IN VITRO STUDIES ON MEDICINALLY IMPORTANT PLANTS OF ZINGIBERACEAE

THESIS

submitted to the ***University of Calicut***
in partial fulfilment of the requirement for the
Degree of Doctor of Philosophy
in ***Botany***

By

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CERTIFICATE

This is to certify that the thesis entitled: **IN VITRO** ***STUDIES ON MEDICINALLY IMPORTANT PLANTS OF ZINGIBERACEAE****, submitted to the University of Calicut by* ***Mr. Mustafaanand P.H.****, for the award of the degree of* ***Doctor of Philosophy in Botany****, embodies the record of the bonafide research work accomplished by him under my supervision and guidance, and the thesis has not previously been formed the basis for the award of any degree, diploma, associateship, fellowship or anyother similar title or recognition.*

It is also certified that Mr. Mustafaanand has successfully completed the qualifying examination prescribed by the University of Calicut, as part of the Ph.D. programme.

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January 14, 2000.

DECLARATION

I, Mustafaanand P.H. do here by declare that the research work entitled : **IN VITRO *STUDIES ON MEDICINALLY IMPORTANT PLANTS OF ZINGIBERACEAE,*** *is an original piece of research work done by me in the Department of Botany, University of Calicut and that it has not previously been submitted for the award of any prize or degree - either in part or full - to any University, Institution or Board, except for the publications in the reputed Journals.*

Calicut University,
January 14, 2000.

(Mustafaanand P.H.)

ACKNOWLEDGEMENTS

It is my profound privilege to express my heart-felt gratitude to **Dr. Aleyamma Thomas**, *Professor of Botany, University of Calicut for suggesting a challenging topic for this research endeavour, her mesmerising guidance, constant encouragement and constructive criticisms - that persisted throughout my stay over here, and even still now.*

I extend my respectful gratitude to **Dr. K. Unnikrishnan** *(Professor & former Head of the Department of Botany) and* **Dr. N. Neelakandan**, *Professor & Head of the Department of Botany, University of Calicut for providing the library and laboratory facilities of the Department, and also their innovative criticism and encouragement through out my work.*

I am highly indebted to **Dr. Sailas Benjamin**, *Lecturer, Department of Botany, University of Calicut for his valuable help and co-operation for compiling the thesis.*

It is my pride to mention **Dr. Sushilkumar**, *Director of Central Institute of Medicinal and Aromatic Plants (CIMAP), and also of National Botanical Garden and Research Institute (NBGRI), Lucknow, Librarians of the Jawaharlal Nehru University, and Delhi University for their help in collecting valuable literature for my research.*

Gratefully I remember all the Faculty members of the Department of Botany, my colleagues and friends for their valuable suggestions and inspiring criticisms.

I would ever remember M/S. **K. Ajay Kumar, P.N. Azeez** *and* **K. Narendran**, *Art and Photography Unit, University of Calicut for their immense help for preparing the Research Photographs.*

I am very much indebted to the University of Calicut for awarding me Junior Research Fellowship of University.

I am so lucky in having Ms. **Abidha Anand**, *my wife, and son* **Badusha P. Anand** *- embodied with boundless patience, co-operation and constant encouragement to make my goal a success.*

To sum up, I surrender myself before the strength of hearty prayers of my parents, family members and well-wishers. And, I should admit the real fact that, it is the **Almighty Allah**, *Who mercifully persuaded me to the limelight this humble expedition.*

Mustafaanand P.H.

PREFACE

This preface is intended to facilitate an easy entry in to the contents in the thesis, and thus to get an overview to the readers. The thesis is comprised of six major chapters *viz;* Introduction, Review of Literature, Materials and Methods, Observation and Results, Discussion, and the last chapter being Summary and Conclusion. Tables and figures are arranged at the respective places, where ever they are mentioned first. All the plates are arranged together as a bundle, next to observation and results. For easy understanding of the plates, detailed footnotes given.

For simplicity, all the Six Chapters are subdivided, according to the relevance and specificity of the contents in the text described at that place. An elaborate bibliography is provided at the end, following the Sixth Chapter, *viz.*, Summary and Conclusion. Major publications emerged during this work have been appended, juxtaposed to the bibliography. Prior to the opening chapter, also enclosed a detailed index and abbreviations used in the text.

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ABBREVIATIONS USED

2,4-D	-	2-4-dichlorophenoxyacetic acid
B5	-	Gamborg *et al.*, medium (1968)
BAP	-	6-benzylaminopurine
CW	-	Coconut water
DMSR		Direct multiple shoot regeneration.
DPX	-	Dextrene plastisizer xylene
EDTA	-	Ethylene dinitrilo tetra acetic acid
FAA	-	Formalin - acetic acid - alcohol
GA_3	-	Gibberellic acid
HCl	-	Hydrochloric acid
IAA	-	Indole-3-acetic acid
IBA	-	Indole-3-butyric acid
KN	-	Kinetin (6-furfuryl amino purine)
MAP	-	Medicinal and aromatic plants
m.ha	-	Million hector
mg/l		Milligram per Litter
MS	-	Murashige and Skoog (1962)
NAA	-	α-Naphthaleneacetic acid
NaOH	-	Sodium hydroxide
PPM	-	Parts per million
SH	-	Schenk and Hildbrand (1972)
Sq.km.	-	Squire kilometre
TBA	-	Tertiary butyl alcohol
UV	-	Ultraviolet
μM	-	Micromolar
WH	-	White's medium (1963)
μm	-	Micrometer (micron)
ψ	-	Psi

CHAPTER I

INTRODUCTION

India has long been considered as the **Paradise of Medicinal Plants,** endowed with its own well-flourished germplasm repositories. These medicinal herbs are the building blocks of our unique and holistic systems of medicine, such as ayurveda, unani, sidha, tebetan, and a host of other ill-popular folk medicinal traditions. Such a vast diversity of plant wealth has been found to mainly be confined to the agro-climatic regions, extending from the ice caps of Himalaya, down to the Arabian sea; from dry arid desert of Rajasthan to the wettest Chirapunji, and the older parts of Gondwana in the north (Thomas, 1994).

Medicinal and aromatic plants (MAPs), which are currently being used commercially, identified as indigenous to different parts of the world. Traditional medicaments, derived from plants through the studies and observations for thousands of years, are now yielding their secrets and finding important roles in modern medicine. Now-a-days, over three-quarters of the world population rely mainly upon plants and plant extracts for the maintenance of their health. Recent studies made by World Health Organisation (WHO) indicates that over 30% of the world plant species have, at one time or other, been used for medicinal purposes. Of 2,00,500 higher plant species reported on earth, over 80,000 are medicinally important (Kumar *et al.*, 1997). In fact, though India is harbouring about 45,000 endemic plant species, only a small share of them are found to be commercially viable.

About 60% of Indian population depend mainly on Ayurvedic medicines for the treatment of common diseases (Nair *et al.*, 1992). India, being a sub-continent, housing a variety of agro-climatic conditions, is much suitable for the cultivation of various medicinal as well as aromatic plants, which demand different environmental conditions. Further, the availability of abundant trained manpower and relatively cheaper cost of production prevailed in India create a condition which is much favourable for it becoming a major international producer and processor of superior medicinal and aromatic plant materials (Kumar *et al.*, 1997).

Medicinal plants contribute 25% of the prescribed drugs (Khadhar, 1992). Since ancient times, many herbaceous species have widely been employed for traditional medicinal purposes. In recent years, screening and indexing of such plants for biological activities resulted in the development of novel therapeutics, useful for the treatment of various ailments of man such as rheumatism, kapha, pitha, blood pressure, cancer, *etc.* (Anonymous,1948, 1959). Certain compounds so obtained showed wonderful effects in treating sterility, lethal cancer, hypoglycaemia, coupled with cardio-vascular effects on central nervous system (Satyavathi *et al.*, 1969). In addition, Indian traditional systems of medicine like Ayurveda and Sidha are also depend chiefly upon medicinal plants as a main source of medicines. Most of the Indian population, especially the inhabitants of the rural areas, use traditional medicines only. With the realisation of the fact that, herbal medicines have no side effects and many more herbals are being reported to be effective against many chronic diseases for which

no allopathic drugs available, and because of these reasons, production of herbal-based medicines have now attained tremendous momentum throughout the world.

In fact, over 170 pharmaceutical companies in the world import crude extracts of MAPs from India. Of this, 90% are contributed by wild species. A World Bank report (1997) discloses that the foreign exchange earned due to the export of crude herbal drugs by India during 1994-95 was $ 53.2 million (Rs.228crores). Medicinal plant related trade in India is worth Rs.550 crore, and herbal medicines of Rs. 900 crore worth are produced annually in India (Banerjee, 1998).

As a result of ever increasing demand for medicinal herbs, the supply of medicinal plants has become dwindled. Except a few cultivated plants, most of the medicinal plants are collected from the wild. With the rapid depletion of Indian forests, availability of raw drugs is also facing severe threat. Ayurveda, like other systems of herbal medicines, has reached a very crucial phase. About 50% of the tropical forests - the treasury of plant and animal diversity - have already been destroyed and the remaining half may not withstand the onslaught of man for another century. In India alone, about 55,2000 sq. km. of forest - that we had in 1975 - have been reduced to 45,7000 sq.km. by 1982 (Khoshoo, 1986). Forests in India have been disappearing at an average of 1.5 m. ha every year; and what left at present is only 8% as against a mandatory 33% of the geographical area. This wanton destruction has rendered almost 3-4 thousand species of Indian plants on the verge of extinction. Sivarajan (1991) had dealt with the causes and consequences of deforestation in India

in detail. Due to increasing demand for raw drugs, accompanied by unscrupulous and unscientific collection of medicinal herbs world over, some of the most valuable plant species are facing extinction.

Of course, if this process of over exploitation continues unbridled, there may have fewer sources of supply of these life saving raw materials, and their scarcity would further generate marketing of substitutes, allies, and false products which will hinder the very existence of our indigenous medicinal systems (Rao, 1991). To overcome these dangers, the following measures have to be adopted on warfoot, at least in the case of the red-dated species.

(1) *In situ* conservation of such medicinal plants in their natural habitats, as sancturies or as gardens.

(2) Artificial selection and improvement of germplasm by suitable inbreeding and outbreeding.

(3) *In vitro* conservation: This is a novel technique, which is attaining greater significance these days. It comprises a wide range of techniques including *in vitro* culture of organs, tissues, cells and protoplasts by adopting recent biotechnological methods to improve the existing stocks and to select the high-yielding varieties for the production of higher titres of active principles, to be used for medicinal purposes.

Some medicinal plants (although highly desirable as herbal source of drugs) cannot be procured in sufficient quantity to meet the demand, because such plants could not be domesticated, and if so, they are slow growing. Secondly, the production of crude drugs is

subject to vagaries of the climate, crop diseases and varying methods of collection and processing, which also influence the availability of raw herbal drugs.

Conservation in natural habitats, though desirable, is becoming increasingly difficult and in certain cases impossible. During such situations, *in vitro* culture offers an effective alternate method for alleviating the problems facing various ayurvedic pharmaceuticals and naturalists. Nevertheless, through this method; conservation of germplasm, induction of variations, bio-transformations, production of secondary metabolites, *etc.* could be achieved with great ease. *Ex situ* conservation by *in vitro* techniques is a part of biotechnology which involves judicious management of the available bio-resources for the welfare of mankind. One such challenging aspects is plant tissue culture (Henle, 1993).

Application of tissue culture techniques unfolds a wide scenario, which is characterized by the totipotency of plant cells, as clearly explained by Haberland (1902) for the first time on palisade tissue of *Lamium purpureum* and *Eichhornia crassipes.* Meristem culture is considered to be the best method for the rapid propagation of plants (Chopra, 1990). It ensures genetic stability in the *in vitro* propagated progeny (D'Amato, 1977), and enables the plants to be free from the various pathogens including viruses, mycoplasmas, viroids, bacteria and fungi (Walkey, 1972; Murashige, 1974). However, the cultured meristems constitute an ideal material for the long-term conservation of germplasm by cryopreservation.

Since the cultivation practices for medicinal plant are being highly conventional and the natural reserves extremely limited, *in vitro* propagation becomes an important tool for rapid multiplication of medicinal plants (Jha & Sen,1984; Ramavat *et al.*, 1991). Meristem culture technique has been extensively employed by researchers and nursery men for the commercial exploitation of many economically important plants. It offers wonderful opportunities in the field of genetic manipulation, somatic embryogenesis and various screening strategies (Bajaj, 1986; George, 1996).

Research in the synseed (synthetic seed) technology increasingly illustrates developmental similarities between zygotic embryos in seeds and somatic embryos produced from cell culture. It is interesting to note that, being immobilised as synseeds, certain seed characteristics must be duplicated in somatic embryos. The possibilities of utilising somatic embryos in the same manner as seeds offer intriguing applications in many areas of agriculture. Somatic embryogenesis would maintain germplasm purity and allow the amount of space required to house valuable collections to be reduced from several acres to just a few square feet of storage space (Redenbaugh, 1993; Redenbaugh *et al.*, 1993).

Plant cell bears potential for the production of secondary products at commercially feasible levels. It has renewed the enthusiasm for the production of secondary metabolites from plant cell cultures. Substances like, alkaloids, sterols, anthraquinones, anthocyanins, flavonoid and terpenes have played a pivotal role in pharmaceutical, cosmetic, perfumery, dyeing and flavour industries.

In addition to their economic importance, many secondary metabolites play ecological and physiological roles in higher plants, either to protect harbouring plants against micro-organisms and animals or to enhance the ability of one plant species to compete with other plants in a particular habitat for better survival (Bell, 1980).

Taking all these aspects into account for the present research endeavour, two important endangered medicinal herbs of South India, *viz., Alpinia galanga,* (*vern*: Mal. Aratha, Eng. Greater galanga) and *Kaempferia rotunda* (*vern*: Mal. Chengazhineer kizhangu, Eng. Indian crocus) belonging to the family Zingiberaceae, which are traditionally being used in the Indian system of medicines, have been selected for a detailed study (Sabu, 1991, Anand & Hariharan, 1998).

Chemical composition of important medicinal compounds and the utility of these MAPs in clinical preparations have scarcely been documented. Chemically, the rhizome of *A. galanga* contains essential oils (0.04% of biomass) , comprising 48% methyl cinnamate and 20-30% cineol with some camphor, and probably *d*-pinene. Leaves also yield a volatile oil (Anonymous, 1948). Rhizome of *K. rotunda,* on steam distillation yields a light yellow volatile oil (0.2% of biomass) with an unpleasant odour. It contains cineol and probably methyl chavicol (Anonymous, 1959).

Clinically, the dried and sliced rhizome of *A. galanga* constitutes a major ingredient in more than 50 Ayurvedic preparations-like *aswagandharishta, chavikasava, balathaila, ashtavargham kashaya, etc.* (Sabu, 1991). These preparations are customarily being used against headache and rheumatic pains. Rhizome in its raw form (being

aromatic, pungent and bitter) improves appetite, taste and voice (Kirtiker & Basu, 1935; Sivarajan & Balachandran, 1994). Tubers of *K. rotunda* have been used in more than 21 Ayurvedic preparations like *asokarishta, anuthaila, chavanaprasam* etc. (Sabu, 1991). The tubers have aromatic, thermogenic, stomachic, anti-inflammatory, sialagogue, emitic and vulnerary (Anonymous, 1959). It is useful in vettiated conditions like vata and kapha, gastropathy, dropsy, inflammations wounds, ulcers, blood clots,and cancerous swellings (Kirtikar & Basu, 1935; Sabu, 1991). The powder extracted from *K. rotunda* is made like paste and is used for healing of fresh wounds.

Being important MAPs, massive collection and unscientific over-exploitation of these wild-growing *A. galanga* and *K. rotunda* resulted in its enlistment among endangered plants, which require immediate attention (Sabu, 1991; Singh & Pruthi, 1998). Hence, conservation of the germplasm of these plants is one of the most important and urgent tasks facing plant scientists of today, and its need is the greatest in the tropics, particularly in tropical Asia, where genetic diversity is great and the existence of many species is threatened. Traditional and aromatic plants are among the foremost in the list of endangered plant species throughout the world. Whenever a plant product is being extracted or processed, germplasm conservation must be incorporated as an integral part of the whole operations. In this context, *in vitro* conservation of such plants should be the lone alternative to achieve this goal with great ease and least problems.

