-	-	,,
2,4-D 1.0 + Kin 0.2	-	-	-	-	,,
2,4-D 1.0 + Kin 0.5	-	-	-	-	,,
2,4-D 1.0 + Kin 1.0	-	-	-	-	,,
2,4-D 1.0 + Kin 2.0	-	-	-	-	,,
2,4-D 1.0 + Kin 3.0	-	-	-	-	,,
2,4-D 1.0 + Kin 4.0	-	-	-	-	,,
2,4-D 1.0 + Kin 5.0	-	-	-	-	,,
2,4-D 0.5 + 2ip 0.25	-	20.0	20.0	6.6	Cream, friable and hairy callus
2,4-D 0.5 + 2ip 0.5	-	26.7	26.7	23.0	,,
2,4-D 0.5 + 2ip 0.75	-	8.3	8.3	8.3	Pale brown, friable and hairy callus
2,4-D 0.5 + 2ip 1.0	-	6.7	13.3	6.6	,,
2,4-D 0.5 + 2ip 2.0	-	15.3	15.3	11.5	,,
2,4-D 0.5 + 2ip 4.0	-	7.1	17.1	5.3	,,

Contd.

Table 4. Continued

1	2	3	4	5	6
2,4-D 0.5 + 2ip 6.0	-	13.3	13.3	6.6	Brown, compact calli
2,4-D 0.5 + 2ip 8.0	-	7.1	7.1	3.5	,,
2,4-D 0.5 + 2ip 10.0	-	-	-	-	--
2,4-D 1.0 + 2ip 0.25	-	25.0	25.0	12.5	Pale brown, friable and hairy callus
2,4-D 1.0 + 2ip 0.5	-	13.3	20.0	6.6	,,
2,4-D 1.0 + 2ip 0.75	-	7.6	15.4	7.7	,,
2,4-D 1.0 + 2ip 1.0	-	13.1	13.0	13.3	,,
2,4-D 1.0 + 2ip 2.0	-	7.1	21.4	16.0	,,
2,4-D 1.0 + 2ip 4.0	-	12.5	12.5	9.4	Pale brown, compact calli
2,4-D 1.0 + 2ip 6.0	-	7.7	7.7	6.9	Brownish compact calli
2,4-D 1.0 + 2ip 8.0	-	2.1	6.7	4.6	,,
2,4-D 1.0 + 2ip 10.0	-	-	-	-	--

- No response

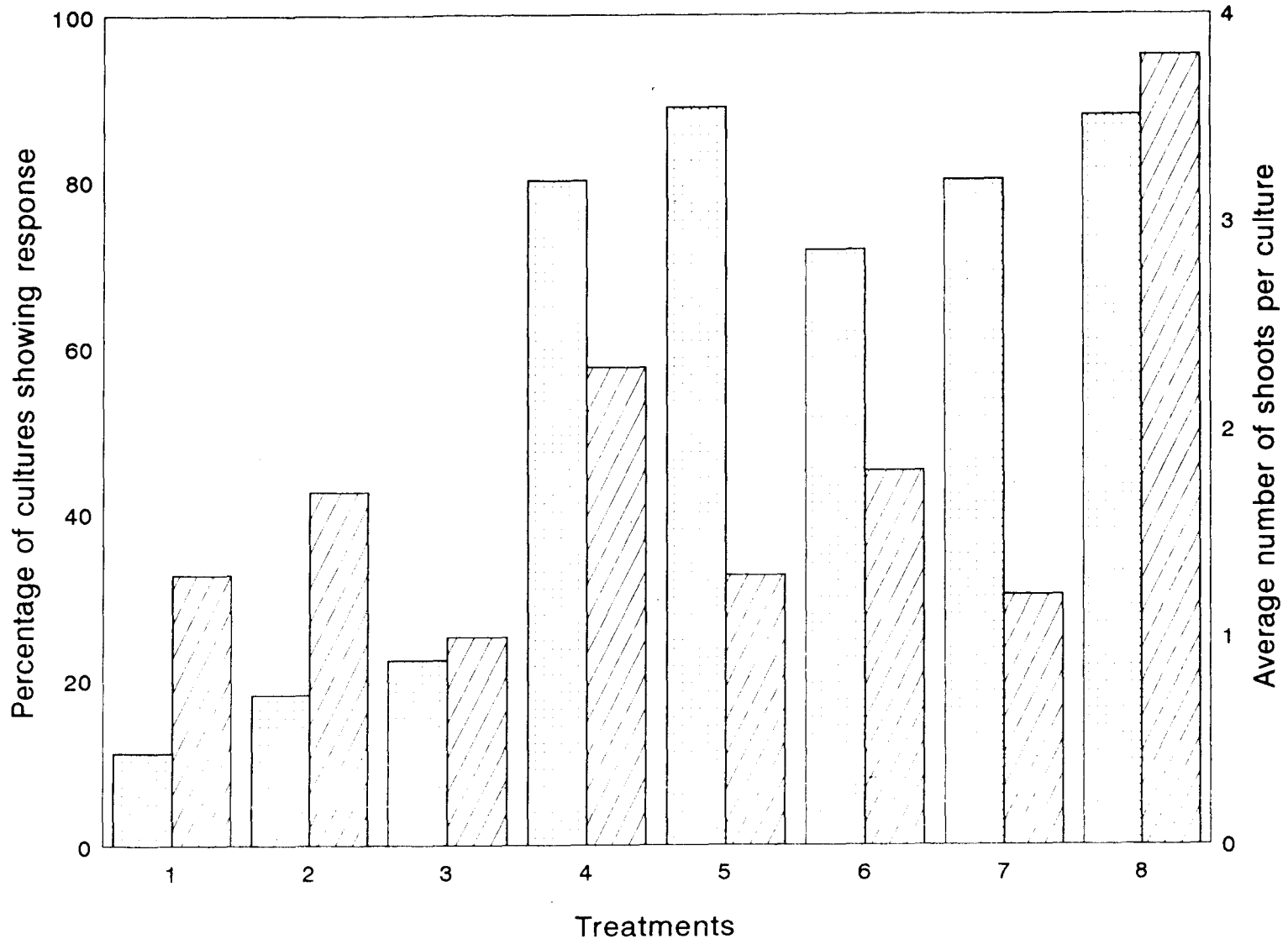


Fig.2. Effect of kinetin on indirect organogenesis

4.3 Indirect organogenesis

4.3.1 Effect of different growth regulator combinations

4.3.1.1 Combined effect of varying levels of BA and 2,4-D on indirect organogenesis

The effect of different combinations of 2,4-D and BA for indirect organogenesis under light and dark conditions is presented in Table 5. Rhizogenesis was recorded when combination of BA 0.25 to 8.0 mg l^{-1} with 2,4-D, 0, 0.1 and 0.2 mg l^{-1} were tried both under light and dark conditions (Plate 5). When BA alone was used at different concentrations (0.25 to 8.0 mg l^{-1}), positive response for shoot initiation was observed. In the absence of 2,4-D, presence of light turned the colour of calli to green at different levels of BA. The cultures incubated in darkness remained cream and friable with thin roots. At 8.0 mg l^{-1} concentration of BA, shoot formation was obtained with an average number of 5.6 shoots per culture and 96.0 per cent of cultures recorded shoot formation. Under dark incubation, 8.0 mg l^{-1} BA induced white, compact, shiny, globular calli. BA at 10.0 mg l^{-1} did not favour callus growth or regeneration with or without 2,4-D.

4.3.1.2 Effect of kinetin

Kinetin was effective in inducing shoot buds from the calli cultured under light (Table 6 and Fig.2). The highest number of shoots (3.8) was obtained with kinetin at 6.0 mg l^{-1} concentration 25 days after inoculation. Rhizogenesis was absent in all the treatments under both light and dark incubation. Under darkness, higher concentrations of kinetin (2.0 to 6.0 mg l^{-1}) induced white, globular, compact calli in culture, which were also observed at kinetin 6 mg l^{-1} maintained under light.

Table 5. Combined effect of varying levels of BA and 2,4-D on indirect organogenesis in *K. galanga*

Basal media - ½MS

(Explant - calli maintained in ½ MS medium supplemented with 2,4-D 1 mg l^{-1})

Treatments (mg l^{-1})	Light				Dark		
	Nature of response	Percentage of cultures showing response	Average No. per culture		Nature of response	Average No. per culture	
			Roots	Shoots		Roots	Shoots
2,4-D 0.2 + BA 0.25	Cream friable calli and green roots	38.5	1	-	Cream, friable, callus and root initiation	3	-
2,4-D 0.2 + BA 0.5	,,	44.4	4	-	,,	3	-
2,4-D 0.2 + BA 0.75	,,	63.6	4	-	Cream, friable calli and thin roots	2	-
2,4-D 0.2 + BA 1.0	Cream friable calli and thin hairy roots	57.1	7	-	Cream, friable calli and thin hairy roots	4	-
2,4-D 0.2 + BA 2.0	,,	58.4	8	-	,,	6	-
2,4-D 0.2 + BA 4.0	,,	55.6	6	-	,,	6	-
2,4-D 0.2 + BA 6.0	,,	37.5	5	-	,,	3	-
2,4-D 0.2 + BA 8.0	Cream friable calli with thin root	12.5	7	-	,,	2	-

Continued

Treatments ($mg\ l^{-1}$)	Light				Dark		
	Nature of response	Percentage of cultures showing response	Average No. per culture		Nature of response	Average No. per culture	
			Roots	Shoots		Roots	Shoots
2,4-D 0.2 + BA 10.0	Calli turned brown and dried	--	-	-	Calli turned brown and dried	-	-
2,4-D 0.1 + BA 0.25	Cream friable calli and green roots	93.0	8	-	Cream friable calli and root initiation	2	-
2,4-D 0.1 + BA 0.5	,,	92.5	7	-	,,	4	-
2,4-D 0.1 + BA 0.75	,,	92.8	8	-	,,	3	-
2,4-D 0.1+ BA 1.0	Cream friable calli and very thin roots	100.0	12	-	,,	7	-
2,4-D 0.1 + BA 2.0	,,	87.5	10	-	,,	8	-
2,4-D 0.1 + BA 4.0	,,	72.0	11	-	,,	9	-
2,4-D 0.1 + BA 6.0	,,	80.0	9	-	,,	4	-
2,4-D 0.1 + BA 8.0	,,	66.6	9	-	Cream, granular calli	-	-

Continued

Treatments ($mg\ l^{-1}$)	Light				Dark		
	Nature of response	Percentage of cultures showing response	Average No. per culture		Nature of response	Average No. per culture	
			Roots	Shoots		Roots	Shoots
BA 0.25	Light green friable callus with roots	28.6	7	-	Cream friable calli	-	-
BA 0.5	Cream friable callus and thin roots	44.4	8	-	Cream friable calli	-	-
BA 0.75	,,	37.5	10	-	Cream friable calli with thin roots	6	-
BA 1.0	,,	57.1	9	-	,,	9	-
BA 2.0	,,	42.8	7	-	,,	5	-
BA 4.0	,,	71.6	5	-	,,	4	-
BA 6.0	,,	80 0	5	-	,,	3	-
BA 8.0	Green, nodular hairy callus and shoot formation	96.0	-	5.6	White, compact shiny, globular calli	-	-
BA 10.0	Turned brown and dead	-	-	-	Turned brown and dead	-	-

Observations taken on 25 DAI

Table 6. Effect of Kinetin on indirect organogenesis in *K. galanga*

Basal medium - ½ MS

(Explant - calli maintained in ½ MS medium supplemented with 2,4-D 1 mg l^{-1})

Treatment No	Treatments (mg l^{-1})	Light				Dark		
		Nature of response	Percentage of cultures showing response	Average No. per culture		Nature of response	Average No. per culture	
				Roots	Shoots		Roots	Shoots
T_1	Kin 0.25	Green nodular calli and shoot buds	11.1	-	1.3	Cream friable calli	-	-
T_2	Kin 0.5	,,	18.1	-	1.7	,,	-	-
T_3	Kin 1.0	,,	22.2	-	1.0	,,	-	-
T_4	Kin 2.0	Green nodular calli, shoot formation and production of white globular structure	80.0	-	2.3	Cream friable calli and production of white globular compact structure	-	-
T_5	Kin 3.0	Green nodular calli & shoots	88.8	-	1.3	,,	-	-
T_6	Kin 4.0	Green nodular calli & shoot buds and white globular structure	71.6	-	1.8	,,	-	-
T_7	Kin 5.0	,,	80.0	-	1.2	,,	-	-
T_8	Kin 6.0	Green noduiar calli, shoot formation and production of white globular structure	87.8	-	3.8	,,	-	-

Observations were taken on 25 DAI

4.3.1.3 Effect of 2ip

The cytokinin 2ip was not effective in inducing indirect organogenesis in *Kaempferia galanga* (Table 7). 2ip at a concentration of 2.0 mg l^{-1} induced cream nodular calli and white globular structures in the presence of light. The percentage of cultures responded was only 18.2. Dark incubation in the same treatment also resulted in induction of white globular structures. At higher concentrations of 2ip (2.0 and 6.0 mg l^{-1}) the calli turned brown without any positive response.

4.3.1.4 Effect of GA_3

GA_3 did not express either promoting or inhibiting effect on indirect organogenesis in combination with 8.0 mg l^{-1} concentration of BA in ½ MS medium under light incubation (Table 8). In the presence of light, all the treatments (GA_3 0.5 to 2.0 mg l^{-1}) induced shoots. Maximum percentage of cultures showing response (93.3) and the highest number of shoots per culture (3.5) were observed with GA 0.5 mg l^{-1}. When the concentration of GA_3 was increased, the number of shoots produced per culture was reduced. Under dark condition only callus growth was recorded. Rhizogenesis was not observed under light or dark culture condition.

4.3.1.5 Effect of other media additives

The effect of varying levels of media additives like silver nitrate and activated charcoal on indirect organogenesis both under light and dark culture conditions are presented in Table 9. When $AgNO_3$ at different levels were tried, only rhizogenesis could be obtained under light and dark incubation. In the presence of light, $AgNO_3$ at 5.0 and 10.0 mg l^{-1} concentration induced green friable calli with

Table 7. Effect of 2ip on indirect organogenesis in *K. galanga*

Basal medium - ½ MS

(Explant - calli maintained in ½ MS medium supplemented with 2,4-D 1 mg l^{-1})

Treatments (mg l^{-1})	Light				Dark		
	Nature of response	Percentage of cultures showing response	Average No. per culture		Nature of response	Average No per culture	
			Roots	Shoots		Roots	Shoots
2ip 0.25	Cream, friable calli	30	-	-	Cream, friable calli	-	-
2ip 0.5	,,	20	-	-	,,	-	-
2ip 1.0	,,	18.2	-	-	,,	-	-
2ip 2.0	Cream, nodular calli and production of white globular structure	18.2	-	-	Cream, friable calli and white globular structure	-	-
2ip 4.0	Turned brown	-	-	-	Turned brown	-	-
2ip 6.0	Turned brown	-	-	-	Turned brown	-	-

Observations were taken 25 DAI

Table 8. Effect of GA_3 on indirect organogenesis in *K. galanga*

Basal media - ½MS

(Explant - calli maintained in ½ MS medium supplemented with 2,4-D 1 mg l^{-1})

Treatments (mg l^{-1})	Light				Dark		
	Nature of response	Percentage of cultures showing response	Average No. per culture		Nature of response	Average No. per culture	
			Roots	Shoots		Roots	Shoots
BA 8 + GA_3 0.5	Green friable callus + shoot formation	93.3	-	3.5	Turned brown	-	-
BA 8 + GA_3 1.0	Green friable calli + shoot	63.3	-	2.6	,,	-	-
BA 8 + GA_3 1.5	Green nodular calli + shoot	64.2	-	2.1	Cream friable calli	-	-
BA 8 + GA_3 2.0	,,	63.0	-	2.0	,,	-	-

Observations were taken on 25 DAI

Table 9. Effect of other media additives on indirect organogenesis in *K. galanga*

Basal media - ½MS

(Explant - calli maintained in ½ MS medium supplemented with 2,4-D 1 mg l^{-1})

Treatments	Light				Dark		
	Nature of response	Percentage of cultures showing response	Average No. per culture		Nature of response	Average No. per culture	
			Roots	Shoots		Roots	Shoots
$AgNO_3$ 1.0 mg l^{-1}	Cream friable calli and root initiation	60	15	-	Cream friable calli and rooting	10	-
$AgNO_3$ 5.0 mg l^{-1}	Green friable calli and green root initiation	46.7	7	-	Cream friable calli and rooting	8	-
$AgNO_3$ 10.0 mg l^{-1}	,,	64.2	6	-	,,	7	-
$AgNO^3$ 15.0 mg L_1	Cream friable calli with the hairy structure	46.7	3	-	Cream friable calli	-	-
$AgNO_3$ 20.0 mg l^{-1}	Turned brown and dead	-	-	-	Turned brown	-	-
Activated charcoal 0.1%	Cream friable calli and root initiation	29.4	2	-	Cream friable calli and rooting	4	-
Activated charcoal 0.2%	Cream friable calli	60.0	-	-	Cream friable calli	-	-
Activated charcoal 0.5%	,,	12.5	-	-	,,	-	-
Activated charcoal 0.8%	,,	25.0	-	-	,,	-	-

Observations were taken 25 DAI

Plate 5. Rhizogenesis on callus cultured in the medium supplemented with 2,4-D 0.2 mg l^{-1} and BA 0.5 mg l^{-1}

Plate 6. White, compact, shiny embryogenic calli induced in MS medium supplemented with NAA 3.0 mg l^{-1} and BA 1.0 mg l^{-1}

green roots. At 20 mg l^{-1} concentration of $AgNO_3$, the calli turned brown and dead later.