I.1. *Relevance of the Present Study:*

Extensive and indiscriminate deforestation have resulted in the extinction of valuable medicinal herbs, along with many others, which may have become useful later. Even the exact ones are not available in sufficient quantities, nor yield to cost effective procurements as they have receded to inaccessible areas. Thus, heavy shortage for the much renowned rejuvenative drugs persists, which are now being adulterated with various sources. Cultivation, collection, productivity and quality of *Alpinia galanga* and *Kaempferia rotunda* have heavily affected many crop-specific problems. Lack of viable seeds and scarcity of free planting material make conventional crop improvement programmes ineffective. Furthermore, *A. galanga* and *K. rotunda* are being vegetatively propagated and vegetative propagules are the products of commerce. Thus, these two plants are ideally suitable for biotechnological manipulations to solve many of the aforesaid problems.

Under these circumstances, *in vitro* techniques of plant biotechnology is an useful tool and is ideally suited for crop improvement, particularly in vegetatively propagated crops like *Alpinia* and *Kaempferia.* Cell and tissue culture techniques can be used for production and multiplication of disease free planting material, creation of genetic variability, development of molecular markers for finger printing genotypes, production of transgenics and subsequently new varieties with desirable traits. *In vitro* techniques like production of synthetic seeds and maintenance of cultures in minimal growth media could be used for conversion and exchange of germplasm.

Development of efficient protocols for the reincarnation of these plants is the most important prerequisite for the meaningful application of biotechnology on them. Moreover, disillusioned with the synthetic western medicines, more and more people are now realising that *natural are better* and are returning to the traditional herbal systems. This green wave is likely to gain momentum in the years to come (Tyler, 1986). Immense possibilities of these age-old Indian systems in achieving the proclaimed goal of **health for all by 2000 A.D.**, must be realised.

With the above background, the present work has been initiated with the following objectives in view:

I. 2. ***Objectives:***

1. To select suitable explant(s) for the effective *in vitro* studies of *A. galanga* and *K. rotunda*.

2. To standardise a reproducible and easy procedure for the sterilisation of the explants for its maximum survival on the medium.

3. To choose the suitable standard medium for further culture studies.

4. To standardise most suitable cytokinin for the direct shoot multiplication of *A. galanga* and *K. rotunda*.

5. To standardise most suitable combination of auxin-cytokinin to induce maximum number of shoots.

6. To standardise the best auxin for *in vitro* rooting of shoots

obtained by direct or indirect methods.

7. To standardise the most suitable concentration of sucrose for the maximum frequency of shoot multiplication.

8. To standardise suitable hormone combination for the viable callus induction on sprout discs of *A. galanga* and *K. rotunda.*

9. To induce caulogenesis and rhizogenesis from the calli by indirect method.

10. To maximise the field establishment rate of plantlets produced by direct multiple shoot induction.

11. To maximise the field establishment of plantlets produced by indirect method.

12. To maximise the field establishment of plantlets produced by somatic embryogenesis.

13. To standardise the best formula for the preparation of calcium alginate beads for synseed production.

14. To study the various stages of development of somatic embryos by histological method.

15. Popularisation of the protocol developed and transfer technology from the lab to field.

***

CHAPTER II

REVIEW OF LITERATURE

Medicinal and aromatic plants (MAPs) have been known for millennia, and all cultures from ancient times to present day being dependent on plants in one way or other as a source of medicine. Rapid denudation of natural green lands, coupled with large scale collection of MAP have led to the decline of the availability of such plants and extinction of some important species. It necessitated the development of suitable techniques for rapid and large scale multiplication of MAPs. Large scale propagation of medicinal plants can be achieved by clonal multiplication or micropropagation - an established technique in plant tissue culture. By culturing organs (shoot tip, axillary buds, leaves, roots, meristems, stem, node or cotyledonary node) of selected high yielding medicinal plants and inducing multiple shoots, genetically uniform offsprings could be obtained. It offers many unique advantages over conventional propagation methods - for instance - rapid multiplication of valuable genotypes, expeditious release of improved varieties, production of disease-free plants, non-seasonal production, germplasm conservation, and also facilitating novel delivery system of propagules generated with the above objectives, through the production of synseeds.

Most of the published reports of the plant tissue culture on herbaceous, ornamental, bulbous plants, corm producing species, ferns and mosses, orchids, woody ornamentals, tree species, coniferous trees, fruit and nut crops, temperate field crops, medicinal plants, cereals and grasses, root and tuber crops, forage and grain legumes, subtropical and tropical vegetables, *etc.* have been reviewed by George

Ohyama (1975), Minocha (1987), Nadel *et al.* (1991); light and photoperiod by Pinker *et al.* (1989), Morini *et al.* (1992), and gelling agents by MacRae and Vanstaden (1990), Pochet *et al.* (1991) were investigated in detail. Plantlets regenerated from shoot apex cultures may occasionally include variants. However, most of the reports imply that genetic stability is preserved in shoot tip cultures (D'Amato, 1985; Murashige, 1974; Morel, 1965). According to D'Amato (1977), this stability is retained due to the fact that the shoot apex provides enough background, on which cell division and DNA replication are rightly controlled and the cells with genetic defects are eliminated because of competition. Developmental stages of an explant as well as its size can greatly influence the success of a culture procedures (George & Sherrington, 1984). Size of the explant determines the survival rates of the cultures - the larger the explant, more the chances of its survival. Similarly, whenever elimination of viruses is one of the objectives; meristem of small size should be used (Kartha & Gamborg, 1975; Hu & Wang, 1983).

Micropropagation of plants is a multi-billion dollar industry, being practised as hundreds of small and large scale expeditions. This method is employed, especially for the propagation of economically important ornamental and foliage plants and recently - along with medicinal and aromatic plants - plantation crops are also getting increased interest (Bajaj *et al.*, 1988, Bajaj, 1986). At present, there are about one thousand plant species, which are found to be feasible for micropropagation (Murashige, 1974; George, 1996). They include the species of *Anthurium, Arachis, Asparagus, Beta, Brassica, Chrysarthemum, Dianthes, Cicer, Gerbera, Gladiolus, Phlox, Prunus Vigna, Zea, etc.* (George, 1996).

In 1974, Murashige had developed the concept of three developmental stages for micropropagation, *viz;* explant establishment, multiplication of the propagules, rooting and hardening for transferring them to soil. This concept has been expanded further, and now accepted that there exist five stages for propagation-like preparative stage to minimise contamination, callus culture, organogenesis, multiplication by sub-culture, and transfer them to green house conditions (Debergh & Zimmerman, 1991).

During the last few years, there has been great interest in propagating MAPs *in vitro* (Bhat *et al.*, 1994; Purohit *et al.*, 1994; Sudha & Seeni, 1996; Pattnaik & Chand, 1996; Sahoo & Chand, 1998). Apical or axillary shoot buds, nodes, shoot tips, leaves, cotyledons or cotyledonary nodes from aseptically germinated seedlings, embryonal axis from zygotic embryos, *etc.* were the chief explants used for these studies. Development of multiple shoots from nodal culture have been reported in *Datura innoxima* (Engrild, 1973); *Adatoda beddomii* (Sudha & Seeni, 1994); *Piper nigrum* and *P. longum* (Bhat *et al.*, 1995) and *Melisa officinalis* (Tavares *et al.*, 1996). All these plants were grown on MS medium containing auxin and cytokinin combination. List of some important medicinal plants, which are being subjected to tissue culture techniques are summarised in Table 1.

Table 1. A profile of plants having noteworthy results in plant tissue culture

	Plant species	Ex-plant used	Results	References
1	*Acorus calamus*	Rhizome buds	Multiple shoots	Harikrishnan *et. al.* 1997
2	*Allium cepa*	Bulbs	Multiple shoots	Kahane *et al.*, 1992
3	*Aloe barbadensis*	Vegetative meristem	Multiple shoots	Natali *et al.*, 1990
4	*Aristolachia indica*	Young leaves, nodes	Multiple shoots	Remashree *et al.*, 1994
5	*Asparagus racemosus*	Callus	Shoots	Kar and Sen, 1985
6	*Calotropis gigantia*	Immature embryos	Callus and shoots	Roy and De, 1990
7	*Cephalis ipecacuanha*	Root segments	Direct multiple shoots	Yoshimatsu and Shimomura, 1994
8	*Coleus parviflorus*	Callus	Shoot regeneration	Bejoy *et al.*, 1990
9	*Clyeome gynandra*	Gynandrophore	Multiple shoots	Naseem and Jha, 1997
10	*Cympopogon flexosus*	Stem segments	Multiple shoots	Nayak *et al.*, 1996
11	*Datura insignis*	Node	Multiple shoots	Cristina *et al.*, 1990
12	*Digitalis thaspi*	Shoot tips	Multiple shoots and direct rooting	Herrera *et al.*, 1990
13	*Eclipta alba*	Nodal segments	Multiple shoots	Franca *et al.*, 1995
14	*Flaveria trinervia*	Leaf callus	Shoots	Sudharsana and Santhamma, 1991
15	*Gardenia jasminoides*	Shoot tips	Multiple shoots	George *et al.*, 1993
16	*Gloriosa superba*	Shoot tips	Multiple shoots	Samarajeewa *et al.*, 1993
17	*Glycyrrhiza glabra*	Node	Multiple shoots	Kukreja, 1998
18	*Gomphrena officinalis*	Callus	Adventitious shoots	Mercier *et al.*, 1992

Contd ...

No.	Plant	Explant	Response	Reference
19	*Gymnema elegans*	Axillary node	Multiple shoots	Komalavalli and Rao, 1997
20	*Gymnema sylvestre*	Nodal explant	Shoots	Reddy *et al.*, 1998
21	*Hemidesmus indicus*	Axillary buds	Shoots	Pattnaik and Debata 1996
22	*Majorana hertensis*	Nodal stem	Multiple shoots	Iyer and Pal, 1998
23	*Mentha piperata*	Leaf and Nodal explants	Multiple shoot	Kukreja, 1996
24	*Naregamia alata*	Leaf callus	Shoots	John *et al.*, 1997
25	*Ornithogalum umbellatum*	Bulb scale	Shoots	Nayak and Sen, 1995
26	*Phytoloca dodecandra*	Shoot tips	Multiple shoots	Demeke and Hughes, 1990
27	*Plumbago rosea*	Node	Direct shoots	Harikrishnan and Hariharan, 1996
28	*Piper* species	Node, internode	Adventitous shoots	Bhat *et al.*, 1995
29	*Piper nigrum*	Shoot tips	Multiple shoots	Philip *et al.*, 1992
30	*Piper betle*	Shoot buds	Multiple shoots	Nirmalbabu *et al.*, 1992
31	*Piper colubrinum*	Leaf callus	Shoot regeneration	Kelkar *et al.*, 1996
32	*Rauwolfia micrantha*	Nodal explants	direct shoots	Sudha and Seeni, 1996
33	*Sesamum indicum*	Nodal explants	Multiple shoots	Gangopadhyay, *et al.*, 1998
34	*Trichopus zeylanicus*	Shoot tips	Shoots	Krishnan *et al.*, 1995
35	*Tridax procumbense*	Node	Multiple shoots	Sahoo and Chand, 1998
36	*Tylophora indica*	Nodal stem	Multiple shoots	Sharma and Chandel, 1992
37	*Vetiveria zizanoides*	Callus	Multiple shoots	Mucciarelli *et al.*, 1993
38	*Vanilla planifolia*	Axillary buds	Multiple shoots	George and Ravishankar, 1997
39	*Withania sonifera*	Leaf callus	Direct shoot regeneration	Abhyankar and Chinchanikar, 1996

II. 2. *Organogenesis:*

Callus cultures were utilised successfully for the regeneration of shoots, roots and somatic embryos in many herbaceous plants, including MAPs. Most frequently, plant regeneration from *in vitro* callus occurs *via* adventitious shoot formation, and rarely through somatic embryos (Murashige, 1974). Very recently, callus induction and organogenesis have been reported in certain important medicinal plants such as *Aristolchia indica* (Remashree *et al.*, 1997); *Piper attenuatum* (Madhusudhanan *et al.*, 1996); *Allium senescens* (Nair & Seo, 1995); *Eclipta alba* (Franca *et al.*, 1995); *Orithogalum umbellatum* (Nayak & Sen, 1995) *Hemidesmus indicus* (Sarasan *et al.*, 1994); *Clitorea ternatea* (Kumar *et al.*, 1993); *Piper longum* (Sarasan *et al.*, 1993); *etc.* These reports seem to be very much successful and reproducible.

II. 3. *Embryogenesis:*

Following the initial reports on the differentiation of embryoids from carrot tissue cultures by Steward *et al.* (1958) and Reinert (1959); considerable data have been accumulated on *in vitro* induction of embryos in cultures of several plants (Ammirato, 1983; Debeaujon & Branchard 1993). Somatic embryogenesis is of considerable interest from several points of view: *viz.*, (i) to understand the physical and chemical requirements for embryoid induction and development (ii) to study the biochemistry underlying this process, and (iii) to raise plantlets on commercial scale, which breed true-to-type *ie,* homozygous.

After carrot becomes a model for studies on embryogenesis in culture, investigations were extended to several other medicinal plants like *Asparagus cooperi* (Ghosh & Sen, 1991); *A. officinalis* (Delbreil & Jullien, 1994); *Apium graveolens* (Collin & Issac, 1991); *Carum carvi* (Furmanowa *et al.*, 1991); *Catheranthus roseus* (Kim *et al.*, 1994); *Cicer arietinum* (Ramana *et al.*, 1996); *Coriandrum sativum* (Kataeva & Popowich, 1993; Kim *et al.*, 1996) *Foeniculum vulgare* (Hunault & Du manoir, 1992); *Gladiolus hort* (Stefanaik, 1994); *H. indicus* (Sarasan *et al.*, 1994); *P. nigrum* (Joseph *et al.*, 1996) and *Quercus acutissima* (Kim *et al.*, 1994). Direct embryogenesis from leaf explants of sugar cane (*Saccharum officinarum*) was reported by Manickavasagam and Ganapathi (1998); *Simaruba glauca* (Rout & Das, 1994); *Trachyspermum ammi* (Sehgal & Abbas, 1994). Recently, somatic embryogenesis by suspension culture of *Triticum aestivum* has been reported by Subhadra *et al.* (1995). An endangered medicinal plant, *Saraca asoka* was also regenerated by this method by Harikrishnan *et al.* (1998) from its cotyledonary node cultures.

II. 4. *Synseed Production:*

Encapsulation (entrapment) of somatic embryos or vegetative parts such as shoot tips and axillary buds for construction of synseeds (synthetic seeds or artificial seeds) has received considerable attention in recent years (Nayak *et al.*, 1998; George & Eapen, 1995; Paulet *et al.*,1993). Redenbaugh *et al.* (1984) were the first to report on synseed production in celery, a valuable spice. More such reports were appeared on various medicinal plants like *Asparagus officinalis* (Uragami *et al.*, 1990) *A. cooperi* ((Ghosh & Sen, 1994); *Cymbidium*

giganteum (Corrie & Tandon, 1993); *Eleusine coracana* (George & Eapen 1995) and *Saccharum* sp. (Paulet *et al.*, 1993).

In 1998, Nayak *et al.* have reported high frequency of plant regeneration from calcium alginate (a common material used for encapsulation) encapsulated protocorm-like bodies of *Spathoglottis plicata*. In fact, encapsulation of somatic embryos in a variety of gel coatings to produce artificial seeds for direct field planting has been achieved with limited success (Ganapathy *et al.* 1994). Being an emerging field, synseed production is achieving great momentum these days.

II. 5. In vitro *Studies in Zingiberaceae:*

Literature survey on *Zingiberaceae* reveals that most of the tissue culture works have been confined to the genera *Elatteria, Curcuma* and *Zingiber,* owing to their significance as plantation crops, yielding valuable spices and condiments. Other medicinal members like *Alpinia, Amomum, Costus, Kaempferia, etc.* are also occupying a prime position in making traditional medicines. However, literature survey provides only a few reports on the i*n vitro* studies on these plants, and in the light of such works, the important aspects have been reviewed on genus-to-genus basis.

II. 5.1. *Alpinia:* *In vitro* studies regarding *Alpinia* have been very much limited. The first ever *in vitro* report of clonal propagation on this genus was on *A. purpurata,* which came from Chang and Criley (1993), and they had demonstrated that axillary vegetative buds on inflorescence bracts that do not normally develop aerial offshoots can

be cultured *in vitro* to increase desirable clones efficiently. In 1995, Illg and Faria had described the micropropagation of the same plant by inoculating inflorescence buds on Murashige and Skoog (MS) medium containing 6-benzyladenine (BA) and naphthalene acetic acid (NAA). The plants so generated could be stored for more than six months in flasks containing deionised water and sucrose. In 1996, Fereol *et al.* described the effects of gamma irradiation on the *in vitro* plantlets of *A. purpurata,* and some plantlets so generated showed morphological abnormalities of leaves or of whole plants. In general, this technique has great potential for increasing the genetic variation of Zingibers.

Recently, *in vitro* clonal multiplication of *A. calcarata* from its young rhizome buds was reported by Agretious *et al.* (1996). Authors have tried different basal media, and maximum success was obtained in MS medium, supplemented with benzylaminopurine (BAP) and indole-3-acetic acid (IAA) with the concentrations of 1.5mg/l and 0.5 mg/I, respectively. Martin *et al.* (1999) could generate callus from the rhizome buds of *A. calcarata* and succeeded in its organogenesis for the generation of viable plantlets, and successfully transferred to the fields after hardening. Anand and Hariharan (1997) were succeeded in the *in vitro* multiplication of *A. galanga* from an excised rhizome bud cultured on MS medium, supplemented with benzylamino purine (BAP) alone or in combination with an auxin, especially indole-3-acetic acid (IAA).

II. 5.2. ***Amomum*:** Only one report is available on the *in vitro* studies of this genus, and was from Sajina *et al.* (1997). They reported the micropropagation of *A. subulatum,* by activating the rhizome buds so as to grow in MS medium by supplementing 3.0% sucrose and 0.5

mg/l kinetin. The sprouted buds were multiplied at the rate of 5-10 shoots per culture in the same medium containing 1.0 mg/I BAP and 0.5 mg/l indole-3-butyric acid (IBA). The *in vitro* generated plantlets could be established in the soil with 90% success.

II.5.3. ***Costus:*** Caulogenesis from rhizome callus of *C. speciosus* had been explained by Jain and Chaturvedi (1985). They had callused the rhizome tissue explants after 30 days incubation in a treatment comprising the SH basal medium, supplemented with adenine sulphate, BAP, IAA and malt extract. In 1991, Roy and Pal reported a rapid multiplication method of *C. speciosus* by *in vitro* rhizome production from the *in vitro* shoots developed from zygotic embryos. Pal and Sharma (1993) conducted the cytological and developmental studies on the callus cultures of this species. In 1996, Pal and Roy carried out a detailed study on the production and histological aspects of culture raised triploid and tetraploid *C. speciosus* plants. Their findings suggest that the culture conditions, probably the supplied nutrients induced nodal rhizome formation. Rhizome formation in this plant is significant, as it behaves as the perennating organ and it accumulates diosgenin, and steroidal saponin like aferoside-A (Lin *et al.*, 1996).

II.5.4. ***Curcuma:*** Nadgauda *et al.* (1978) regenerated multiple shoots from vegetative buds of turmeric *(C. longa)* on MS medium, supplemented with coconut milk, inositol, kinetin and BAP. Studies by Kuruvinashetty *et al.* (1982) had led to the standardisation of micropropagation techniques for turmeric and a high multiplication rate (7 to 10 per explant) was observed. Nadgauda and Mascaranhas

(1986) have proposed techniques for screening high curcumin-containing plants raised through callus cultures, and a positive correlation of curcumin concentration in the seedlings and in the mature rhizomes was established, which provides a basis for the selection in the laboratory of turmeric plants with high curcumin content.

Yasuda *et al.* (1988) reported successful callus induction and multiplication using MS medium with NAA, 6-benzyl adenine (BA) and kinetin, from the rhizome explants of *C. aromatica, C. domestica* and *C. zedoaria.* Balachandran *et al.* (1990) cultured rhizome buds of *Curcuma* sp. on MS medium, supplemented with BAP and kinetin, for the production of multiple shoots, and the *in vitro* plantlets were successfully established in the field and were reported to be morphologically uniform. A method for short-term *in vitro* conservation of *Curcuma* germplasm was also described in that report. Rhizome explants of *C.amada* produced shoots and roots simultaneously, suitable for field propagation and conservation, when cultured in B5 medium containing naphthaleneacetic acid (NAA) and BAP (Barthakur & Bordoloi, 1992).

II. 5.5. *Eletteria:* *E. cardomomum* (cardamom) has been referred to as the queen of spices, and in India it is a major representative of a few spices in which micropropagation system has commercially been exploited on large scale. Callus induction and organ differentiation in cardamom was reported by Rao *et al.* (1982) for the first time, but no details regarding the transfer of the plantlets to the field have been presented. Nadgauda *et al.* (1983) described the conditions tested for

in vitro multiplication of three high yielding cardamom varieties and their subsequent transfer to the field. The average multiplication rate per shoot was observed as 1:3. Kumar *et al.* (1985), using immature panicles of cardamom to derive plantlets directly without the intervention of callus or embryoids on MS medium supplemented with NAA, BAP, calcium pantothenate, folic acid, kinetin and coconut water.

Clonal propagation of different genotypes of cardamom, using rhizome buds has been described by Priyadarshan *et al.* (1988). The differential response of the genotypes to the same medium (MS) was also discussed, with IAA, BAP and kinetin as growth regulators. Reghunadh and Gopalakrishnan (1991) have standardised *in vitro* techniques for the production of high rate of shoot proliferation in cardamom, using modified MS medium. As per their reports, the method of shoot callus culture and subsequent organogenesis could efficiently be used to evolve stress tolerant cardamom varieties. Recently, encapsulation of embroys or shoot tips in sodium alginate was successfully carried out in cardamom (Kumar *et al.*, 1998).

II.5.6. ***Kaempferia:*** Although this genus has been declared as endangered (Sabu, 1991), only very few *in vitro* studies have been conducted so far with a view to preserve and utilise it economically. The first ever *in vitro* report on this genus (*K.galanga*) had been from Vincent *et al.* (1991, 1992 a, b), from our laboratory which described the various aspects of plantlet regeneration from callus, embryogenesis and micropropagation. Besides these reports, in 1997, Anand *et al.* have initiated a pioneering work on *K. rotunda* and succeeded in its

in vitro propagation taking rhizome bud as explants. They could generate an average of 8.0 shoots per culture on MS medium.

II.5. 7. ***Zingiber:*** *In vitro* clonal propagation studies on ginger was initiated by Hosoki and Sagawa (1977) on MS medium supplemented with BA, and morphogenesis was observed without any multiple shoot formation and with 60-70% survival of plantlets was recorded. Nadgauda *et al.* (1980) studied the conditions for obtaining multiple shoots from two varieties of ginger, and achieved 90-100 % plantlets survival in the field. Pillai and Kumar (1982) and Sato *et al.* (1987) explained the micropropagation of Zingiber. Kulkarni *et al.* (1984) subjected the ginger callus to culture filtrate of Pythium and subsequently regenerated plantlets indicating the possibility of creating vareties *in vitro.* Roller tube culture, a technique similar to gyratory shaking was applied to ginger, (Noguchi and Yamakawa, 1988) and the multiple bud clumps obtained when transferred to solid medium yielded better plants which could be acclimatised easily. A linear relationship between rate of multiplication and concentration of BAP was observed. Plants produced *in vitro* were easily established in the soil with almost 100 % survival. However, multiplication rate was lower than those reported by Bhagyalakshmi and Singh (1988). Callus induction and bud regeneration have reported in ginger by Kuruvina Shetty *et al.* (1982) and Ilahi and Jabeen (1987).

Micropropagation techniques for ginger were also reported by Inden *et al.* (1988) as well. Sakamura and Suga (1989), Wang (1989), Saradha and Padmanabhan (1989) were also explained micropropagation of ginger. Rapid clonal propagation and short term

conservation of ginger germplasm *in vitro* have been achieved by Balachandran *et al.* (1990) and Dekkers *et al.* (1991). Plant propagation by regeneration of ginger callus was reported by Malamug *et al.* (1991).

Nirmal Babu *et al.* (1992a,b) reported the conservation of floral buds of ginger into vegetative buds and plantlets directly by tissue culture and *in vitro* plant regeneration from leaf derived callus in ginger. The medium used was MS with BAP and 2,4-D. *In vitro* formation of roots and rhizomes from anther explants of ginger have been explained by Ramachandran and Chandrasekaran Nair (1992). The plant regeneration either by organogenesis or embryogenesis, and or both was explained by Kackar *et al.* (1993).

Sharma and Singh (1995) reported *in vitro* micro-rhizome production of ginger. Sharma *et al.* (1994) have reported that the shoot buds of ginger can be encpasulated in calcium alginate beads to make synthetic seeds. These seeds were germinated *in vitro* and thus could be used as planting material and for exchange. *In vitro* rhizome formation and their germination was also reported by various workers (Sakamura *et al.*, 1986; Sakamura and Suga, 1989; Bhat *et al.*, 1994). Microrhizome of *Zingiber officinale* was successfully produced from tissue culture derived shoots by transferring them to liquid MS medium, supplemented with 1 mg/l BAP, 2.0 mg/l calcium pantothenate, 0.2 mg/l GA_3 and 0.5 mg/l NAA (Sharma and Singh, 1995).

Most recently, embryogenesis and plant regeneration from ovary derived callus cultures of ginger were reported by Nirmal Babu *et al.* (1996). One to two week old ovary produced profuse callus, when

cultivated on MS supplemented with 2,4-D +BAP. Production of synthetic seeds was reported by Ravindran *et al* (1996), by encapsulating somatic embroys and shoot buds. *In vitro* pollination were conducted to overcome the pre-fertilisation in *Zingiber officinale* was achieved using callus derived from shoot bud primordia, grown on MS medium (Rout & Das ,1997). Organogenesis was maximum on the medium supplemented with 5.0 mg/l BAP, 1.0mg/l NAA and 100mg/l adenine sulphate.

Encapsulation of embryoids and shoot tips in sodium alginate was successfully carried out in cardomom as well (Ganapathy *et al.* 1994; Ravindran *et al.*, 1996). The production of synseeds were reported in several more species like ginger and vanilla, Ravindran *et al.*(1996) and Sharma *et al.* (1994), respectively.

II. 6. In vitro *Studies on Endangered and Rare Plants:*

Recently plant tissue culture is used for rapid multiplication of endangered species, in which conventional methods prevents limitations (Kartha, 1985). Conservation and multiplication of rare and endangered medicinal plants through tissue culture have acquired greater importance in recent years, because of major threat to germ plasm of medicinal plants. The important achievements include micropropagation of *Chlorophytum borivilianum* (Purohit *et al.* 1994); barberin synthesis by callus cultures of *Coscinium fenestration* (Nair *et al.* 1992); callus regeneration of *Coptis teeta* (Tandon & Rathore 1992); micropropagation *Coleus forkohlii* (Sharma *et al.* 1991); *Eremostachys superba* (Sunnichan & Shivanna 1998); *Gloriosa* sp. (Finnie & Van Staden, 1989); *Gymnema elegans* (Komalavalli & Rao 1997); *Leontochir*

ovallei (Lu *et al.* 1995) and *Picrorhiza kurroa* (Upadhyay *et al.*1989). *In vitro* propagation of *Rauwolfia micrantha* (Sudha & Seeni 1996) and rapid propagation through shoot tip cultures of *Trichopus zeylanicus* were explained by Krishnan *et al.* (1995). Mathur *et al.* (1989) cultured axial shoot buds of *Valeriana wallichii* and successfully encapsulated the tips obtained from multiple shoots. Purohit and Dave (1996) developed an *in vitro* procedure to multiply the endangered tree species *Sterculia urens* and Harikrislınan *et al.* (1998) reported propagation of another tree species, *Saraca asoka*. *Woodfordia futicosa* was micropropagated through shoot tip and nodal culture on SH medium, supplemented with 0.2mg/l BAP by Krishnan and Seeni (1994).

II.7. ***Modern Trends in PlantTissue Culture:***

In modern biotechnological approaches, the twin areas of plant cell culture and genetic engineering (recombinant DNA technology) have brought us to the verge of a major revolution in the culture and propagation of plants and breeding, for superior varieties. Tissue culture technology combined with classical breeding procedures would serve to attain the precise breeding goals envisaged at the commencement. Protoplast culture has also opened up possibilities for introducing new genetic characteristics to a plant cell using a vector, often through the direct DNA up take techniques.

II. 8. ***Conclusion:***

General procedures for the tissue culture operation for any plant materials would become common. Though popular media formulae

are available; selection and activation among and in such media may be required. Marginal variations in the concentration of hormones and their combinations may pose yet another severe challenge in developing a suitable protocol for any novel initiative. In fact, back references may not be a perfect solution to these questions. However, it is anticipated that such painstaking achievements would certainly be a backbone to go deep into the present initiative.

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CHAPTER III

MATERIALS AND METHODS

The materials and methods adopted in the present study have been described in brief. For convenience, the entire aspects are being subdivided under different heads.

III.1. *Source of Explants:*

For the present study, mother plants of *Alpinia galanga* (Linn) Willd and *Kaempferia rotunda* Linn. were procured from the herbal garden of the Aryavaidya Sala, Kottakkal, Malappuram, Kerala, and maintained on garden pots at the Department of Botany, University of Calicut. For experimental purpose, healthy rhizomes of these plants were harvested and washed with 0.2% Dithane M-45 for 30 minutes, under constant stirring. After repeated washing, these rhizomes were dried in shade, and planted in plastic trays, containing sterile sand and maintained in the green house of the Department garden. The rhizomes of different age (collected after 1-5 months of cultivation in the trays), bearing sprouts were used for the culture experiments. The mother sprouts, which used as explants were categorized in to two parts: the upper dome-shaped sprout bud (simply, bud) and its basal rounded ring-like portion, the sprout-disc (simply, disc). Young leaves, flower buds, root tips, *etc.* were also collected from the same mother plants and used for culture directly, after proper sterilization.

III. 2. *Preparation and Sterilisation:*

The sprouting buds along with the attached rhizome were excised and washed in running tap water for 30 minutes, and the outer scale leaves were removed carefully. The explants were surface-dried

by blotting with sterile filter paper, and then treated with 0.2% Dithane M-45 for 20-25 minutes in a 250 ml conical flask. Subsequently, three to four washings were done using sterile double distilled water to remove the traces of the fungicide used. The expalnts were then treated with mercuric chloride ($HgCl_2$) solution (0.05 - 0.2%) for 10-20 minutes and washed thoroughly in sterile distilled water. After $HgCl_2$ treatment, the explants were treated with 70% ethanol for 10-25 seconds, and then washed thoroughly with sterile distilled water twice. Except the innermost leaf primordium, all other leaves and attached rhizome tissues were trimmed off to get buds of about 1.0-1.5 cm^3 size before inoculation. The sterile explants thus obtained were inoculated asceptically on autoclaved and pre-cooled culture medium on laminar-air flow chamber (Thermadine, India).

III.3. ***Detergents and Fungicides:***

For surface sterilisation, detergents - like teepol, extran and soap water and fungicides like Bavistin (0.2%) Dithane M-45 (.2%) and the sterilant $HgCl_2$ (0.05 - 0.2%) were used in the present study.

III. 4. ***Antibiotics:***

To eliminate bacterial cartaminations, antibiotics like - ampicillin or erythromycin (10 - 100 mg/l) was added to the medium, when fresh explants were used. It was not added to the medium in subsequent cultures.

III. 5. ***Chemicals:***

Chemicals used for the present study were of analytical grade, and were obtained from Sigma Chemicals Company, USA; E-Merck (India) Ltd., Hi-media, Bombay; Qualigens (India) and British Drug House (BDH), India.

III. 6. ***Glassware:***

Standard Borosil and Corning glassware were used for the culture experiments. Culture tubes (25 x 200 mm, 25 x 150 mm & 32 x 200 mm) and conical flasks (100 - 250 ml) were used throughout the work.