Rhizogenesis could also be obtained at 0.1 per cent level of activated charcoal. All the treatments involving activated charcoal induced callus growth. In the presence of light, activated charcoal at 0.8 per cent concentration induced green friable calli whereas in all the other cases cream friable calli were produced.

4.3.2 Effect of different liquid media combinations on indirect organogenesis/ embryogenesis

None of the treatments tried could induce indirect organogenesis in suspension culture (Table 10). Eventhough callus growth was observed in combination of 2,4-D 1.0 mg l^{-1} and BA 1.0 mg l^{-1} in half strength MS medium and NAA 3.0 mg l^{-1} in ½ MS, they turned brown 30 DAI. The combination of 2,4-D 1.0 mg l^{-1} and BA 0.5 mg l^{-1} in ½ MS medium produced granular structures (33.3%). But further regeneration could not be obtained from these structures.

4.4 Somatic embryogenesis

4.4.1 Induction of somatic embryogenesis

4.4.1.1 Effect of different basal media and culture conditions

The response of different basal media and culture conditions on induction of somatic embryoids from the callus are presented in Table 11. For induction of somatic embryogenesis, full strength MS medium was more favourable than ½ MS. Combinations of BA 1.0 mg l^{-1} with NAA 3.0 mg l^{-1} and 1.0 mg l^{-1} in MS medium induced embryogenic calli (48.2% and 28.6% respectively) (Plate 6). But in half strength MS medium the same treatments induced a mixture of embryogenic and

Table 10. Effect of different liquid media combinations on indirect organogenesis/ embryogenesis in *K. galanga*

Treatments (Growth regulators in mg l^{-1})	Type of response		Percentage of cultures showing response
	15 DAI	30 DAI	
½ MS	--	--	--
½ MS + 2,4-D 0.5 + BA 0.5	--	--	--
½ MS + 2,4-D 0.5 + BA 1.0	--	--	--
½ MS + 2,4-D 0.5 + BA 2.0	--	--	--
½ MS + 2,4-D 0.5 + BA 4.0	--	--	--
½ MS + 2,4-D 0.5 + BA 6.0	--	--	--
½ MS + 2,4-D 1.0 + BA 0.5	Globular structures	Turned brown	33.3
½ MS + 2,4-D 1.0 + BA 1.0	Callus growth	Turned brown	33.3
½ MS + 2,4-D 1.0 + BA 2.0	--	--	--
½ MS + 2,4-D 1.0 + BA 4.0	--	--	--
½ MS + 2,4-D 1.0 + BA 6.0	--	--	--
½ MS + NAA 1.0	--	--	--
½ MS + NAA 3.0	Callus growth	Turned brown	33.3

- No response

Table 11. Effect of different basal media and culture conditions on induction of somatic embryogenesis in *K. galanga*

Starting material - White compact callus

Treatment (Growth regulators in mg l^{-1})	Dark			Light		
	Prcentage of cultures induced			Percentage of cultures inducing		
	Embryogenic with early stages of embryoids	Nonembryogenic calli	Mixed calli	Embryogenesis with early stages of embryoids	Nonembryogenic calli	Mixed calli
½ MS + NAA 1.0 + BA 0.5	0	100	0	0	100	0
½ MS + NAA 1.0 + BA 1.0	0	41.7	58.3	0	75	25
½ MS + NAA 1.0 + BA 1.5	0	83.3	16.7	0	100	0
½ MS + NAA 1.0 + BA 2.0	0	100	0	0	100	0
½ MS + NAA 3.0 + BA 0.5	0	100	0	0	100	0
½ MS + NAA 3.0 + BA 1.0	0	87.5	12.5	0	90	10
½ MS + NAA 3.0 + BA 1.5	0	100	0	0	100	0
½ MS + NAA 3.0 + BA 2.0	0	100	0	0	100	0
MS + NAA 1.0 + BA 0.5	0	78.8	22.2	0	87.5	12.5
MS + NAA 1.0 + BA1.0	28.6	0	71.4	0	91.7	8.3
MS + NAA 1.0 + BA 1.5	0	85.8	14.2	0	91.7	8 3
MS + NAA 1.0 + BA 2.0	0	90.0	10.0	0	100	0
MS + NAA 3.0 + BA 0.5	0	100	0	0	100	0
MS + NAA 3.0 + BA 1.0	48.2	0	51.8	0	75	25
MS + NAA 3.0 + BA 1.5	0	25.0	75.0	0	100	0
MS + NAA 3.0 + BA 2.0	0	100	0	0	100	0

Observations were taken 30 DAI

Plate 7. Callus showing tendency for embryogenesis and organogenesis simultaneously

Plate 8. The callus showing tendency for organogenesis and embryogenesis simultaneously in medium supplemented with NAA 3.0 mg l^{-1} and BA 1.5 mg l^{-1}

Table 12. Effect of different growth regulators on induction of somatic embryogenesis in *K. galanga*

Treatments (mg l^{-1})	Percentage of cultures inducing		
	Embryogenesis with initial stages of embryoids	Nonembryogenic calli	Mixed calli
1	2	3	4
½ MS + BA 0.25	0	100	0
½ MS + BA 0.5	0	100	0
½ MS + BA 0.75	0	100	0
½MS + BA 1.0	0	100	0
½ MS + BA 2.0	0	100	0
½ MS + BA 4.0	0	100	0
½ MS + BA 6.0	0	100	0
½ MS + BA 8.0	0	4	96
½ MS + Kin 0.25	0	89	11
½ MS + Kin 0.5	0	82	18
½ MS + Kin 1.0	0	78	22
½ MS + Kin 2.0	0	20	80
½ MS + Kin 3.0	0	12	88
½ MS + Kin 4.0	0	29	71
½ MS + Kin 5.0	0	20	80
½ MS + Kin 6.0	0	13	87
½ MS + NAA 1.0	0	100	0
½ MS + NAA 2.0	0	100	0
½ MS + NAA 3.0	0	94.5	5.5
½ MS + NAA 5.0	0	100	0

Contd.

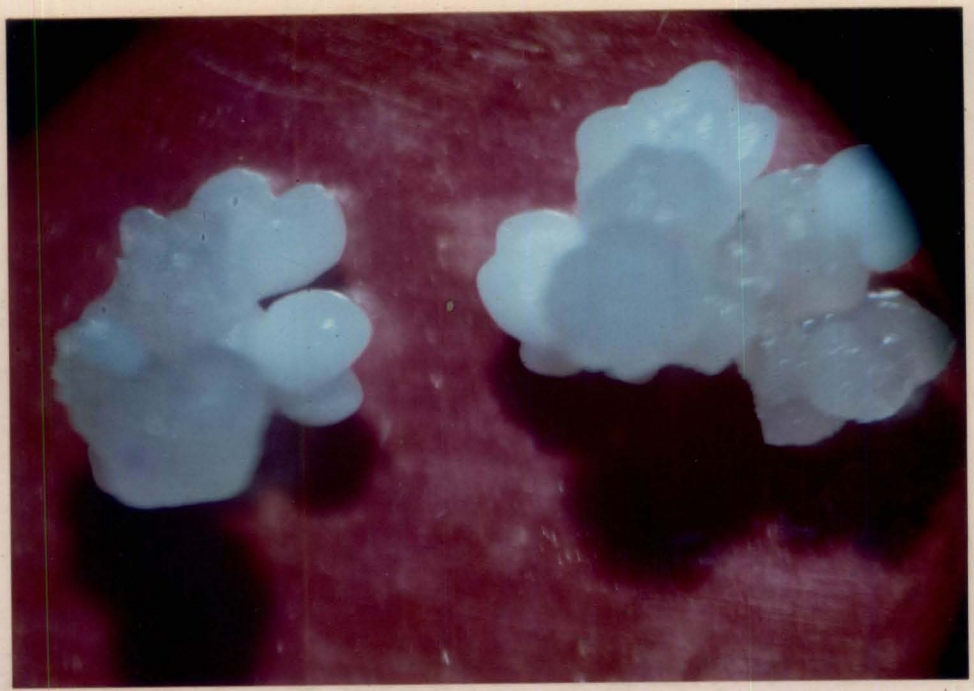
Table 12. Continued

1	2	3	4
½ MS + NAA 0.5 + BA 0.5	0	100	0
½ MS + NAA 0.5 + BA 1.0	0	100	0
½ MS + NAA 0.5 + BA 1.5	0	100	0
½ MS + NAA 0.5 + BA 2.0	0	100	0
½ MS + NAA 1.0 + BA 0.5	0	100	0
½ MS + NAA 1.0 + BA 1.0	0	41.7	58.3
½ MS + NAA 1.0 + BA 1.5	0	83.3	16.7
½ MS + NAA 1.0 + BA 2.0	0	100	0
½ MS + NAA 3.0 + BA 0.5	0	100	0
½ MS + NAA 3.0 + BA 1.0	0	87.5	12.5
½ MS + NAA 3.0 + BA 1.5	0	100	0
½ MS + NAA 3.0 + BA 2.0	0	100	0
MS + NAA 0.5 + BA 0.5	0	100	0
MS + NAA 0.5 + BA 1.0	0	91	9
MS + NAA 0.5 + BA 1.5	0	100	0
MS + NAa 0.5 + BA 2.0	0	100	0
MS + NAA 1.0 + BA 0.5	0	78.8	22.2
MS + NAA 1.0 + BA 1.0	28.6	0	71.4
MS + NAa 1.0 + BA 1.5	0	85.8	14.2
MS + NAA 1.0 + BA 2.0	0	90.0	10.0
MS + NAA 3.0 + BA 0.5	0	100	0
MS + NAA 3.0 + BA 1.0	48.2	0	51.8
MS + NAA 3.0 + BA 1.5	0	25.0	75.0
MS + NAA 3.0 + BA 2.0	0	100	0