III. 7. ***Preparation of the Medium:***

Different nutrient media like B5, MS, SH and WH (Table 2) were tested for their potentiality of supporting the explant during various experiments. For the different media formations, fresh stocks were prepared once in a month. Separate stock solutions were prepared according to standard methods of George and Sherrington (1984) and stored in the refrigerator at 2-4°C. Separate stocks were prepared for macro and micro-nutrients, vitamins and amino acids. Stocks of calcium chloride, ferrous sulphate, Na_2EDTA (ethylene diamine tetra acetic acid) and glycine were also prepared separately. Glass double distilled water was used for all operations and necessary media were formulated from basic ingredients. Appropriate concentrations of different elements and growth regulators were added before making the final dilution to required volume.

Prior to autoclaving, the pH of all media was adjusted to 5.8 using either 0.1 N sodium hydroxide solution (NaOH) or 0.1 N hydrochloric acid (HCl). For solidifying the culture medium, bacteriological grade agar (0.8%) was used. About 20-25 ml of medium were dispensed in to each culture tube conical flask. The containers were sealed with cotton plugs made of non-absorbent cotton, covered with chease cloth/alluminium foil/polypropylene caps, and autoclaved for 20 minutes at 121°C and 15 ψ pressure.

III. 8. *Growth Regulators:*

The basal medium was altered by adding different concentrations and combinations of auxins and cytokinins, either singly or in combinations.

III. 8.1. *Auxins:*

The major auxins, *viz.,* indol-3-acetic acid (IAA); α-naphtalene acetic acid (NAA); indol-3-butryric acid (IBA), and 2,4-dichlorophenoxyacetic acid (2,4-D) were used in this study at a concentration 0.25 to 4 mg/l or, otherwise it is specified.

III. 8.2. *Cytokinins:*

Two major cytokinins such as, 6-benzylaminopurine (BAP) and 6-furfurylaminopurine (KN) at a concentration of 0.1 - 6 mg/l were used in the experiment. In addition to these cytokinins, tender coconut water (CW) at a concentration of 5 - 30 ml/l was also used.

III.8.3. *Gibberellic Acid (GA_3):*

In addition to auxins and cytokinins; the growth regulator, gibberellic acid (GA_3) was also used at the concentrations of 1.0 mg/l - 5 mg/l. GA_3 was microfiltered for sterilisation and added to the autoclaved medium.

III. 9. Culture Experiments:

9.1. *Asceptic Techniques:*

All procedures demanding asceptic conditions were carried out on an aseptic laminar-air flow cabinet. All requirements needed for inoculation were exposed for half an hour to UV radiation on the cabinet. The culture instruments were flamed while handling, using rectified spirit. All the cultures were maintained in the culture room at 25 $\pm$ 2°C and 16/8 hour photoperiod, under the white fluorescent tubes (1500-2000 lux).

III. 9.2. *Direct Multiple Shoot Regeneration:*

For the establishment of direct shoot multiplication in *A. galanga* and *K. rotunda* from their sprout-buds, MS medium supplemented with different concentrations of cytokinins (BAP, KN or CW) or various combinations of auxins (IAA, IBA or NAA) and cytokinins were used. All the cultures were incubated under culture conditions mentioned elsewhere. All experimental data were recorded weekly on each individual treatment, and also optimum concentration of growth regulators facilitating maximum shoot production and development.

III. 9.3. *Effect of Different Sucrose Level on Direct Shoot Regeneration:*

Without altering the other nutrient compositions of standard MS medium various levels of sucrose (1-5%) were added to detect the most suitable concentration of sucrose for direct multiple shoot induction from rhizome sprout buds of *A. galanga* and *K. rotunda*.

III. 9. 4. *Indirect Multiple Shoot Induction:*

A protocol for indirect caulogenesis and rhizogenesis from the sprout discs through callus was developed for *A. galanga* and *K. rotunda* by using MS nutrient medium containing different growth regulators such as auxins (NAA and 2, 4-D) and cytokinins (BAP and KN). Depending upon the nature of the plant species and size of the explants, growth regulators were used either singly or in combinations. Callus obtained in different combinations were sub-cultured for further growth and redifferentiation (organogenesis). The calli have been maintained indefinitely by serial sub-culture of 3 weeks interval.

III. 9.5. *Somatic Embryogenesis:*

The morphogenic potentiality of callus, obtained from disc explants were tested for the establishment of somatic embryogenesis. To realize these objectives, the callus obtained on high auxin/low cytokinins media were transferred to the medium with less auxin/high cytokinin and subsequent transfer to hormone free medium. Embryogenesis resulted and the plantlets so obtained were transferred to the field successfully.

III. 10. ***Rooting of* In Vitro *Shoots:***

Sufficiently grown shoots (10-12 cm) bearing 2-3 leaves were excised from primary cultures and transferred to MS (½ or full strength) medium containing IBA or NAA at the concentrations ranging from 1.0 to 4.0 mg/l. About 15-20 ml of the medium was dispensed to 25 x 200 mm culture tubes or 250 ml clonical flasks before autoclaving. The cultures were examined periodically and morphological changes, growth and percentage of response were recorded.

III. 11. ***Hardening and Field Transfer of the Plantlets:***

In vitro - raised plantlets with well developed leaves and roots were excised from primary cultures and washed carefully using single distilled water for removing all the traces of medium adhering to the roots. Plantlets were pre-treated by dipping in 0.2% Bavistin for 2-3 minutes and transplanted in polythene bags or plastic trays, containing a mixture of sterile garden soil and sand in 1:1 ratio. The transplanted plantlets were kept in humid chamber for 2-3 weeks for hardening and establishment under laboratry conditions. These plantlets were transferred to the field. The growth of the transplanted plantlets were recorded in weekly intervals.

III. 12. ***Histological Studies:***

To study the different stages of embryogenesis, somatic embryos were subjected to histological studies. The materials were fixed in formalin-acetic acid- alcohol mixture (FAA, 1:1:3) (Johansen, 1940). The materials were dehydrated through a graded series of

tertiary butyl alcohol (TBA) followed by embedding in to molten paraffin (58-60°C). Serial sections of 5 to 10 μM thick were prepared for photomicroscopy using rotary microtome (Leitz, Germany).

The serial sections fixed on microslides were then subjected to staining process. Deparaffinisation of the slides were carried out with xylene and the sections were passed through a downward series of TBA and finally rinsed with distilled water. The slides were stained using 0.1% Heidenhein's haematoxylin (Jensen, 1962), employing the mordant ammonium ferric sulphate. Counter staining was carried out, using saffranin. After staining, the slides, were dehydrated through upward series of TBA and xylene, and finally mounted on Dextrene plastisizer xylene (DPX) mountant.

III. 13. ***Synseeds:***

The globular or turpedo-shaped somatic embryos obtained from callus culture, were encapsulated in calcium alginate beads. For encapsulation of the same, 1-5% solution of sodium alginate was prepared, containing the ingredients of MS medium (without $CaCl_2$) with 1.0 or 2.0 mg/l BAP, autoclaved after adjusting the pH to 5.8. After cooling, the somatic embryos were mixed with the above preparation and dropped in to calcium chloride solution (25 -150 μM) using a glass column fitted on a stand under laminar hood.

The drops set as small transparent beads when left in the same $CaCl_2.H_2O$ solution for 30 minutes for curing. The beads were removed and washed with one or two changes of sterile water. All

these experiments were done at asceptic condition. The synseeds were stored in petri-plates or in MS medium at 3-4°C.

III. 14. *Photographs:*

The sections were observed using Carl-Zeiss microscope and photomicrograph of different developmental stages were taken using Olympus camera (Asahi Pentax 35 mm SLR). Photographs of culture tubes and flasks were taken by Bellows close-up camera (Leica DBP, Wtzlar-Germany).

III. 15. *Growth Measurements:*

Growth measurements consisted of number of shoots/roots produced, length of shoots, percent of culture, response, callus induction, organogenesis/ embryogenesis, *etc.* Visual rating was also carried out on same experiments. Detailed description of growth responses is included in the respective sections.

III. 16. *Statistical Analysis:*

Observations were recorded for a number of cultures developing multiple shoots/roots, length of plantlets, amount of callus produced at different intervals. The most promising treatments for various observations were repeated to confirm its reproducibility. At least 10 replica were made for each treatment, and in all cases the results were confirmed. A completely randomised design was used for statistical analysis. Mean and standard deviations were calculated using the method of Misra and Misra (1983).

Table 2. Formulations of Tissues Culture media used (Composition mg/l)

Components	MS	B5	WH	SH
Macronutriats				
NH_4NO_3	1650	--	--	
KNO_3	1900	2500	80	2500
$CaCl_2$ - $2H_2O$	440	150	--	200
$MgSO_4$ - $7H_2O$	370	500	720	400
KH_2PO_4	170	--	--	--
$(NH_4)_2 SO_4$	--	134	--	--
NaH_2PO_4 H_2O	--	150	16.5	--
$Ca(NO_3)_2 4H_2O$	--	--	300	--
KCl	--	--	65	--
Na_2SO_4	--	--	200	--
$NH_4H_2PO_4$				300
Micro nutrients				
KI	0.830	0.750	0.75	1.000
H_3BO_3	6.200	3.000	1.50	5.000
$MnSO_4$ 4 H_2O	22.300	10.000	7.00	10.00
$ZnSO_4$ $_7H_2O$	8.600	2.000	8.000	1.000
$Na_2M_0O_4$ $_2H_2O$	0.250	0.250	--	0.100
MOD_3	--	--	--	--
$CuSO_4$ $5H_2O$	0.025	0.025	--	0.200
$CaCl_2$ $6H_2O$	0.025	0.025	--	0.100
Na_2 EDTA	37.300	37.300	--	20.000
$FeSO_4$ $7H_2O$	27.800	27.800	--	15.000
$Fe_2(SO_4)_3$	--	--	2.50	-
Organic constituents				
Myo -inositol	100	100	0.1	1000.0
Nicotinic acid	0.5	1	0.50	5.0
Pyredoxin HCl	0.5	--	--	0.5
Thiamin HCl	0.1	10	0.1	5.0
Glycine	2	--	3	--
Sucrose	30,000	20,000	20,000	30,000

***

CHAPTER IV

days for the induction of growth. The same morphogenic responses were noticed in the rhizome buds (60%) and discs (40%) of *K. rotunda* in an average of 35 days (Tab: 3). Hence, in subsequent studies, only rhizome buds and discs of both the plants were used as the explants. In fact, rhizome discs were used only for indirect shoot multiplication. For all other studies rhizome buds were used.

Table 3 . Result of preliminary experiments with different explants of *A. galanga* and *K. rotunda* on standard MS medium

Source		Explant used	Percentage of response	Days taken for response	Nature of response
	1	Young leaf discs	10	50-55	Only swelling of leaf epidermis
	2	Young flower buds	30	50-55	Explants becoming swollen and gradually dead
A. galanga	3	Root tips	10	50-55	Massive nature
	4	Young rhizome sprout buds	50	35-40	Healthy sprout emerged out and size increased
	5	Sprout discs	40	35-40	Surface growth of cut end
	1	Young leaf discs	20	50-55	Epidermis swells
	2	Root tips	10	50-55	Massive nature
K. rotunda	3	Young rhizome sprout buds	60	30-40	Sprouts emerged out
	4	Sprout discs	40	30-40	Surface growth of cut end

IV.1.1. *Size of the Explants:*

Size of the explant was found to be a crucial factor in the survival rate of the culture. When larger explants were used, the chances for contamination were more, and use of smaller size (~ 1.0 cm^3) was suitable to enhance establishment percentage (Fig: I, Plate I

A, B). When rhizome sprouts of about 1.0 cm size were used, both *A. galanga* and *K. rotunda* showed best results. The percentage of establishment of *A. galanga* was 50 and that of *K. rotunda* showed still higher value *i.e.*, 60% (Fig. 1).

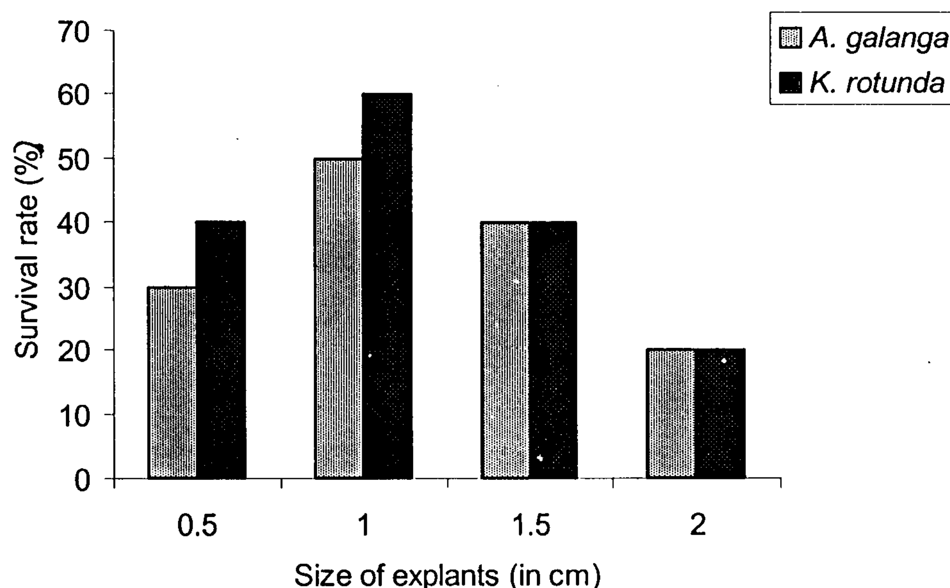

Figure 1. Survival of the rhizome sprouts on standard MS medium, after 35 days of incubation both in *A. galanga* and *K. rotunda*, when explants of different sizes used

Similarly, when rhizome discs were used for the culture, 40% of the discs showed surface growth at cut ends. Discs of ~ 1.0 cm^3 were responded very well (Fig: 2). When the size of the discs increased upto 2 cm, the bacterial and fungal contaminations were high, with very low survival rate (Fig. 2). In fact, maximum survival rate was only 40% in both the specimens, even the size of the explant maintained as ~ 1.0 cm^3. On the basis of these preliminary results, size of the explants (rhizome sprouts and discs of both plants) was maintained ~ 1.0 cm^3 for further studies.

OBSERVATION AND RESULTS

An efficient and reproductive protocol has been developed through asceptic cultures for the micropropagation of *Alpinia galanga* and *Kaempferia rotunda.* Different experiments were conducted on these very important medicinal plants to induce direct multiple shoots, callus, organogenesis and somatic embryogenesis. The following aspects were studied in detail and the results are being discussed with relevant recent works done in medicinal and aromatic plants (MAPs) elsewhere. Initial studies were conducted on standard MS-agar medium after surface sterilization of explant using the detergent extran (4% for 20 minutes) and 0.1% mercuric chloride ($HgCl_2$) for 15 minutes.

IV.1. *Explant Selection:*

Various explants were used in this study, and the most responsive ones were selected by trial and error methods. For each experiment, 10 replica were carried out. Percentage of survival, days taken for the induction of response and nature of morphogenesis were the main parameters investigated. A detailed profile of these aspects are shown in Table 3.

Among different explants used, buds of young rhizome sprouts and their discs of both plants (prepared as described under materials and methods) were more efficient than other explants used in terms of morphogenic percentage within minimum incubation time (Tab:3). About 50% buds of *A. galanga* produced heatlhy emergences in 35-40

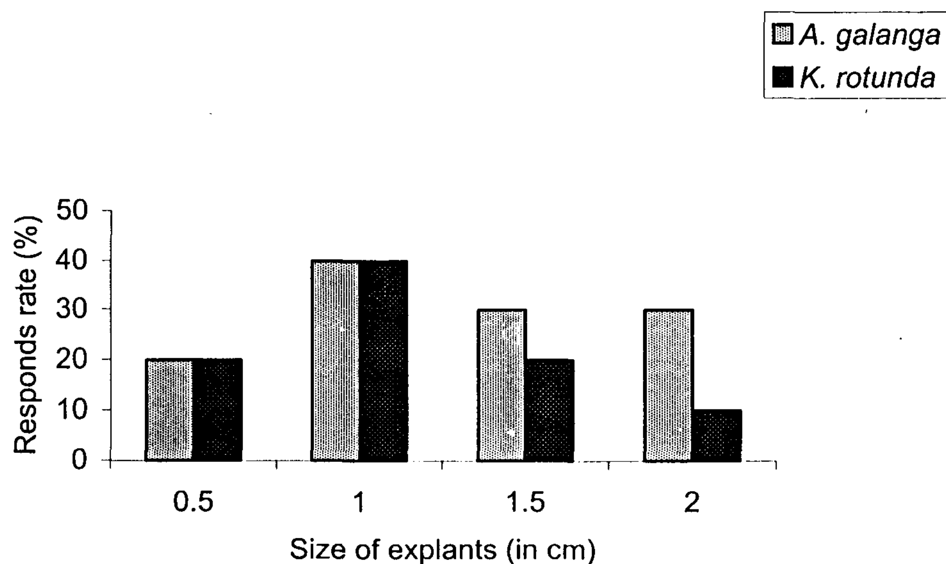

Figure 2. Response percentage of the rhizome discs on standard MS medium, after 38 days in *A. galanga* and 35 days in *K. rotunda*, when explants of different sizes used.