Observations were taken on 25 DAI

Plate 9. Callus showing globular embryoid

Plate 10. Torpido stage of embryoid



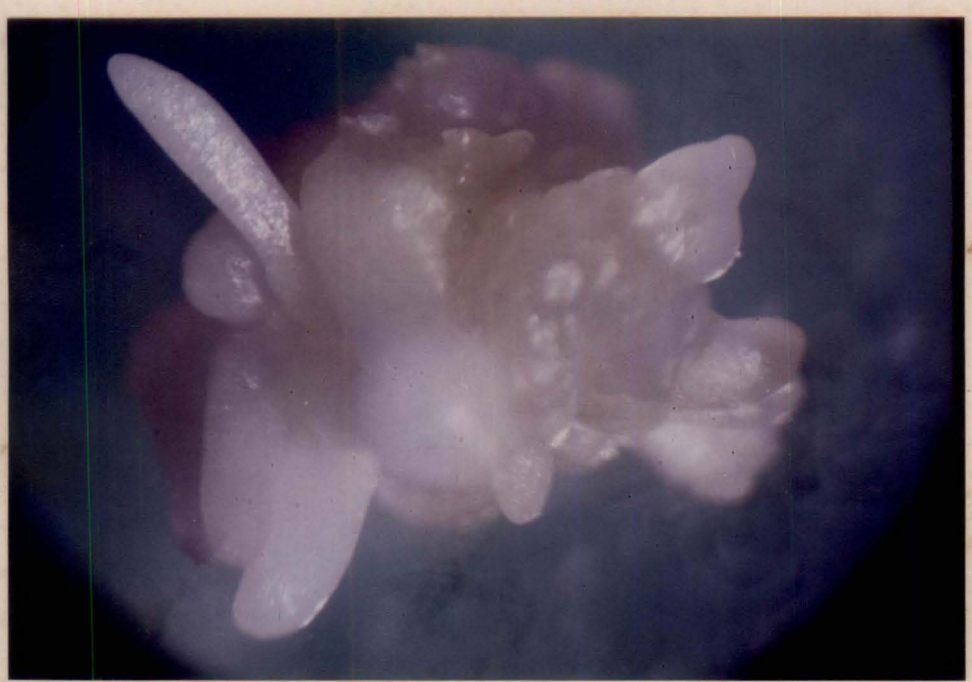
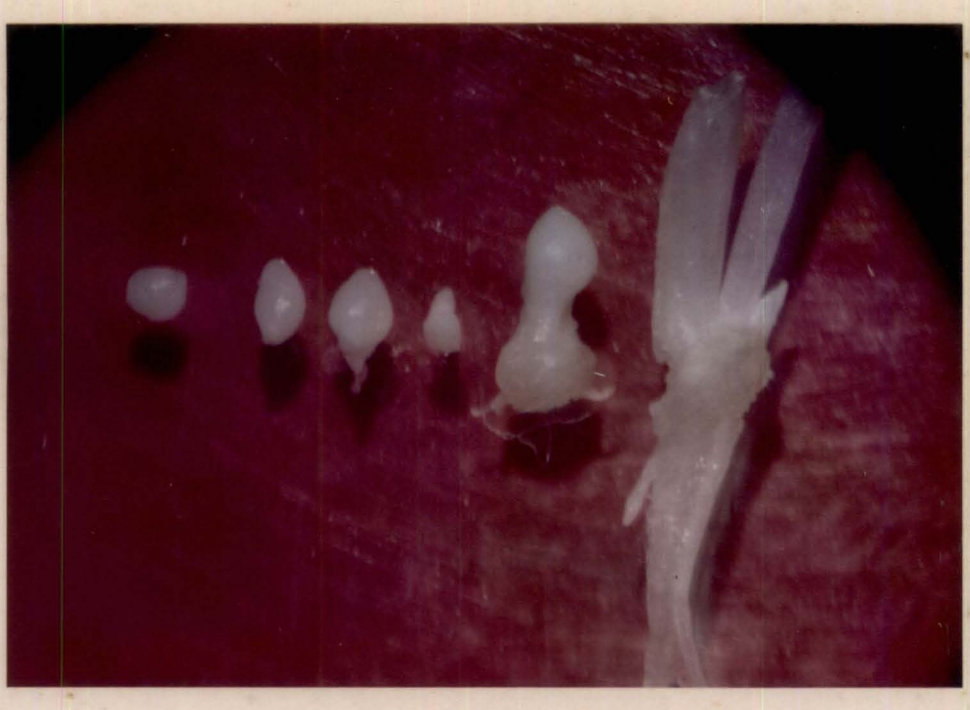


Plate 11. Different stages of embryogenesis

Plate 12. Longitudinal section of embryoid and shoot originating from callus



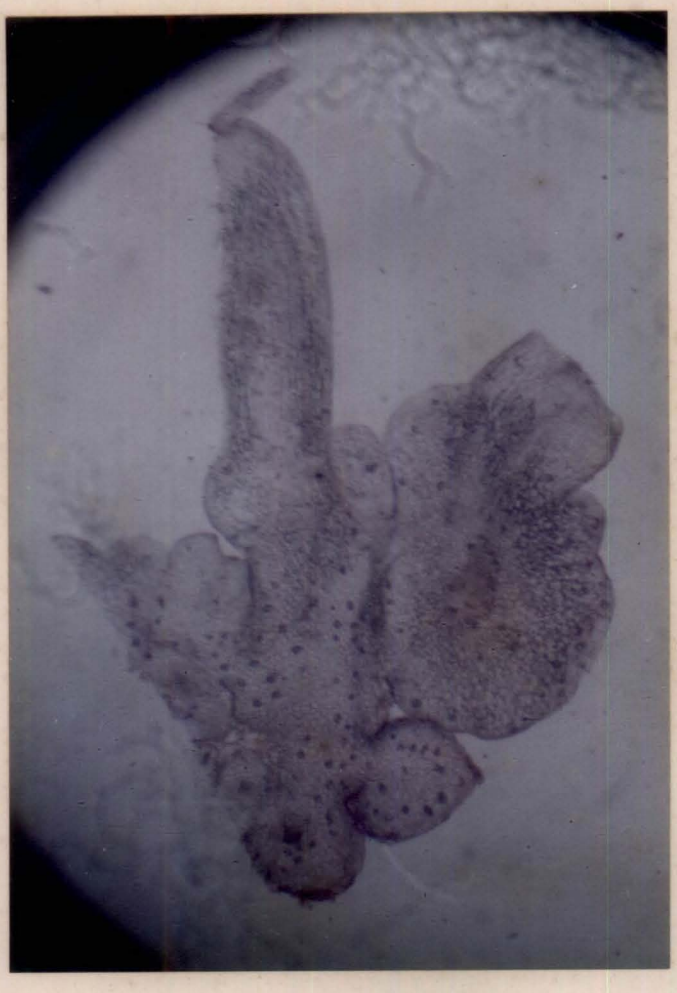


Plate 13. Plantlet regeneration from embryoid

Plate 14. *Kaempferia* plantlets evolved through indirect embryogenesis/ organogenesis

(3.0 and 1.0 mg l^{-1}) induced embryogenic calli with early stage of embryoids in culture (Plate 9). Maximum response (embryogenic 48.2% and mixed calli 51.8%) was recorded in combinations of NAA 3.0 and BA 1.0 mg l^{-1} in MS medium. Among the various levels of BA tried (0.5 to 2.0 mg l^{-1}), 1.0 mg l^{-1} concentration was found to be the best with all the levels of NAA for the induction of embryogenesis.