IV.1.2. *Age of the explants:*

The explants were collected from rhizomes of varying age (2 months to 5 months) and of the various aged specimens tried (Fig: 3), explants collected from 2 months old rhizomes were more responsive in the case of *A. galanga,* while in *K. rotunda;* the explants collected from 3 months old rhizomes were more suitable for culture studies.

IV.2. *Sterilisation:*

For surface sterilisation a multi-step approach had been employed, so as to standardise most suitable sterilants and their optimum concentrations, the explants of both plants were subjected to an initial treatment with the detergents, followed by mercuric chloride and ethanol. Inborn contaminants were removed by antibiotics

treatment. As mentioned in the beginning of this chapter initial studies were conducted using an arbitrary concentration of extran and

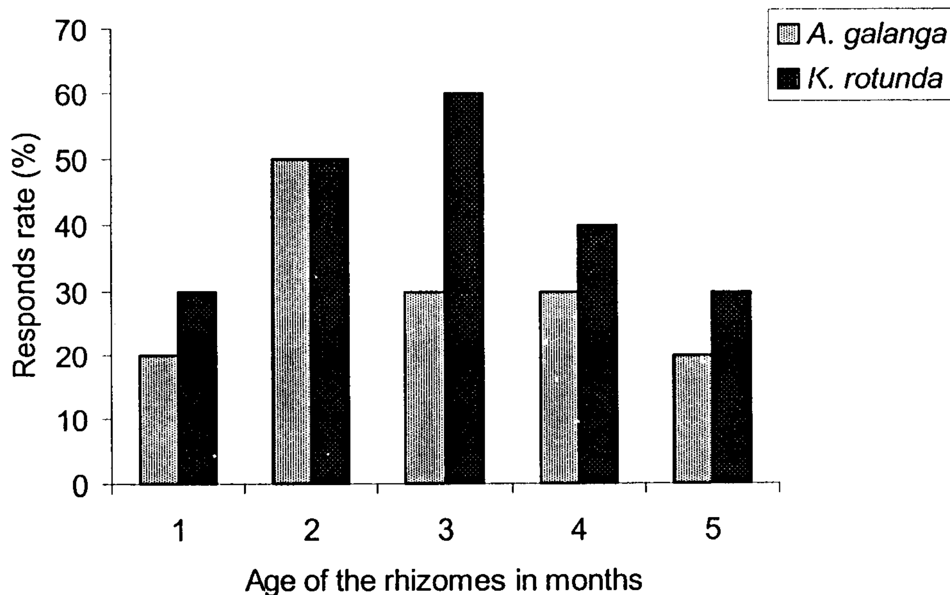

Figure 3. Survival rate of the different months old rhizome bud explants of *A. galanga* and *K. rotunda*.

$HgCl_2$. The individual effects of various detergents and sterilants were experimented in detail.

IV.2.1. *Use of Detergents:*

Use of detergents was a crucial factor in determining survival rate of the culture. In the present study the detergents like teepol, extran and soap water at different concentrations (2-7%) were used. Of them, extran 5% (30 min) was more satisfactory than the other two detergents used (Tab: 4).

IV.2.2. *Use of Mercuric Chloride:*

To remove the fungal contaminants, mercuric chloride solution with specific percentage was found to be critical. Of the various percentage of solution used, treatment with 0.1% $HgCl_2$ solution for 10 minutes produced satisfactory results in *A. galanga,* while 0.15% for 15 minutes was necessary for *K. rotunda.* At these optimum concentrations,the survival percentage of the explants was 40 and 50 in *A. galanga* and *K. rotunda,* respectively (Fig: 4).

Table 4. The effect of different detergents on explants of *A. galanga* and *K. rotunda* after a treatment of 30 min.

Explant	Detergents (%)											
	Teepol				Extran				Soap water			
	1	3	5	7	1	3	5	7	1	3	5	7
A. galanga	0	0	10	10	0	10	20	20	0	0	10	10
K. rotunda	0	0	0	10	0	10	20	20	0	10	10	10

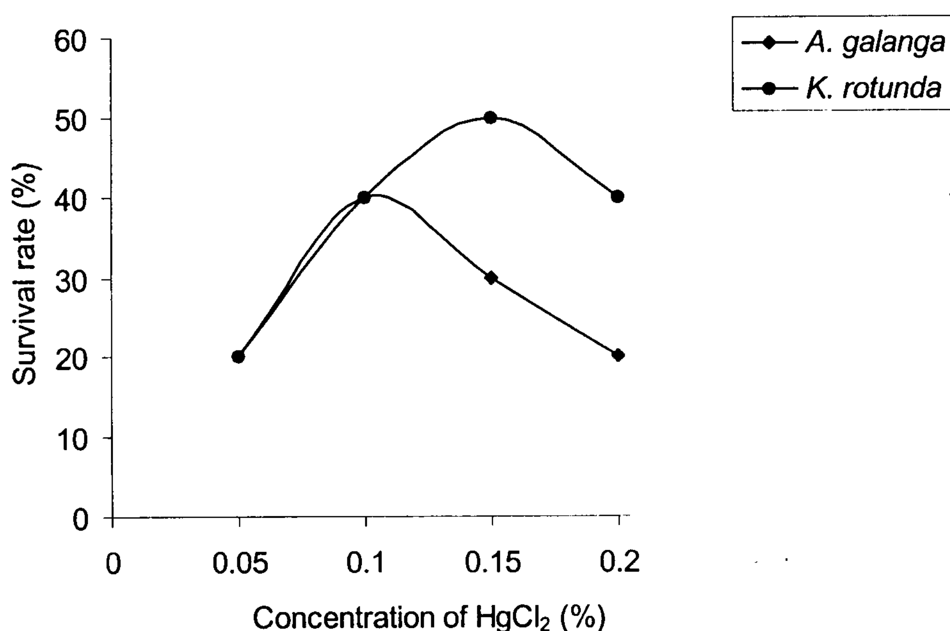

Figure 4. Survival rate of the 5% extran pre-treated explants of *A. galanga* and *K. rotunda*, when various concentrations of $HgCl_2$ used.

IV.2.3. *Ethanol Treatment:*

Final rinsing of the $HgCl_2$ treated explants with 70% alcohol was found as effective to reduce the contamination to maximum. In this experiment, it was noticed that the explant exposure time was also very critical to the survival of the culture. In *A. galanga,* the optimum exposure time was 15 seconds and in *K. rotunda,* it was about 20 seconds (Fig: 5).

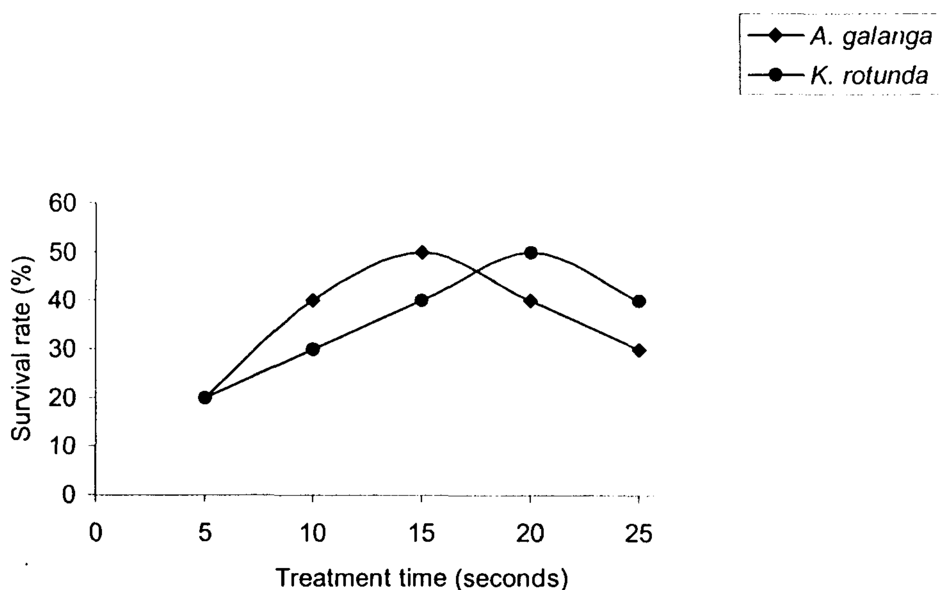

Figure 5. Response percentage of *A. galanga* and *K. rotunda*, when $HgCl_2$ pre-treated explants, further treated with 70% ethanol in different time intervals.

IV.2.4. *Antibiotics Treatment:*

Foregoing culture trials showed that even effective surface sterilisation could not eradicate the bacterial contaminants. Hence, to

remove them, different antibiotics were incorporated in to the culture medium, only when the fresh explants were used for experiments. Addition of antibiotics like ampicillin or erythromycin (25-100 mg/l) was showed better results. Of this, 50 mg/l erythromycin was most effective for *A. galanga* and 75 mg/l was effective for *K. rotunda* (Fig: 6). The best survived ones were subjected to further studies.

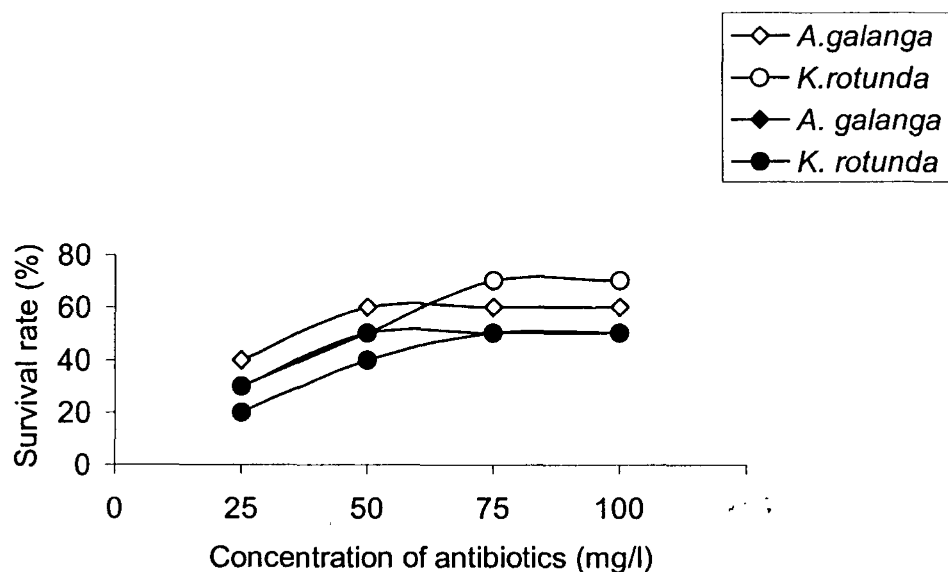

Figure 6. Survival rate of the explants of *A. galanga* and *K. rotunda*, when different concentrations of erythromycin (hollow symbols) and ampicillin (solid symbols) were added respectively, in to the medium.

IV.2.5. *Combined Effect of Detergents and Sterilants:*

The combined effect of various detergents and sterilants were shown in Fig:7 From the figure, it was understood that the multi-step approach for the sterilisation was successful for obtaining maximum

survival of *A. galanga* and *K. rotunda* rhizome explants. The survival percentage was 60 and 70 in *A. galanga* and *K. rotunda,* respectively (Fig: 7).

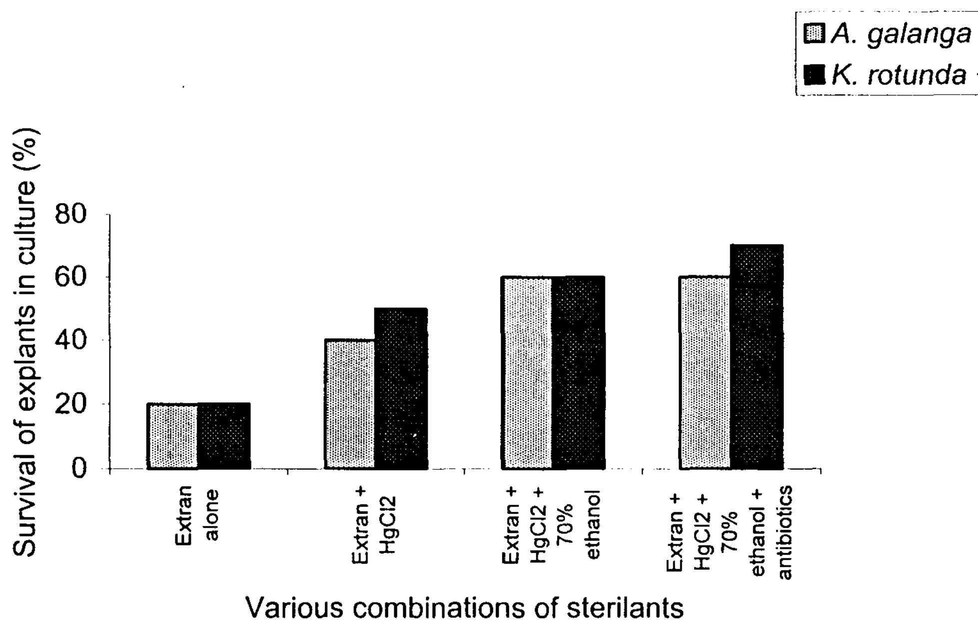

Figure 7. Compilation of the best results obtained after independent studies using different combinations of sterilants.

IV.3. *Effect of Hormone-free Basal Medium:*

The mineral salt composition of the basal medium significantly affected the regeneration capacity of the explants. To select the best medium, the explants (rhizome sprouts) were cultured on different nutrient media like B5, MS, SH and WH. The establishment rate was high on MS medium and the response of the explant was 50 and 60 for *A. galanga* and *K. rotunda,* respectively (Fig: 8). By using full strength MS medium, the explants showed response in 30-35 days, while the response in all other media was slow and took 40-45 days for the

initiation of proliferation. Because of quick response in MS medium, that was used for further studies (Fig: 8 & Plate 2 A, B).

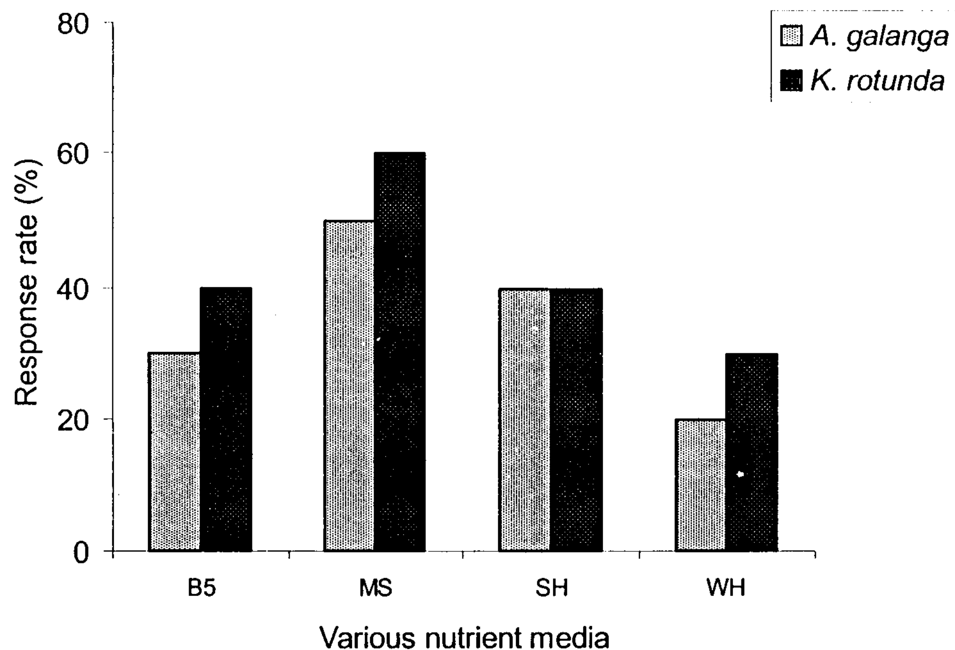

Figure 8. Growth response percentage of fully sterillized rhizome buds on various basal media, without hormone, after 60 days of inoculation.

IV.4. *Direct Multiple Shoot Regeneration:*

The explants (sprout buds), were used to induce multiple shoots from *A. galanga* and *K. rotunda*. Hormone-free medium could not show improved effect on direct shoot induction from rhizome buds. To rectify this barrier, different growth regulators were tested in the medium. Cytokinins in the form of BAP, KN and CW were added individually or in combinations within them or with auxins - like IAA, NAA and IBA in different concentrations in the medium.

IV.4.1. *Effect of Different Cytokinins:*

Various cytokinins like BAP, KN and CW were used in various

combinations to test their potentiality to induce direct multiple shoots. Of various concentrations of BAP used (0.50 mg/l to 3.0 mg/l) for shoot induction, a lower concentration of BAP (1.0 mg/l) was effective for *A. galanga*, while in *K. rotunda*, the effective optimum concentration was 2.0 mg/l (Plate 3A,B & Tabs: 5, 6). The frequency of response was 60% in both, and the explants responded by three weeks of inoculation. An increase in the concentration of BAP, from the optimum level, adversely affected shoot multiplication (Tabs: 5, 6).

When KN (0.50 to 3.0 mg/l) was substituted for BAP with MS medium, efficiency of shoot induction was comparatively low (Tab: 5). As shown in Plate 3 C and D, in *A. galanga*, 50% of the explants produced an average of 3.0 shoots/culture at 1.0 mg/l KN (Tab: 5 & Plate 3 C). Vitality of the sprouts was not so good as that obtained in the presence of BAP (Plate 3 A, B). Experiment with *K. rotunda* also proved the inefficiency of KN for multiple shoot induction, and 2.0 mg/l KN could induce 3-4 shoots/explant after 25-30 days of inoculation (Tab: 6 & Plate 3 D).

Potentiality of the MS medium, supplemented with CW, for inducing shoots in both *A. galanga* and *K. rotunda* was also tried. Upon visual rating, it was found that CW had no promotive effect on multiple shoot induction over BA or KN (Fig: 9). Most of the trials showed same results in terms of the number of shoots, as with KN. In fact, the medium with CW showed a maximum of 40% shoot induction frequency and was 10% less against KN.

Table 5. Effect of basal MS medium containing different cytokinins on direct shoot multiplication in *A. galanga*

Medium	Cytokinin mg/l	Average No. of Shoots per culture	±SD	Average length of Shoots in cm	±SD	% of response
MS	-	1.6	0.51	3.9	0.39	50
	BAP					
MS	0.25	1.6	0.51	3.9	0.84	50
MS	0.5	2.4	0.66	7.8	0.25	50
MS	**1**	**3.5**	**0.35**	**10.3**	**1.78**	**60**
MS	3	1.6	0.51	6.1	0.90	50
MS	3	1.4	0.51	5.1	1.02	40
	KN					
MS	0.25	1.4	0.51	3.6	0.39	50
MS	0.5	1.8	0.42	6.5	0.47	50
MS	1	3.0	0.63	8.5	0.88	50
MS	2	2.4	1.43	3.0	0.69	40
MS	3	1.4	0.51	3.4	1.02	40
	BAP + KN					
MS	0.25+0.25	1.2	0.42	3.4	0.39	30
MS	.50+.50	1.5	0.84	5.7	0.54	40
MS	1.00+1.00	1.0	0.21	3.8	0.54	20

Data represents an average of 10 treatments
Growth period 60 days
Basal medium +3 percent Sucrose +0.8 percent Agar
SD = standard deviation.

Table 6. Effect of MS media, BAP and KN on direct multiple shoot induction in *K.rotunda*

Medium	Cytokinin mg/l	Average No. of shoots per Culture	± SD	Average No. of roots per culture	± SD	%of response
MS	-	1.4	0.51	1.4	0.52	60
	BAP					
MS	0.5	1.6	0.51	2.2	0.79	50
MS	1	2.4	0.51	2.8	0.79	50
MS	1.5	3.4	0.99	3.8	0.42	60
MS	**2**	**4.0**	**0.97**	**3.6**	**0.52**	**60**
MS	2.5	2.4	0.51	3.4	0.84	50
MS	3	2.2	0.42	2.4	1.27	40
MS	KN					
MS	0.5	1.4	0.51	1.4	0.52	40
MS	1	1.8	0.42	2.8	0.79	50
MS	1.5	3.0	0.51	1.4	0.52	50
MS	2	3.5	0.21	1.8	0.42	50
MS	3	2.2	0.42	1.4	0.52	40
	BAP+KN					
MS	0.50+0.50	2.4	0.51	2.6	1.23	30
MS	1.00+1.00	3.0	0.66	2.5	0.87	60
MS	1.50+1.50	2.8	0.78	2.0	1.97	20

SD standard deviation
Data represents mean value of 10 treatments
Medium supplemented with 3% sucrose + 0.8% Agar

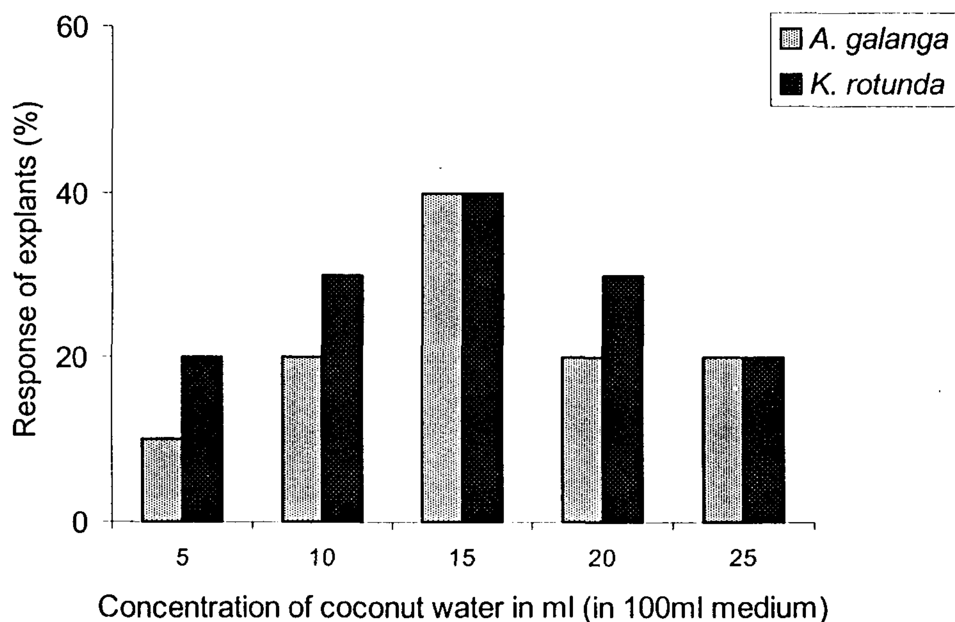

Figure 9. Response percentage of the explants of *A. galanga* and *K. rotunda*, on MS medium containing CW at various concenterations.

IV.4.2. *Synergistic Effect of BAP + KN:*

The synergistic effect of BAP + KN was also tested to assess its potentiality in multiple shoot induction. It was found that coupling of BAP + KN had adversely affected *A. galanga* and produced lesser number of shoots (1-2), with very low response percentage (40%) (Fig: 10). However, in *K. rotunda,* the synergistic effect of BAP + KN produced a promotive effect, and was in par with the results obtained with BAP alone. Of the various combinations tried, 1.0 mg/l BAP + 1.0 mg/l KN produced an average number of 3.0 shoots/explant and the percentage of response was 60 (Fig: 10 & Tabs: 5, 6).

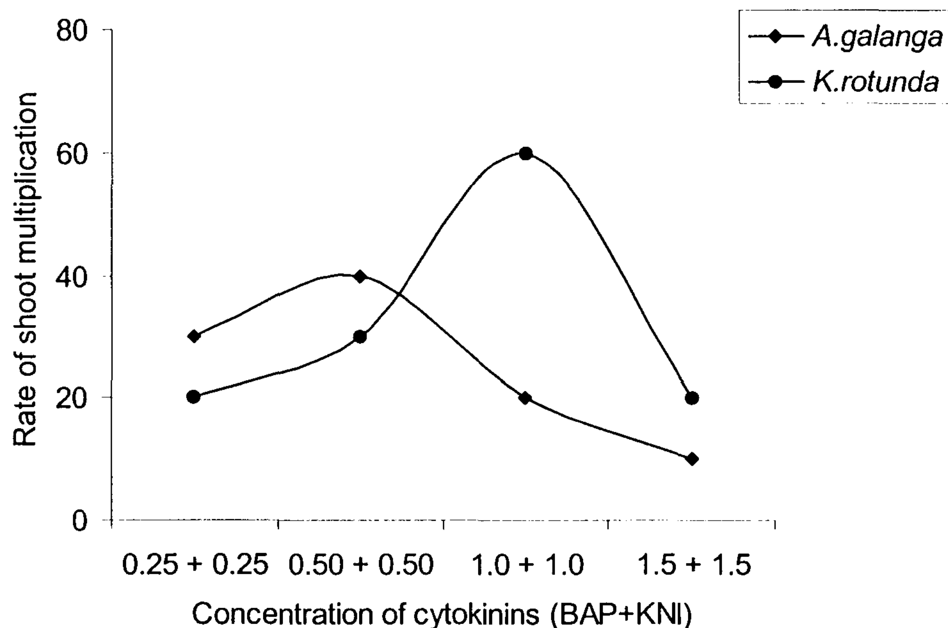

Figure 10. Responts of *A. galanga* and *K. rotunda*, rhizom buds on various concentrations of BAP+KN.

From the above study it was proved that individual application of BAP (1.0 mg/l or 2.0 mg/l) in both *A. galanga* and *K. rotunda* was most effective than KN or CW or BAP + KN.

IV.4.3 *Interaction of Cytokinins/Auxins:*

To enhance the rate of shoot multiplication, MS medium was supplemented with different concentrations and combinations of cytokinins (BAP or KN) along with auxins (IAA, NAA or IBA). Upon visual rating, it was noticed that high cytokinin/low auxin ratio enhanced the rate of shoot multiplication.

IAA was found to be the most effective auxin in combination with BAP (MS + BAP + IAA) for the induction of direct multiple shoots (Tabs: 7, 8 & Plate 4). Of the different combinations tried,

Table 7. Effect of different auxins and cytokinis on direct multiple shoot Induction from sprout bud of *A. galanga* on MS basal medium

Growth hormone Mg/l		% of response	Average No. of shoots per culture	±SD	Average Length of shoots in cm	±SD
Auxin	Cytokinin					
IAA	BAP					
0.1	0.5	50	2.6	0.52	6.9	0.52
0.25	0.75	70	4.8	0.79	12.2	0.72
0.5	**1**	**80**	**6.9**	**0.79**	**13.4**	**0.97**
0.75	1.5	60	4.6	0.84	10.6	0.62
1	2	50	3.4	0.52	10.0	0.47
1	3	40	2.4	0.52	8.4	0.39
IBA	BAP					
0.25	0.5	50	3.2	0.42	9.2	0.54
0.50	1	60	4.0	0.84	11.0	0.58
0.75	2	40	3.6	0.52	8.6	0.62
1	3	40	3.2	0.42	7.5	0.47
NAA	BAP					
0.25	0.5	50	2.8	0.79	6.7	0.72
0.5	1	60	3.4	0.67	7.7	0.63
0.75	2	50	3.2	0.79	8.2	0.42
1	3	40	2.8	1.23	5.2	0.26

Contd ...

IAA	KN					
0.25	0.5	50	1.6	0.52	7.2	0.79
0.5	1	60	2.6	0.52	9.4	0.40
0.75	2	40	2	0.67	8.4	0.66
1	3	30	1.4	0.52	7.6	0.62
IBA	KN					
0.25	0.5	40	1.8	0.79	4	0.67
0.5	1	50	2.6	0.52	5.2	0.42
0.75	2	50	2	0.67	5.4	0.52
1	3	30	1.4	0.52	3.8	0.42
NAA	KN					
0.25	0.5	40	1.4	0.52	4.6	0.40
0.5	1	50	2.4	0.52	4.4	0.78
0.75	2	40	1.8	0.79	4.6	0.84
1	3	40	1.8	0.79	4.8	0.79

Data represents an average of 10 replicates.
Growth period 60 days
MS Basal medium + 3% sucrose + 0.8% Agar.
SD. standard deviation

Table 8. Effect of different auxins and cytokinins on direct multiple shoot and root induction from sprout buds of *K.rotunda* on MS basal medium

Auxin mg/l	Cytokin mg/l	% of response	Average No. of shoots per culture	±SD	Average No. of roots per culture	±SD	Average length of Shoots per culture	±SD
IAA	BAP							
0.10	0.50	50	3.4	0.52	6.2	0.79	10.0	0.88
0.25	1.00	60	5.2	0.79	10.4	1.08	13.0	0.94
0.50	**2.00**	**90**	**8.0**	**0.92**	**16.8**	**1.79**	**14.0**	**1.23**
1.00	3.00	70	4.5	0.52	9.2	0.79	10.4	0.90
IBA	BAP							
0.10	0.50	50	3	0.94	3.2	0.79	6.6	0.62
0.25	1.00	60	2.6	0.52	3.0	0.67	6.2	0.42
0.50	2.00	70	2.7	0.59	3.0	0.72	6.0	0.33
1.00	3.00	40	1.6	0.52	2.2	0.42	6.4	0.39
NAA	BAP							
0.10	0.50	40	1.4	0.52	4.6	0.52	4.6	0.39
0.75	1.00	50	2.0	0.47	5.0	0.67	5.8	0.26
0.50	2.00	60	2.9	0.72	4.8	0.88	6.0	0.58
1.00	3.00	50	1.4	0.52	7.6	0.42	4.0	0.42
IAA	KN							
0.10	0.50	30	1.6	0.82	1.4	0.52	7.2	0.86
0.25	1.00	50	1.6	0.52	1.6	0.52	4.6	0.40
0.50	2.00	50	2.0	0.68	2.8	0.79	6.5	0.62
1.00	3.00	40	1.4	0.92	2.2	0.79	5.8	0.72

contd....

IBA	KN							
0.10	0.50	30	1.2	0.42	1.6	0.92	6.4	0.62
0.25	1.00	50	2	0.67	2.4	0.52	6.6	0.78
0.50	2.00	40	3	0.67	2.8	0.79	6.0	0.58
1.00	3.00	30	1.4	0.52	1.4	0.82	8.4	0.72
NAA	KN							
0.10	0.50	30	1.4	0.52	2.2	0.79	5.8	0.26
0.25	1.00	40	2.0	0.67	3.0	0.67	6.0	0.86
0.50	2.00	50	3.0	0.95	4.6	0.52	6.2	0.77
1.00	3.00	40	1.2	0.42	3.2	0.42	4.0	0.86

Data represents an average of 10 replications
MS supplemented with 3% sucrose + 0.8% Agar
Growth period 60 days
SD: standard deviation

1.0 mg/l BAP + 0.5 mg/l IAA induced an average number of 6.9 shoots in *A. galanga* (Plate 4 A,B & Tab: 7). In *K. rotunda,* 2.0 mg/l BAP + 0.5 mg/l IAA was emerged as optimum concentration for best results. At this concentration, explant produced an average of 8.0 shoots/culture (Plate 4 C & Tab: 8). Moreover, *in vitro* shoots of *K. rotunda* produced roots in the shoot inducing medium (Plate 4 C).

Other formulae tried were: MS + BAP + IBA., MS + BAP + NAA., MS + KN + IAA and MS + KN + IBA/NAA (Tabs: 7,8). The results obtained in most of the experiments were not so promising when compared to the effects of MS + 1.0 mg/l BAP + 0.5 mg/l IAA for *A. galanga,* and MS + 2.0 mg/l BAP + 0.5 mg/l IAA for *K.rotunda* (Tabs: 7,8). However, MS + 1.0 mg/l KN + 0.5 mg/l IAA in *A. galanga* (Plate 4 D), and MS + 2.0 mg/l KN + 0.5 mg/l IAA in *K. rotunda* (Plate 4 E) could produce promising results.