4.4.2 Maturation of somatic embryoids

The effect of different basal media with varying levels of sucrose and growth regulator combinations on maturation and germination of somatic embryoids are presented in Table 13 and Fig.3. It was observed that the percentage of cultures producing mature embryoids and number of plantlets per culture were comparable in both MS and half strength MS basal media. But average number of embryoids matured per tube (7.4) was higher in MS medium than in ½ MS with same levels of sucrose (3%). Somatic embryoids at different stages of development (globular, cotyledonary and torpido forms) were observed in the cultures (Plate 10, 11 and 12). The best medium for embryoid maturation and germination was MS medium with five per cent sucrose in which the average number of embryoids matured and the number of plantlets obtained per tube were 7.5 and 6.1 respectively (Plate 13 and 14). Eventhough, the percentage of cultures producing mature embryoids (83.3) was highest in MS medium with BA 1.5 mg l^{-1} concentration, the average number of mature embryoids produced per tube and number of plantlets per culture were less. The plantlets produced were later maintained in MS medium with three per cent sucrose.

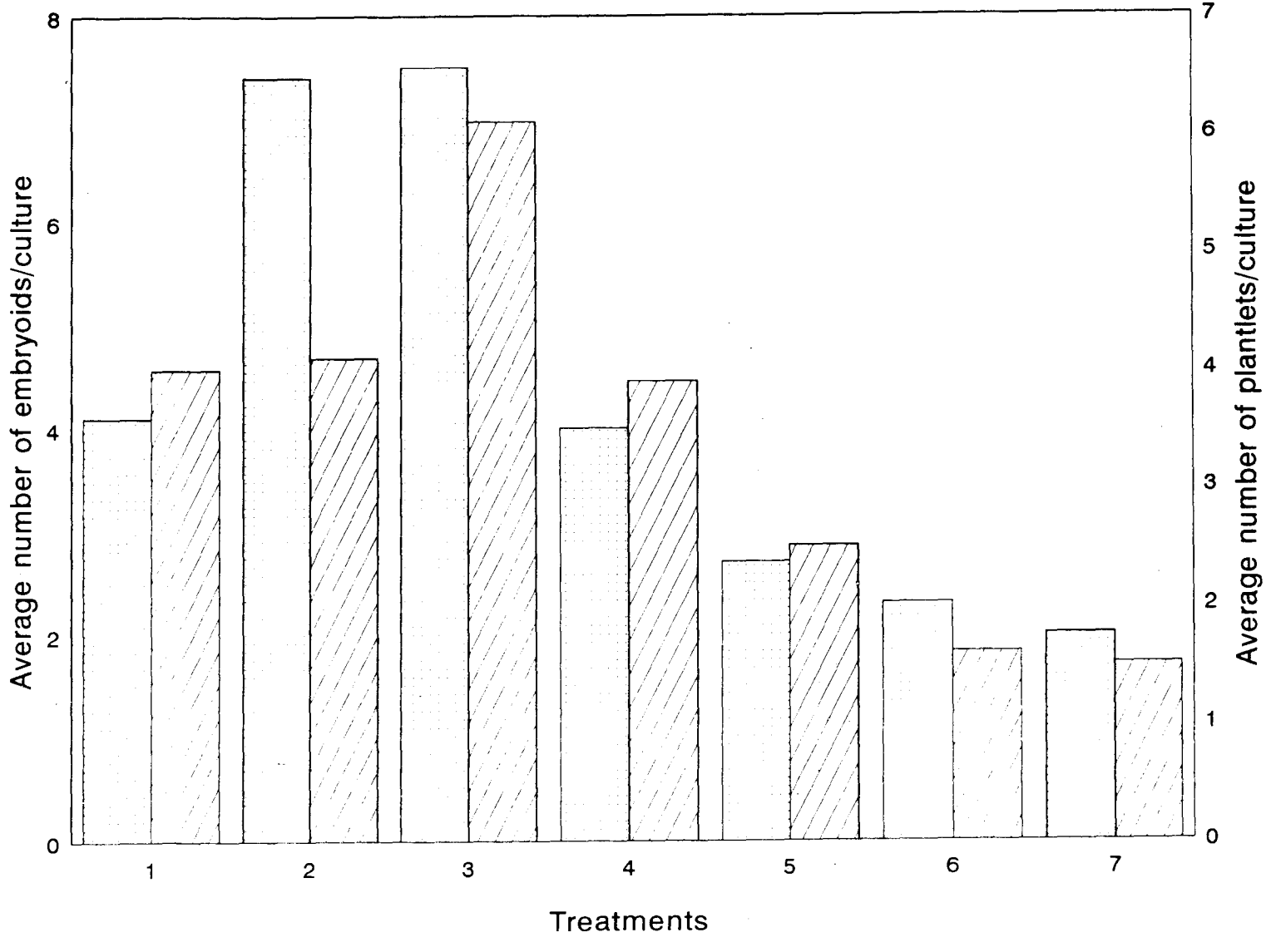


Fig.3. Effect of different media combinations on maturation and germination of somatic embryoids

Table 13. Effect of different media combinations on maturation and germination of somatic embryoids in *K. galanga*

Treatment No.	Treatment details (Growth regulators in mg l^{-1})	Percentage of cultures producing mature embryoids	Average No. of embryoids/ culture	Number of plantlets per culture
T_1	½ MS (3% sucrose)	62.5	4.1	4.0
T_2	MS (3% sucrose)	63.6	7.4	4.1
T_3	MS (5% sucrose)	66.0	7.5	6.1
T_4	MS (5% sucrose + BA 0.5)	53.8	4.0	3.9
T_5	MS (5% sucrose) + BA 1.0	25.0	2.7	2.5
T_6	MS (5% sucrose) + BA 1.5	83.3	2.3	1.6
T_7	MS (5% sucrose) + BA 2.0	20.0	2.0	1.5

Observations were taken 30 DAI

Fig. 4. Flow chart for indirect embryogenesis in *K. galanga*

Stage I

Calli
Maintained in
½ MS + 2,4-D 1 mg l^{-1}
(cream friable calli)

Stage II

½ MS + BA 8.0 mg l^{-1}
(compact, shiny, globular calli)

Stage III

MS + NAA 3 + BA 1.0 mg l^{-1}
(globular embryoids)

Stage IV

MS (5% sucrose)
(mature embryoids)
plantlets (6.1/tube)

Stage V

MS (3% sucrose)
(maintenance of plantlets)

Plate 15. Comparison of plants propagated through conventional method (1) indirect embryogenesis (2) and direct organogenesis (3) in pots


1
2
3

4.5 Field evaluation

Observations recorded on the percentage survival of plants in the field, leaf area, leaf orientation and number of tillers are presented in Table 14 and 15 and Plate 15. The data recorded on growth parameters, like leaf area and number of tillers were statistically analysed and presented in Table 13.

4.5.1 Percentage survival of plants

Maximum survival of plants were obtained for conventionally propagated plants (100%). It was followed by plants obtained through clonal propagation (90%). The survival of plants obtained through indirect organogenesis and somatic embryogenesis were 72.2 per cent and 77.74 per cent respectively.

4.5.2 Leaf orientation

Plants obtained through indirect organogenesis and somatic embryogenesis had erect leaves while conventionally propagated and clonally propagated plants had horizontally oriented leaves.

4.5.3 Leaf area

Maximum leaf area per leaf was recorded in conventionally propagated plants (31.80 cm^2) which was significantly superior to all other plants. It was followed by clonally propagated ones. The leaf area of plants obtained through indirect organogenesis and somatic embryogenesis was on par.

Table 14. Tissue culture derived plants Vs conventionally propagated plants with respect to leaf area and number of tillers in *K. galanga*

Treatments	Leaf area (cm^2)				Number of tillers			
	30 DAP	60 DAP	90 DAP	Mean	30 DAP	60 DAP	90 DAP	Mean
T_1 (Clonally propagated)	14.7	13.88	18.25	15.61^{b}	2.70	5.40	19.60	5.90
T_2 (Indirect organogenesis)	12.36	13.75	18.67	14.93^{bc}	4.70	8.0	13.30	8.67
T_3 (Somatic embryogenesis)	10.44	10.72	17.29	12.81^{bc}	2.40	5.5	12.9	6.93
T_4 (Conventional method)	15.10	38.73	41.56	31.80^{a}	3.4	5.8	8.9	6.03
Mean	13.15	19.27	23.94		3.30	6.16	11.16	
Treatment	SEm±		0.87			SEm±		0.20
	CD		3.2*			CD		0.93*
DAP	SEm±		0.76			SEm±		0.22
	CD		2.77*			CD		0.81*

* Significant at 1% level

Table 15. Tissue culture derived plants Vs conventionally propagated plants of *K. galanga* with respect to survival percentage and leaf orientation

Treatments	Surival of plants (%)	Leaf orientation
T_1 (Clonally propagated)	90.00	Horizontal
T_2 (Indirect organogenesis)	72.20	Erect
T_3 (Somatic embryogenesis)	77.78	Erect
T_4 (Conventional method)	100.00	Horizontal

4.5.5 Number of tillers

Plants obtained through indirect organogenesis had maximum number of tillers (8.7) and this was significantly superior to all other treatments. It was followed by plants obtained through somatic embryogenesis which was again superior to clonally propagated plants.

Discussion

DISCUSSION

The results obtained from the present study on indirect organogenesis and embryogenesis in *K. galanga* are discussed in this chapter.

5.1 Production of axenic plants as explant source

Axenic plants were raised as a source of explant from rhizome sprouts. Treatment with 0.1 per cent mercuric chloride for seven minutes was effective in making the explants free of contaminants. Among the different basal media tried for culture establishment, ½ MS and MS media were identified as ideal ones (Table 1). MS medium with desired salt composition is widely used for enhanced release of axillary bud in many crops. Vincent *et al.* (1992a) obtained shoot proliferation in *K. galanga* when cultured on modified MS medium. Modified MS medium has been identified for axillary bud release in various zingiberaceous crops like *Alpinia purpurata* (Illg and Faria, 1995), turmeric (Nadgauda *et al.*, 1978), cardamom (Nadgauda *et al.*, 1983 and Raghunath, 1989) and ginger (Inden *et al.*, 1988; Choi, 1991b and Dogra *et al.*, 1994). In the present study, MS medium with half the strength inorganic nutrients induced higher percentage of sprouting compared to full MS medium. Illahi and Jabeen (1987) also found that half the strength of MS inorganic nutrients to be more favourable for ginger micropropagation than its full strength. Considering the economic aspect also ½ MS medium was profitable. Hence, half strength MS medium was used for large scale production of axenic cultures as explant source.

5.2 Callus induction

5.2.1 Standardisation of basal media and explants

Among the different explants tried for callus induction, callusing percentage was the highest (88.9) for pseudostem bit explants after 60 days of inoculation. Choi (1991c) used explants of pseudostem containing one leaf blade for getting callus mediated organogenesis in ginger. In the present study callusing percentage was the highest for pseudostem base after 30 days of inoculation. But later, the callusing percentage was not increased, because the remaining explants started sprouting.

Among the different basal media tried for *Kaempferia galanga*, cultures were found established only in MS and MS with half the concentration of inorganic salts; the highest being in half MS basal medium. Geetha *et al.* (1990) obtained callus production from leaf segments of black pepper in modified half MS medium.

5.2.2 Effect of growth regulators

5.2.2.1 Effect of auxins

Pseudostem bits were cultured on half strength MS medium with various levels of 2,4-D and NAA for induction and proliferation of callus. 2,4-D was found superior to NAA for inducing calli and for its further proliferation. Maximum callus index was recorded with 10 mg l^{-1} 2,4-D and the calli produced at all the levels of 2,4-D used were cream coloured and friable with good morphogenic potential.

A number of workers have reported the use of 2,4-D for production and maintenance of calli. Babu *et al.* (1992b) obtained callus growth and proliferation at concentration 9.0 to 22.6 μm of 2,4-D in ginger. Callus inducing property of 2,4-D

at higher levels was also reported by Mucciarelli *et al.* (1993) in *Vetiveria zizanioides*. Bregitzer *et al.* (1995) reported callus induction and growth in 2,4-D containing medium at 20.4-27.1 μm concentration in barley.

With NAA at 3 mg l^{-1}, callus induction was observed after 30 days of inoculation. Further proliferation of calli was also good at this level of NAA. The calli produced were with good morphogenic potential and rhizogenesis was found to be initiated in the cultures. Higher concentration of NAA (5 mg l^{-1}) was found to delay callus production.

5.2.2.2 Effect of growth regulator combination

Among the various growth regulator combinations attempted, the highest callusing percentage (92.3) and callus index (193.9) were recorded with 2,4-D 1.0 mg l^{-1} and BA 0.25 mg l^{-1} concentration. The calli induced were cream coloured, friable and hairy with good regeneration capacity. But higher concentration of BA with 2,4-D inhibited callus growth and the calli produced turned brown with poor morphogenic potential. Addition of kinetin was found to nullify the callus inducing property of 2,4-D. Addition of 2ip also reduced the effectiveness of 2,4-D for callus induction.

Skoog and Miller (1957) have reported that the levels of plant growth regulating substances in the culture medium, particularly high auxin and low cytokinins, often lead to callus formation. Vincent *et al.* (1991) could obtain white friable calli from the cut ends of the young rhizome sprouts of *K. galanga* within 25 DAI on MS medium supplemented with 1 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA. The favourable effect of the combination of 2,4-D with lower levels of BA has been

reported in various monocots like *Panicum virgatum* (Denchev and Conger, 1995), *Poa pratensis* (Vander Valk *et al.*, 1995). At higher levels of BA, the calli produced turned brown. Browning effect of BA at higher levels has also been reported by Vander Valk *et al.* (1995) in Kentucky blue grass.

5.3 Indirect organogenesis

5.3.1 Effect of different growth regulator combinations

Combinations of cytokinins like BA, kinetin and 2ip were tried with 2,4-D at 0.2, 0.1 and 0 level. Level of 2,4-D was fixed at low concentration because of the negative relationship between concentration of 2,4-D and the regeneration potential (Golds *et al.*, 1993; Ziauddin and Kasha, 1990).

5.3.1.1 Combined effect of varying levels of BA and 2,4-D

Incorporation of BA alone at different concentrations (0.25 to 8 mg l^{-1}) in ½ MS medium showed positive response for shoot initiation from callus under light conditions. At 8.0 mg l^{-1} concentration of BA, shoot formation was obtained. The effect of BA alone (4.4 μm) in MS medium for callus regeneration has been reported by Nair and Seo (1993) in *Allium senescens* var. minor. They reported better response at lower level of 2,4-D (4.5 μm). In the present study, in order to ensure uniformity, the calli maintained in ½ MS medium supplemented with 1 mg l^{-1} 2,4-D were used as starting material to study the influence of growth regulator on regeneration. So eventhough shoot formation was obtained in the medium devoid of 2,4-D, there may be residual effect of 2,4-D in the cultures. Studies of Vander Valk *et al.* (1995) showed that the addition of low concentration of BA to 2,4-D containing callus induction medium promotes somatic embryogenesis

and strongly increases the percentage of shoot forming callus in *Poa pratensis*. The favourable effect of higher concentration of BA in 2,4-D containing medium has also been reported in ginger by Babu *et al.* (1992b).

Combinations of 2,4-D with lower levels of BA induced rhizogenesis in cultures both under light and dark conditions. Mathur (1993) has reported that auxin alone or a high auxin:cytokinin ratio were responsible for the continued callus proliferation or callus growth accompanied by rhizogenesis.

5.3.1.2 Effect of kinetin

Miller *et al.* (1956) reported that kinetin stimulated cell division in cells that other wise might have become multinuclear. Kirkham and Holder (1981) investigated the effect of kinetin on callus water potential. Addition of kinetin was found to increase the callus water potential by increasing the cells turgor potential and thereby reducing the water uptake from the surrounding medium. In the present study, kinetin was found effective in inducing shoot buds from the calli cultured under light and the number of shoots produced were maximum in the presence of kinetin at 6.0 mg l^{-1} concentration at 25 DAI. However, the average number of shoots produced per culture was less in the presence of kinetin than that produced in the presence of BA (8.0 mg l^{-1}). This was in accordance with the findings of Babu *et al.* (1992a) who obtained organogenesis in the form of small shoot primordia at 23.2 μm concentration and well developed shoots at higher concentration of 44.4 μm in ginger. Mucciarelli *et al* (1993) also reported the favourable effect of kinetin (9.3 μm) with lower concentration of 2,4-D (0.9 μm) for shoot formation and embryogenesis. In the present study, kinetin did not induce rhizogenesis in any of the treatments.

5.3.1.3 Effect of 2ip

In *K. galanga*, 2ip was found to be not effective for inducing indirect organogenesis under light or dark incubation. However, some white, shiny compact globular calli were produced at a concentration of 2 mg l^{-1}. Cailloux (1978) reported formation of buds, in shoot tip cultures of *Gynura sarmenta*, when BAP, kinetin and 2ip were used separately. The favourable effect of 2ip for somatic embryogenesis has been reported by Kipnis *et al.* (1994) in Nerine (Amaryllidaceae).

5.3.1.4 Effect of GA_3

In *K. galanga*, GA_3 did not express either enhancing or inhibiting effect on indirect organogenesis even when in combination with BA. Under light incubation well developed shoots were formed in all the combinations, but the percentage and average number of shoots produced were less than that with BA alone (8 mg l^{-1}).

5.3.1.5 Effect of other media additives

The effect of varying levels of media additives like silver nitrate and activated charcoal on indirect organogenesis was tried both under light and dark conditions. Rhizogenesis could be obtained with activated charcoal 0.1 per cent level and silver nitrate at 0.5, 10.0 and 15.0 mg l^{-1} concentration. However, shoot formation was not observed in any of the treatments. In the presence of light, $AgNO_3$ at 5.0 and 10.0 mg l^{-1} concentration induced green friable calli with green roots. The inhibiting effect of ethylene which is produced by cultured plants, tissues and organs

on organogenesis is already established in carrot (Wochock and Wetherell, 1971; Tisserat and Murashige, 1977). Silver ions are known to overcome the action of ethylene on whole plants (Beyer, 1976). Coleman *et al.* (1980) found that if tomato leaf discs were placed for 30 minutes in sterile $AgNO_3$ solution (1.7-17 mg l^{-1}), more roots were produced when they were afterwards placed on a medium containing 0.9 mg l^{-1} IAA. The inhibition of organogenesis in the presence of activated charcoal may be attributed to the absorption of phytohormones.