Nevertheless, of the two cytokinins and three auxins tested in combinations, it was found that BAP along with IAA was effective than others (Tabs: 7,8 & Plate 4 A-C). This combination was used in further cultures of *A. galanga* and *K. rotunda* for the direct multiple shoot induction.

IV. 4.4 *Sub-culture:*

Small clumps or single shoot were excised from the multiple shoots developed in MS+ BAP + IAA combination and sub-cultured on to the medium with the same hormonal combinations (Tabs: 7, 8). Shoot development during sub-culture was same as that of the primary culture (Tabs: 7, 8 & Plate 4 B, C) and an interval of 20 days

was found effective for consecutive sub-cultures.

IV. 4.5 In vitro *Rooting*: *In vitro* obtained shoots of *A. galanga* produced roots in root inducing medium, containing IBA or NAA (1.0 - 3.0 mg/l) in 15 days incubation (Tab: 9 & Plate 5 A). Combined effect of IBA/NAA did not show any significant results (Tab: 9).

Since *K. rotunda* shoots produced roots in the shooting medium, no further treatment was required for root induction (Tab: 8 & Plate 4 C,E). However, those shoots in *K. rotunda,* which did not produced roots were further studied, using rooting hormone.

Half strength MS medium having 2.0 mg/l NAA was more suitable than IBA (Tab: 10 & Plate 5 B), with the development of about 10-12 roots and the frequency of response was 80%. The same concentration of IBA produced only 6 roots per plant (50% decrease). When the concentration of NAA was increased upto 2.5 mg/l, slight callusing was also found, associated with rooting (Tab: 10).

In both the materials, 40 days old shoots were used for root induction studies. When MS medium was supplemented with 1.0 mg/l NAA or IBA, both *A. galanga* and *K. rotunda* did not produce roots. However, over 50% of the shoots of *A. galanga,* in the presence of auxins at 1.5 mg/l induced roots in 2 weeks (Tab: 9). When auxin level was increased to 2.5 mg/l as optimum treatment, 80% of the shoots produced an average of 10.8 roots (Tab: 9 & Plate 5 A). In the presence of higher concentration of auxin (NAA above 2.5mg/l), callusing was also noticed (Tab: 9). Of the two auxins tested, NAA was more effective than IBA (Tabs: 9,10 & Plate 5 A,B). When *in vitro*

Table 9. Effect of IBA and NAA on *in vitro* rooting of cultured shoots of *A.galanga*

Medium	Auxin	Nature of response	Average No. of roots	$\pm$ SD	% of response
MS	IBA				
MS	1	-	-	-	-
MS	1.5	R	2.8	0.78	50
MS	2	R	4.8	0.42	60
MS	2.5	R	3	0.66	70
MS	3	R,C	1.5	0.56	60
	NAA				
MS	1	-	-	-	-
MS	1.5	R	3.6	0.51	50
MS	2	R	8.4	0.51	50
MS	**2.5**	**R**	**10.8**	**0.78**	**80**
MS	3	R,C	5.8	0.42	50
MS	4	RC	1.5	0.68	30
	IBA+NAA				
MS	0.5+0.5	-	-	-	-
MS	1.00+1.00	R	6.0	0.66	50
MS	1.25+1.25	R	2.8	0.78	50
MS	1.50+1.50	R, C	5.6	0.42	40
MS	-	R	1.5	0.56	30

Data represent an average of 10 treatments
R = Roots; C = Callus; - = No response; SD = Standard Deviation.
Growth period 20 days.
MS Basal medium + 3% sucrose + 0.8% Agar.

Table 10. Effect of IBA and NAA on root induction from *in vitro* raised shoots of *K.rotunda*

Medium	Auxin	% of response	Average No. of shoots per culture	± SD	Nature of response
½MS	IBA				
½MS	1.00	-	-	-	-
½MS	1.50	60	4.4	0.51	R
½MS	2.00	80	6.0	0.66	R
½MS	2.50	70	5.2	0.42	R
½MS	3.00	50	4.4	0.51	R
	NAA				
½MS	1.00	-	-	-	-
½MS	1.50	70	7.4	0.51	R
½MS	**2.00**	**80**	**11.2**	**0.78**	**R**
½MS	2.50	60	8.6	0.51	R, C
½MS	3.00	50	7.2	0.42	R, C

R -roots C -Callus formation, SD -Standard deviation
Data represents an average of 10 treatments
Growth period 30 days - No response
½ MS Basal medium + 3% sucrose + 0.8% Agar

raised shoots of *A. galanga* (without roots) excised and transferred to MS medium without hormone, produced only one or two roots even after 60 days of culture (Plate 5 C).

IV. 4. 6 *Deflasking and Hardening:*

Well developed plantlets (60 days old) with leaves and roots transferred to soil using standard techniques described in the materials and methods (Plate 6 A-D). Survival rate of the hardened *in vitro* raised plantlets in the field was improved using pre-treatment of the plantlets by dipping in the fungicide, Bavistin 0.2% for 2-3 min (Tab:11). It was noted that a minimum of 20 days were needed for 70% survival of *A. galanga,* and 15 days enough for the 80% survival of *K. rotunda.*

Table 11. Establishment percentage of *in vitro* raised shoots of *A. galanga* and *K. rotunda* after hardening

Plant	No. of shoots hardened	Survived	Infected	Days used for hardening	%
A. galanga	10	2	7	5	20
	10	4	6	10	40
	10	6	4	15	60
	10	**7**	**3**	**20**	**70**
	20	14	6	25	70
K. rotunda	10	3	7	5	30
	10	5	5	10	50
	10	**8**	**2**	**15**	**80**
	20	16	4	20	80

IV.4.7 *Effect of Different Nutrient Media:*

Considering the efficiency of different hormonal concentrations on MS medium, the same hormonal combinations were tested in other media described elsewhere (Figs: 11,12). Explants of (1.0 cm) *A. galanga* and *K.rotunda* were inoculated on to these media for studying their influence on shoot bud induction and proliferation. It was observed that the morphogenic responses were varied in different nutrient media. In comparison, SH, B5 and WH media showed decreasing performance over MS medium (Figs: 11,12). This piece of work also proves that MS medium is superior to other media used in the present study.

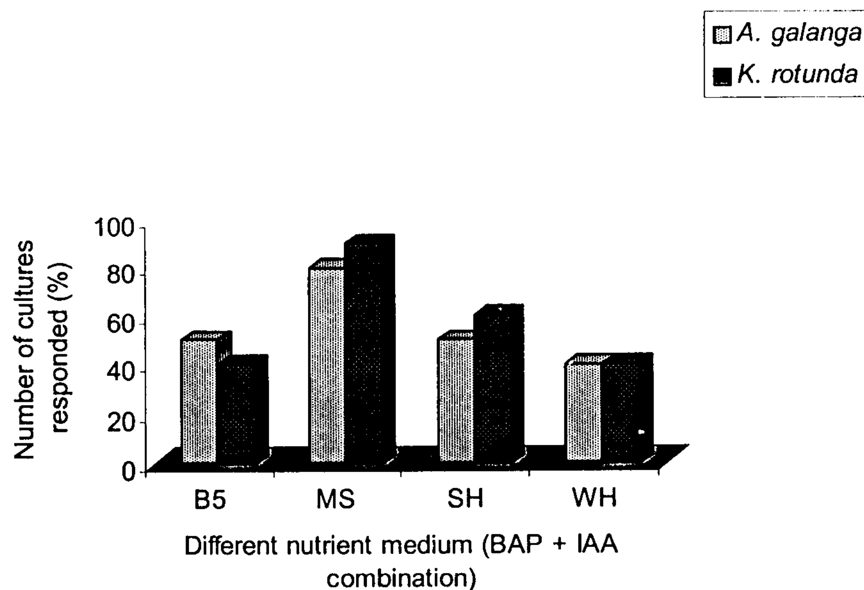

Figure 11. Percentage of shoot induction response of the explants of of *A. galanga* and *K. rotunda*, on different media, when 1.0 mg/l BAP + 0.5mg/l IAA and 2.0mg/l BAP + 0.5mg/l IAA added respectively.

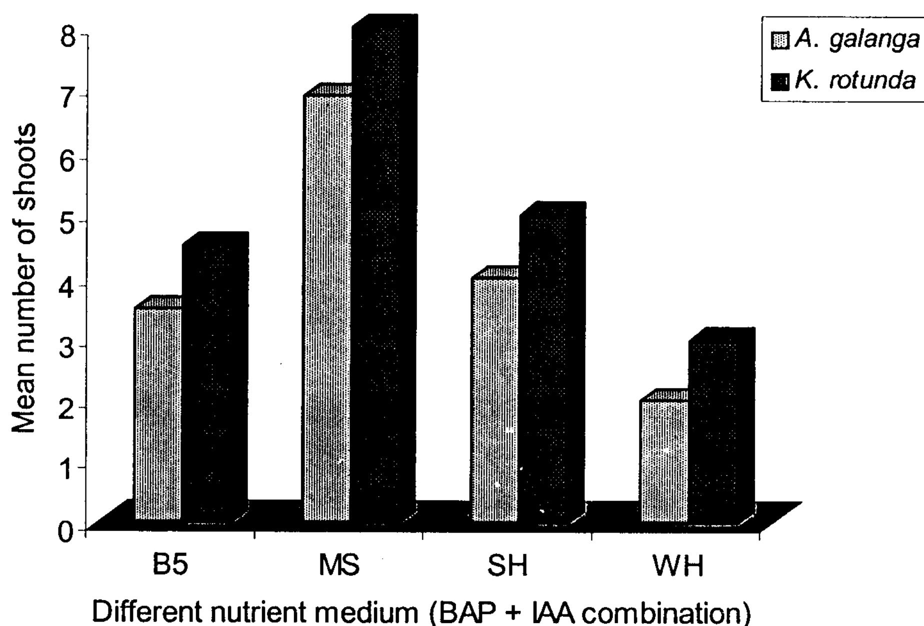

Figure 12. Effect of various nutrient media for the induction of multiple shoots in the presnce of 1.0 mg/l BAP + 0.5mg/l IAA in *A. galanga* and 2.0mg/l BAP + 0.5mg/l IAA in *K. rotunda*.

IV.4.8. *Effect of Sucrose Concentration:*

To study the effect of sucrose on the *in vitro* multiplication of shoots from explants (sprout buds) of *A. galanga* and *K. rotunda,* different levels of sucrose (1-5%) were incorporated on to the basal MS medium. Only sucrose concentration of the medium was altered, keeping all other components and hormonal concentrations (standardised) constant. Shoot bud proliferation was noticed and the results were presented graphically (Figs: 13,14). Optimum result was obtained when MS medium was fortified with 3% sucrose and the percentage of response were 80 and 90 in *A. galanga* and *K. rotunda,* respectively (Plate 4 A, C). An average of 6.9 and 8.0 shoots, respectively were produced. When, in both materials, sucrose concentration was reduced to 2%, a decrease in the number of shoots (4.5 and 5, respectively) and frequency (60%) was observed. When the

sucrose level increased upto 4%, the number of shoots produced were more or less the same as obtained with 2% sucrose level, and the percentage of response was further decreased to 50% in *A. galanga* and increased to 70% in *K. rotunda* (Fig: 13). However, further increase (above 4%) or decrease (below 2%) of sucrose concentration again resulted drastic variations in the number of shoots and response percentage (Fig: 13,14).

Upon 5% level of sucrose, the *in vitro* raised shoots showed stunted growth and less shoot elongation (Plate 7 A, B). In them, the number of shoots per explant was 2 or 3 and percentage of response was only 40%. At 1% sucrose level, the *in vitro* raised plants developed narrow shoots and leaves (Plate 7 C, D & Figs: 13,14) with 40% response in both specimens.

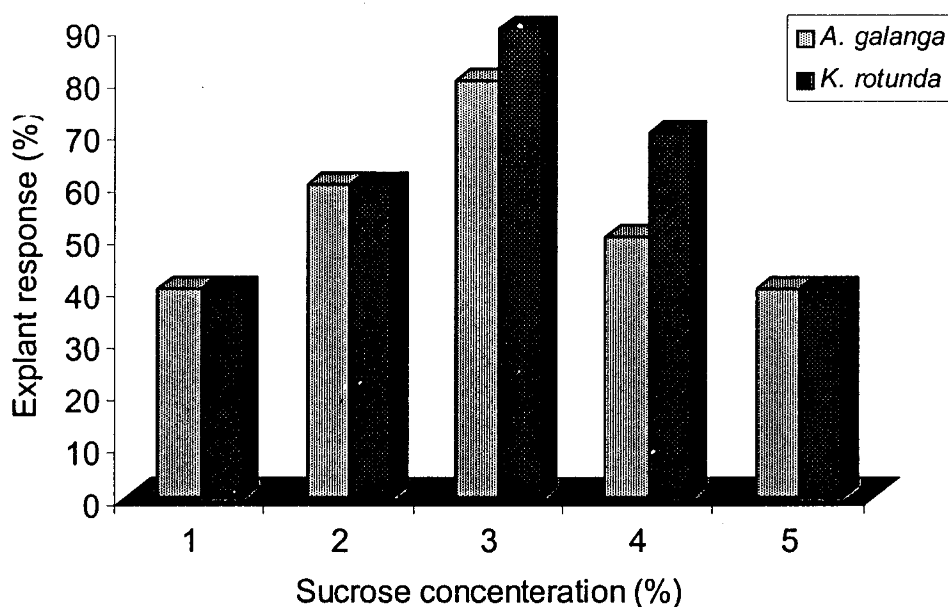

Figure 13. Percentage of response of *A. galanga* and *K. rotunda* on MS hormone medium, containing different sucrose concenterations.

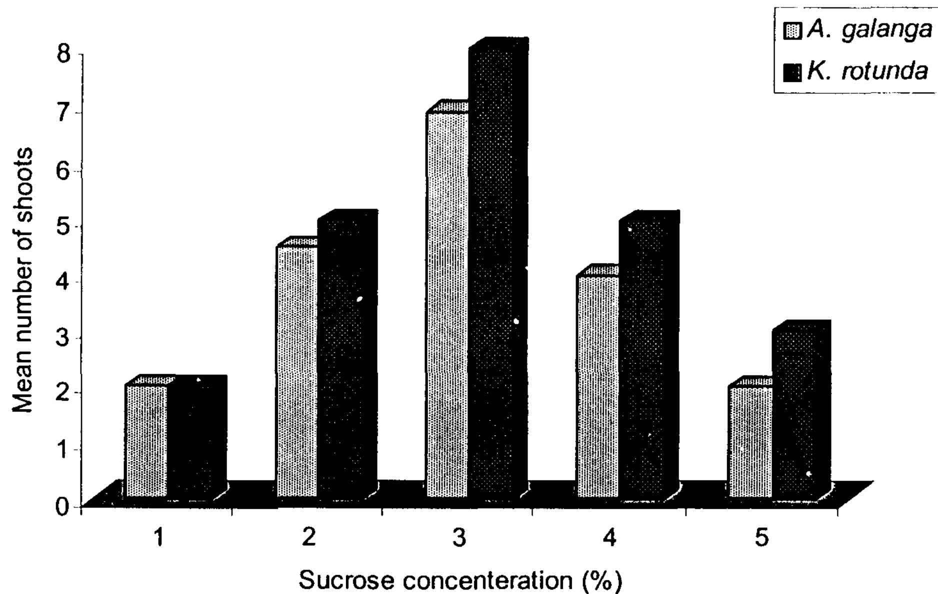

Figure 14. Effect of different concentrations of sucrose on multiple shoot induction of *A. galanga* and *K. rotunda*, under different sucrose concenterations on MS-hormone medium.

IV.5. *Indirect Shoot Multiplication:*

Through callus, multiple shoot induction from the rhizome discs of both *A. galanga* and *K.rotunda* was the main focus of this study. For the induction of callus and subsequent indirect shoot multiplication, rhizome discs (1.0 cm^3) of *A. galanga* and *K. rotunda* were inoculated on MS medium supplemented with different auxins (NAA or 2,4-D) or auxins in combinations with cytokinins (BAP or KN). The efficiency of these hormones was tested individually or in combinations (Tabs: 12,13). The suitable combinations were selected for further experiment.