Fridborg and Eriksson (1975) suggested that activated charcoal (AC) removed growth regulators, particularly auxin from the medium. Paranjothy and Othman (1982) had to use 10 times the level of auxin that would other wise have been effective to initiate callus of oilpalm in presence of activated charcoal.

5.3.2 Effect of different liquid media combinations on indirect organogenesis/ embryogenesis

The calli were inoculated into the liquid ½ MS medium containing different combinations of auxins and cytokinins and kept under continuous shaking at 100 rpm. But none of the treatments tried could induce indirect organogenesis in culture. The combination of 2,4-D 1.0 mg l^{-1} and BA 0.5 mg l^{-1} in ½ MS medium induced globular structures (33.3%). But further regeneration could not be obtained from these structures.

5.4 Somatic embryogenesis

5.4.1 Induction of somatic embryogenesis

5.4.1.1 Effect of different basal media and culture condition

The compact embryogenic calli produced in the regeneration medium

containing 8.0 mg l^{-1} BA were transferred to different media combinations for inducing somatic embryoids. MS medium was found to be the best basal medium for embryoid induction and the tendency was observed for the induction of shoot as well as embryoid simultaneously. MS medium as such or modified was used for induction of somatic embryogenesis by many workers [Vincent *et al.* (1992b) in *Kaempferia galanga*, Vander Valk *et al.* (1992) in *Allium cepa, Allium porrum* and *Allium fistulosum* and Kacker *et al.* (1993)]. The peculiarity of the medium is the presence of high levels of nitrogen in the form of ammonium nitrate, which is ideal for both the embryo initiation and maturation. The requirement of nitrogen in the case of carrot somatic embryos can be satisfied by high concentration of inorganic nitrogen in the form of nitrate (Reinert, 1967).

Malepszy (1988) reported that the first stage of embryogenesis in cucumber took place in dark. In the present study also it was shown that dark incubation was ideal for induction of somatic embryogenesis.

Stickland and Sunderland (1972) reported that light may alter the oxidation of IAA by peroxidase enzymes, by regulating the levels of co-factors and inhibitors of enzymes. Marcotrigiano and Stimart (1981) found that in the light hypocotyls of *Paulocunia* required 3 mg l^{-1} IAA in the medium to produce shoots at the maximum rate whereas under continued darkness, only 1 mg l^{-1} IAA was necessary. The presence of an auxin in the induction medium seems essential for the tissue to develop embryos (Fujimura and Komamine, 1980). Hence in the present study, the high rate of induction of somatic embryos under dark incubation is justified.

5.4.1.2 Effect of different growth regulators

The calli cultured at 8.0 mg l^{-1} concentration of BA produced white, shiny, globular, compact embryogenic calli after 25 days of inoculation. Embryogenic calli was also induced in the presence of kinetin, but the percentage of embryogenic calli produced was less than that induced in the presence of 8.0 mg l^{-1} of BA. With increase in the concentration of kinetin used, there was corresponding increase in the percentage of embryoid induction. When the embryogenic calli was transferred to medium containing NAA and BA, early stages of embryo formation was noticed. Of the different combinations tried NAA 3.0 ppm with BA 1.0 ppm in MS medium was found to be the best for embryoid formation in *K. galanga*. Von Arnold and Eriksson (1981) and Von Aderkas *et al.* (1989) in conifer and Bhaskaran and Smith (1989) in sorghum reported that both an auxin and cytokinin were required in the culture medium. Though 2,4-D in the medium induced large number of nodular embryogenic callus, embryogenesis observed only when the auxin 2,4-D was replaced with NAA. Lazzeri *et al.* (1987) reported the effect of NAA on somatic embryogenesis in soybean.

Vincent *et al.* (1992) obtained globular embryoids in MS medium supplemented with NAA (1.0 mg l^{-1}) and BA (0.1 mg l^{-1}) in *Kaempferia galanga*. Production of somatic embryoids was the highest in *Simarauba glauca* at a combination of BA (11.1 μm) and NAA (13.42 μm) as reported by Rout and Das (1994). Similar result was also reported in *Aconitum heterophyllum* by Giri *et al.* (1993).

5.4.2 Maturation of somatic embryoids

From the induction medium, the early stages of somatic embryoids were

subcultured into different media combinations for maturation. After 20 days of inoculation, MS medium with five per cent sucrose produced the highest number of mature embryoids which were found loosely attached to the mother callus. Maturation and germination of somatic embryoids occurred in hormone free medium as reported by Bhaskaran and Smith (1989) in sorghum. Embryo development and maturation were promoted by high percentage of sucrose in MS medium as reported by Levi and Sink (1992) in Asparagus and Kipnis *et al.* (1994) in Nernine (6% sucrose).

The combination of MS (5% sucrose) with BA 1.5 mg l^{-1} concentration resulted in high percentage of cultures producing embryoids but with lesser number of embryoids per culture. Chee (1991) has reported somatic embryo maturation in cucumber in the presence of cytokinins like BA.

The mature embryos subcultured into fresh MS medium (5% sucrose) developed into individual plantlets which after 30 days culture period were ready for transplantation.

The plantlets were transferred to small polythene bags and kept in the net house. After acclimatization, the plants were transferred to pots containing potting mixture.

5.5 Field evaluation

The field evaluation studies revealed that the plants produced through somatic embryogenesis and indirect organogenesis had variation from those produced through conventional methods in all the parameters studied. It is believed that only clones derived from meristem, shoot tip and bud cultures are generally

phenotypically homogeneous, thereby indicating genetic stability (Hu and Wang, 1983). If an intermediary callus phase is involved, as in indirect organogenesis and somatic embryogenesis, the frequency of genetic changes is increased (Hu and Wang, 1983). Nadgauda (1982) isolated variants with high curcumin content from callus cultured plantlets of turmeric. Some phenotypic variants were also detected among the regenerated plants of barley most originating by epigenetic changes (Ruiz *et al.*, 1992).

In the present study, plants regenerated from callus showed maximum number of tillers. It has been observed that a high ratio of cytokinin to auxin lead to axillary and shoot proliferation in cultures. This may be the reason for maximum number of tillers in plants regenerated in medium containing high cytokinin (BA 8.0 ppm).

Thus, there may be an increased rate of somaclonal variation in plants regenerated through somatic embryogenesis and indirect organogenesis which can be exploited for crop improvement in *Kaempferia galanga* especially since other conventional methods of creating variation are ineffective.

Summary

SUMMARY

The present investigations were carried out during 1994-'96 in the Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara with the objective of standardising the technique of indirect organogenesis and embryogenesis in *Kaempferia galanga* and to evaluate the field performance of the plantlets as compared with conventional propagules. For raising axenic plants as explant source, rhizomes were collected from the medicinal plant garden attached to the All India Co-ordinated Research Project on Medicinal and Aromatic Plants at Vellanikkara. The salient findings of the investigation are presented below.

1. Among the different basal media tried for culture establishment of *Kaempferia galanga* rhizome buds, ½ MS and MS media were identified as ideal ones. Percentage of sprouting was higher in ½ MS compared to MS.

2. Pseudostem bits were identified as the best explant for callus induction.

3. Of the different basal media tried for callus induction, cultures were found to establish only in MS and ½ MS media.

4. 2,4-D was found to be the most effective auxin for callus induction and proliferation from pseudostem bit explant.

5. Incorporation of NAA at 3 mg l^{-1} concentration was effective for induction of calli with good morphogenic potential.

6. Combination of 2,4-D (1 mg l^{-1}) with BA (0.25 mg l^{-1}) in the medium was ideal for induction of callus with morphogenic potential.

7. Higher concentrations of BA with 2,4-D inhibited callus growth.

8. Addition of kinetin was found to nullify the callus inducing property of 2,4-D.

9. Addition of 2ip reduced the effectiveness of 2,4-D for callus induction.

10. Caulogenesis was recorded from the calli cultured in a medium devoid of auxin and having cytokinin.

11. Incorporation of BA (8.0 mg l^{-1}) in ½ MS medium incubated under light gave maximum shoot initiation from the calli.

12. Combination of 2,4-D with lower levels of BA induced rhizogenesis.

13. Kinetin was found to be effective in inducing shoot buds from the calli cultured under light and the number of shoots produced were maximum in the presence of kinetin at 8 mg l^{-1} concentration.

14. 2ip was found to be not effective for inducing indirect organogenesis.

15. GA_3 did not express any effect on indirect organogenesis.

16. Incorporation of silver nitrate in the medium (5.0 and 10 mg l^{-1}) induced green friable calli with green roots.

17. Activated charcoal at 0.1 per cent level induced rhizogenesis in culture.

18. Suspension culture did not induce organogenesis or embryogenesis from the calli.

19. Incorporation of BA (8.0 mg l^{-1}) in the regeneration medium induced white, shiny, compact embryogenic calli in the peripheral region.

20. MS medium was found to be the best basal medium for embryoid induction.

21. Regeneration through organogenesis and embryogenesis was observed simultaneously in the cultures.

22. Dark incubation was ideal for induction of somatic embryogenesis.

23. Kinetin was effective in induction of embryogeneic calli in the culture, but the percentage of embryogeneic calli produced was less than that induced in the presence of 8.0 mg l^{-1} of BA.

24. The presence of auxin and cytokinin simultaneously was required for induction of embryogenesis in *Kaempferia galanga*.

25. MS medium supplemented with NAA (3 mg l^{-1}) and BA (1 mg l^{-1}) was the best medium for inducing somatic embryogenesis.

26. MS medium with sucrose at five per cent level was the best medium for maturation and germination of somatic embryoids.

27. The number of tillers produced per plant was the highest for those produced through indirect organogenesis and embryogenesis.

28. The leaf area per leaf of conventionally propagated plants was significantly superior to those of tissue culture derived plants.

29. Percentage of plants survived in the field was high for conventionally propagated plants.

30. The leaf orientation of plants produced through indirect organogenesis and embryogenesis was erect whereas leaves of conventionally propagated plants were horizontally oriented.

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* Originals not seen

Appendices

ANNEXURE-I

Composition of different basal media tried for *in vitro* culture of *Kaempferia galanga*

a) MS medium (Murashige and Skoog, 1962)

Macronutrients	mg l^{-1}	Micronutrients	Mg l^{-1}
KNO_3	1900	$MnSO_4.4H_2$	22.30
NH_4NO_3	1650	$ZnSO_4.7H_2O$	8.60
$CaCl_2.2H_2O$	440	H_3BO_3	6.20
$MgSO_4.7H_2O$	370	KI	0.83
KH_2PO_4	170	$CuSO_4.5H_2O$	0.025
		$Na_2MoO_4.2H_2O$	0.25
		$CaCl_2.6H_2O$	0.025
		$FeSO_4.7H_2O$	27.80
		$Na_2EDTA.2H_2O$	37.30

Organic constituents	mgl^{-1}
Myo-inositol	100
Thiamine-HCl	0.1
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Glycine	2.0

b) B5 medium (Gamborg *et al.*, 1968)

Macronutrients	mg l^{-1}	Micronutrients	mg l^{-1}
KNO_3	2500	$MuSO_4$	10.0
$CaCl_2.2H_2O$	150	$ZuSO_4.7H_2O$	2.0
$MgSO_4.7H_2O$	250	H_3BO_3	3.0
$NaH_2PO_4.H_2O$	150	KI	0.75

$(NH_4)_2SO_4$	134	$CuSO_4$	0.025
		$Na_2MoO_4.2H_2O$	0.025
		$CaCl_2$	0.25
		NaFeEDTA	0.28

Organic constituents	mg l^{-1}
Myo inositol	100.0
Thiamine-HCl	10.0
Nicotinic acid	1.0
Pyridoxine-HCl	1.0

c) SH medium (Scheuk and Hildebrandt, 1972)

Macronutrients	mg l^{-1}	Micronutrients	mg l^{-1}
KNO_3	2500	$MnSO_4$	10.0
$CaCl_2.2H_20$	200	$ZnSO_4.7H_2O$	1.0
$MgSO_4.7H_2O$	400	H_3BO_3	5.0
$NH_4H_2PO_4$	300	KI	1.0
		$CuSO_4.H_2O$	0.2
		$Na_2MoO_4.2H_2O$	0.1
		$CoCl_2.6H_2O$	0.1
		$FeSO_4.7H_2O$	15.0
		$Na_2EDTA.2H_2O$	10.0

Organic constituents	mg l^{-1}
Myo-inositol	1000
Thiamine-HCl	5.0
Nicotinic acid	5.0
Pyridoxine-HCl	0.5

ANNEXURE-II

Combinations of growth regulators tried for callus induction in *Kaempferia galanga*

Basal media	Auxin (mg l^{-1})	Cytokinin mg l^{-1}
½ MS (3% sucrose)	2,4-D 0.1	--
	2,4-D 0.5	--
	2,4-D 1.0	--
	2,4-D 2.0	--
	2,4-D 4.0	--
	2,4-D 6.0	--
	2,4-D 8.0	--
	2,4-D 10.0	--
	NAA 0.1	--
	NA 0.5	--
	NA 1.0	--
	NAA 2.0	--
	NAA 3.0	--
	NAA 5.0	--
	2,4-D 0.5	BA 0.25, 0.5, 0.75, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0
	2,4-D 0.5	Kin 0.2, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0
	2,4-D 0.5	2ip 0.25, 0.5, 0.75, 1.0, 2.0, 4.0, 6.0. 8.0, 10.0
	2,4-D 1.0	BA 0.25, 0.5, 0.75, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0
	2,4-D 1.0	Kin 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0
	2,4-D 1.0	2ip 0.25, 0.5, 0.75, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0

ANNEXURE-III

Combinations of growth regulators tried for indirect organogenesis in *K. galanga*

Basal medium	Auxin	Cytominin	GA_3
½ MS (with 3% sucrose)	2,4-D 0	BA 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10	
	2,4-D 0.1	BA 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10	
	2,4-D 0.2	BA 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10	
	-	Kin 0.25, 0.5, 1, 2, 3, 4, 5, 6	
	-	2ip 0.25, 0.5, 1, 2, 4, 6	
	-	BA 8	0.5, 1.0, 1.5, 2.0

ANNEXURE-IV

Concentrations of other media additives used for indirect organogenesis in *K. galanga*

Basal medium	Media	Additive
½ MS (3% sucrose)	$AgNO_3$ (%)	1.0, 5.0, 10.0, 15.0, 2.0
	Activated charcoal (%)	0.1, 0.2, 0.5, 0.8

ANNEXURE-V

Growth regulator combinations tried for suspension culture in *K. galanga*

Basal medium	Auxin	Cytokinin
½ MS	-	-
½ MS	2,4-D 0.5	-
½ MS	2,4-D 1.0	-
½ MS	2,4-D 0.5	BA 0.5, 1.0, 2.0, 4.0, 6.0
½ MS	2,4-D 1.0	BA 0.5, 1.0, 2.0, 4.0, 6.0
½ MS	NAA 1.0	-
½ MS	NAA 3.0	-

INDIRECT ORGANOGENESIS AND EMBRYOGENESIS IN *Kaempferia galanga* L.

By
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ABSTRACT OF A THESIS

Submitted in Partial fulfilment of the
requirement for the degree

Master of Science in Horticulture

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1997

ABSTRACT

A study was taken up in the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara, during 1994-'96, to standardise the technique of indirect organogenesis and embryogenesis in *Kaempferia galanga* and to evaluate the field performance of the plantlets as compared with conventional propagules. Axenic cultures were raised from rhizome buds as explant source for callus induction.

Pseudostem bits, leaf bits and pseudostem base were tried as explants for inducing calli. Profuse callusing could be induced from pseudostem bit explant in ½ MS medium supplemented with 2,4-D and low concentrations of BA. The calli induced were creamy and friable with good morphogenic potential.

Shoot regeneration was obtained from the calli in ½ MS medium supplemented with BA 8.0 mg l^{-1} with an average of 5.6 shoots per culture tube. Kinetin was also effective in inducing shoots from the calli and maximum number of shoots were produced at kinetin 6 mg l^{-1} concentration. Only rhizogenesis was observed with combinations of 2,4-D with lower levels of BA and with silver nitrate (5.0 and 10 mg l^{-1}) and activated charcoal (0.1%). Suspension cultures were not effective for inducing organogenesis/embryogenesis.

Compact, shiny, white embryogenic calli were induced in the peripheral region of cultures inoculated in ½ MS + KIN. Under dark incubation, numerous embryoids were induced in MS medium supplemented with NAA (3 mg l^{-1}) and BA (1 mg l^{-1}). MS medium with sucrose at five per cent level was the best medium for

maturation and germination of embryoid. All the stages of somatic embryoids were observed in the cultures.

The plantlets produced through indirect organogenesis and embryogenesis were hardened and successfully planted out. The tissue culture derived plants were compared with conventionally propagated plants in the field. The percentage of survival and leaf area per leaf of tissue culture derived plants were low compared to conventionally propagated plants. But the number of tillers produced per clump was very high in plants produced through indirect organogenesis and embryogenesis and the leaf orientation was erect.

The results of present study reveal that there is scope of successful indirect organogenesis and embryogenesis in kaempferia and there exists significant variation between tissue culture derived and conventionally propagated plants and there is scope of exploiting this variation to complement the limited natural variability in this crop.