Table 12. Effect of auxins and cytokinins for the induction of callus from rhizome disc of *A. galanga* on MS basal medium

Auxin mg/l	Cytokinin mg/l	% of response	Amount of callus formation	Days taken to initiate callus formation
2,4-D				
1.00	--	--	--	
2.00	00	40	++	25-30 days
2.50	00	50	+++	25-30 days
3.00	00	40	+	25-30 days
4.00	00	--	--	
NAA				
1.0		--	--	
2.0	0.0	60	R	15-20 days
2.5	0.0	70	R	15-20 days
3.0	0.0	50	++	30-35 days
4.0	0.0	--	--	--
2,4-D	BAP			
1.0	0.10	--	--	--
1.5	0.25	50	++	10-15 days
2.0	**0.50**	**70**	**++++**	**Embryogenic callus**
2.5	0.75	60	+++	10-15 days
3.0	1.00	40	+	10-75 days
NAA	BAP			
1.0	0.10	--	--	--
2.0	0.25	70	R	15.20 days
3.0	0.50	60	++ R	30-35 days
4.0	1.00	--	++	
NAA	KIN			--
1.0	0.10	--	--	--
2.0	0.25	50	R	15-20 days
3.0	**0.50**	**60**	**+++**	**25-30 days**
4.0	1.00	50	+	Organogenetic

Basal medium MS + 3.0% sucrose + 0.8% Agar
Data taken after 60 days incubation
_ No callusing. R - roots
+ very slight callus formation
++ low callus formation
+++ moderate callus formation
++++ intense callus formation

Table. 13 Effect of 2,4-D, NAA and BAP on callus induction from rhizome discs of *K. rotunda* on MS medium

Auxin mg/l	Cytokinin mg/l	Amount of callus	% of response	Nature of response	Days taken to induce
2,4-D					
1.00	00	--	--	--	--
2.00	00	+	50	Non embryogenic callus	25 -30 day
2.50	00	+++	60	Non embryogenic callus	25-30 days
3.00	00	+	40	Non embryogenic callus	25-30 days
4.00	00	--	--	--	--
NAA					
1.00	00	--	--	--	--
2.00	00	--	50	R	15-20 days
2.50	00	++	60	R, C	30-35 days
3.00	00	+	40	R,C	30-35 days
4.00	00	--	--	--	--
2,4-D	BAP				
1.00	0.50	--	--	--	--
2.00	0.50	++	60	Embryogenic callus	15 - 20 days
2.50	**0.50**	**++++**	**80**	**Embryogenic callus**	**10-15 days**
3.00	0.50	+++	70	Embryogenic callus	10-15 days
NAA	BAP	--	--	--	--
1.00	0.50				
2.00	0.50		50	R	15- 20 days
2.50	0.50	+	40	R, C	15-20 days
3.00	0.50	+	40	R,C	15-20 days

C - Callus, R -roots
_ No callusing
+ Very slight callus formation
++++ intense callus formation

Data taken after 60 days
++ Low callus formation
+++ moderate callus formation
MS + 3% sucrose + 0.8% Agar.

IV.5.1. *Effect of Auxin alone:*

IV. 5. 1.1 **MS + NAA:** The potentialities of both the explants (sprout discs) for callus induction were tested by inoculating them on MS medium, fortified with different concentrations of NAA (1-4 mg/l). At lower concentrations, NAA was found to be ineffective for callus induction, but instead of callusing roots were produced from the basal region of the explants (Tabs: 12,13). At higher concentrations (above 2.5 mg/l), NAA induced very little callus, and the callus was resulted only after 30 days of incubation. Texture of the callus was spongy and friable, which on prolonged culture, changed to dark brown in colour and died subsequently (Plate 8 A,B).

IV. 5. 1.2. **MS + 2,4-D:** For the induction of callus, explants inoculated on MS medium containing various levels of 2,4-D (1-4 mg/l). It was found that 2-3 mg/l produced a moderate amount of callus in both the plants from the cut ends of the explant in 25-30 days. In fact, no sufficient amount of callus formation was noticed in any concentration of the hormone tested (Tabs: 12,13). The callus obtained was water-soaked and sticky with very poor chlorophyll, which on further sub-culture became dead (Plate 8 C,D). In *K. rotunda,* hairy roots were developed, and perished subsequently along with callus (Plate 8 D).

IV.5.2. *Interaction of Auxins and Cytokinins:*

Callusing was not observed in any case of cytokinin treatment under direct shoot multiplication, hence its independent effect on callusing was not tested in these piece of study. The MS basal medium supplemented with high auxin and low cytokinin resulted callus

induction in both the explants (Plate 9 A,B & Tabs: 12,13). The combined effect of auxin and cytokinin triggered the callus formation and rapid proliferation of callus was noticed within 10-15 days of incubation(Tabs: 12, 13). The calli were much vital, and sub-cultured for further studies.

IV 5.2.1: MS + 2,4-D + BAP: Of the various combinations of 2,4-D along with BAP tested, intense callusing was noticed in *A. galanga,* when MS medium supplemented with 2.0 mg/l 2,4-D + 0.5 mg/l BAP. In contrast, maximum result was obtained in *K. rotunda,* when 2.5 mg/l 2,4-D + 0.5 mg/l BAP used (Tabs: 12, 13). In both plants, the callus produced were in the form of compact nodules with large cells and the colour was cream-white and glossy (Plate 9 A,B). The callus produced were either embryogenic or organogenic in nature, which further sub-cultured on different hormone media for various morphogenic studies (Plate 9 A,B).

IV 5.2.2: MS + NAA + KN: Further, a notable organogenic callus was obtained from *A. galanga,* in the presence of 3.0 mg/l NAA + 0.5 mg/l KN on MS medium (Plate 9 C,D & Tab: 12). The above callus was yellowish-green and occasionally small shoot primordia were developed (Plate 9 C). When this callus was further sub-cultured on MS medium containing 3 mg/l BAP + 0.25 mg/l NAA, healthy shoots were obtained (Plate 9 D).

IV 5.2.3: MS + NAA + BAP: Coupling of NAA + BAP on MS medium produced very little callus with prominent roots (Plate 9: E & F). Plate 9 E shows a prominent root with very little glossy,white callus, when 3 mg/l NAA and 0.5 mg/l BAP was supplied. However, the quantity of

callus in *K. rotunda,* further reduced and the number of roots increased when MS medium was supplied with 2.5 mg/l NAA and 0.5 mg/l BAP. (Plate 9 F).

In conclusion, embryogenic calli were produced both in *A. galanga* and *K. rotunda,* only when MS was supplemented with 2,4-D + BAP, and *A. galanga,* produced organogenic calli on MS containing NAA + KN.

IV.5.3. *Organogenesis:*

Two months old primary calli of *A. galanga,* which obtained on MS + NAA + KN (Tab:12) was transferred to a similar low auxin/high cytokinin medium for organogenesis and subsequent transfer to a hormone-free medium resulted rhizogenesis (Plate 9 C, D & 10 A,C).

Caulogenesis was noticed on the callus, when transferred to the MS medium having 0.25 mg/l NAA + 3.0 mg/l BAP or KN (Tab:14). An increased concentration of BAP (5.0 mg/l) could initiate 10-15 shoots on *A. galanga* from each culture, after 20 days of sub-culture (Plate 10 A). Addition of same amount of KN also produced significant results (Tab: 14). Further increase in concentrations of both hormones produced negative results (Tab: 14).

Addition of GA_3 on to the shoot inducing medium enhanced the shoot regeneration capacity of the callus with few roots (Plate 10 B). MS medium supplemented with 0.25 mg/l NAA + 5.0 mg/l BAP + 2.0 mg/l GA_3 showed 80% proliferation to yield an average of 22.5 shoots/culture. Further increase in GA_3 level showed negative results (Tab: 14).

Table 14. Effect of various concentrations of an auxin, cytokinins and GA_3 for morphogenic responses of *A.galanga* calli on MS basal medium

Growth hormones mg/l		% of response	Average No of shoots/culture	±SD	Morphogenic response
Auxin	Cytokinin				
0	1	60	-	-	Slow Callus growth
0	2	50	-	-	Slow Callus growth
0	3	40	-	-	Slow Callus growth
	KN				
0	1	60	-	-	Slow Callus growth
0	2	60	-	-	Slow Callus growth
0	3	50	-	-	Slow Callus growth
NAA	BAP				
0.25	1	50	-	-	Green shoot primodia developed
0.25	2	60	8.4	±0.78	Shoot elongates
0.25	3	60	7.5	±1.43	Shoots elongates
0.25	5	70	12.5	±1.98	Well developed plant lets
0.25	6	60	10.0	±2.51	Well developed plant lets
NAA	KN				
0.25	1	40	-	-	Green shoot primodia developed
0.25	3	60	7.5	±0.51	Shoots elongates
0.25	5	70	10.5	±1.98	Well developed shoots
NAA	BAP+GA3				
0.25	5.0+1.0	70	18.2	±1.79	Well developed shoots
0.25	**5.0+2.0**	**80**	**22.5**	**±1.53**	**Well developed shoots and small roots**
0.25	5.0+3.0	60	20.2	±1.52	Well developed shoots
NAA	KN+GA3				
0.25	5.0+2.0	60	17.4	±2.3	Well developed shoots

Mean of 10 treatments
MS Basal medium 3% sugar 0.8% Agar.
SD - standard deviation.
Growth period 60 days.

The shoots produced *via* indirect method (through callus) produced roots on hormone-free MS medium (Plate 10 C). The shoots without roots were transferred to root inducing medium containing 1.5 mg/l NAA, 90% of the plantlets produced profuse roots in 10-15 days (Plate 10 D). The rooted plantlets were subjected to hardening and then transferred to the field with 70% survival (Plate 11 A,B).

IV.6. ***Somatic Embryogenesis:***

The embryogenic calli of *A. galanga* (Plate 9 A) and *K. rotunda* (Plate 9 B) developed on MS + 2,4-D + BAP formulae (Tabs: 12, 13) were transferred to low auxin/high cytokinin medium and subsequent subculture on to hormone free medium resulted embryogenesis (Plates: 12 ,13 & Tabs: 15,16).

When the calli of *A. galanga* and *K. rotunda* were transferred to basal MS medium having only cytokinin (BAP or KN), resulted slow callus growth (Tabs: 15,16). However, when the calli were transferred to a medium fortified with 0.25 mg/l 2,4-D + 3 mg/l BAP or KN, globular embryos were produced with simultaneous development of shoot and root poles (Plate 12 A, D). In *A. galanga,* upon increasing the level of cytokinin from 3.0 mg/l to 5.0 mg/l and complete removal of 2,4-D from the medium, resulted plantlet formation from embryos (Plate 12 B,C). In fact, calli of *K. rotunda* produced both embryogenesis and caulogenesis simultaneously (Plate 12 D). When that actively growing clump (Plate 12 D) was transferred to MS medium, containing 6.0 mg/l BAP, profuse shoots and roots were developed (Plate 12 E,D).Various stages of the development of the embryos were clearly observed and recorded (Plate 13 A-E).

Table 15. Effect of 2,4-D, BAP and KN on somatic embryogenesis and plantlets formation from rhizome disc derived callus of *A. galanga* on MS basal medium

Growth regulations	Concentrati on mg/l	Morphogenic response	Percentage of response	No.of embryos per culture
BAP	1.0	Slow callus growth	50	--
"	2.0	Slow callus growth	60	--
"	3.0	Only shoot developed	30	--
KN		Slow callus growth	50	--
"	1.0	Slow callus growth	60	--
"	3.0	Only shoot developed	30	--
2,4-D +BAP	0.25 + 2.00	Slow callus growth	50	
	0.25 + 3.0	Globular Embryo developed	10	1-2 embryos
BAP	+ 5.0	Embryos developed in to plants	20	1 or 3 embryos developed
2,4-D + KN	0.15 + 2.0	Embryos developed	10	1-2 Embryos
	0.25 + 3.0	globular embryos developed	10	1-2 Embryos
KN	+ 5.0	Embryos developed into small platlets	10	1-2 Embryos
Hormone free	00	Embryos developed in to plantlets	20	1-2 plantlets developed

MS + 3% sucrose + 0.8% Agar
Data taken after 60 days in cullus
Growth rate measured by visual observation.

Table 16. Effect of 2,4-D, BAP and KN on somatic embryogenesis and plantlets formation from rhizome disc derived callus of *K. rotunda* on MS basal medium

Growth regulations	Concentrati on mg/l	Morphogenic response	Percentage of response	No.of embryos per culture	Shoo t rage
BAP	2.00	Slow callus growth	60	-	-
	2.00	Slow callus growth	60	-	-
	3.00	Slow callus growth	60	-	-
2,4-D + BAP	0.25+ 3.00	globular embryos developed	60	-	-
	0.25 + 4.00	Shoots and roots developed from embryos	40	2-4	8-10
BAP	6.00	Well developed shoots and embryos	50	2-4	15-20
2,4-D + BAP	0.25 + 3.00	Shoots and embryos, developed	50	1-3	10-15
2,4-D + BAP	0.25 + 4.00	Shoots and roots developed from embryos	40	1-3	10-15
KN	6.00	Well developed shoots and embryos	40	1-3	10-15
Hormones free	--	Embryos and Shoots developed very well	60	4-6	20-25

MS + 3% sucrose + 0.8% Agar
Data taken after 60 days
Growth rate measured by visual observation.

IV.7. *Hardening of Plantlets Developed from Somatic Embryos:*

Only 10-20 percentage of plantlets developed from somatic embryos were established in the field (Tab: 17 & Plate 14 A-D). The plantlets obtained from somatic embryos were subjected to hardening for different days and found that about 30 days treatment should be provided for proper development (Tab: 17).

Table 17. Survival percentage of plantlets produced by embryogenesis of *A. galanga* and *K. rotunda*

Source	No. of plantlets hardened	Hardening days	No. of plants established	Plants infected	% of establish-ment
A. galanga	5	20 days	0	5	0
	10	30 days	**1**	**9**	10
K. rotunda	10	20 days	1	9	10
	10	30 days	2	8	20

In *K. rotunda,* the plantlets produced through caulogenesis (from callus) (Plate 12 E) produced roots in shoot inducing medium, hardened properly and transferred to field with 70% survival (Plate 15 A,B).

IV.8. *Histological Studies:*

Histological studies during the embryogenesis of *K. rotunda* revealed that its nodular callus budded off globular embryos and by further development both shoot pole and root poles were established (Plate 16 A,B). The longitudinal section of such a budding callus showed the development, of shoot apex encircled by leaf primordia (Plate 16 C).

IV.9. *Encapsulation of Embryos:*

Medium-textured beads were prepared by dropping 3% sodium alginate in MS medium (without $CaCl_2$) containing the embryos, in to 100 μM $CaCl_2$ solution (Tab: 18 & Plate 17 A,B). After curing, spherical synseeds with centered embryos were stored at 4°C in petriplates containing normal MS medium without $CaCl_2$. Some beads washed with distilled water and cultured on MS medium without hormone. After 2 weeks, the synseeds showed sign of regeneration (Plate 17 C).

Table 18. Selection of beads having medium texture

	Calcium chloride in μM			
Sodium alginate	25	50	100	150
1%	F	F	F	S
2%	F	S	S	S
3%	S	S	M	SH
4%	SH	SH	SH	H
5%	H	H	H	H

F - Fragile; S - Soft, M - Medium, SH - Semi hard, H -Hard

***

PLATES

Plate I: Explants of *A galanga* and *K. rotunda* prepared for surface sterilisation

A. Explants of *A. galanga,* collected from 2 months old (pot-raised) rhizomes. d-disc, b - bud.

B. Explants of *K. rotunda,* collected from 3 months old - rhizomes. d-disc, b-bud.

PLATE – 1

Plate 2: Explants of *A. galanga* and *K. rotunda* cultured on hormone free MS medium.

A. *A. galanga* rhizome buds cultured on MS hormone-free medium (after 40 days)

B. *K. rotunda* rhizome buds cultured on MS hormone-free medium (after 35 days)

PLATE – 2

Plate 3: Direct multiple shoot induction from rhizome bud explants of *A. galanga* and *K. rotunda* on MS + cytokinin medium.

A. Direct shoot regeneration from *A. galanga* rhizome bud on MS + BAP (1.0 mg/l), after 40 days.

B. Direct shoot induction of *K. rotunda* rhizome bud on MS + BAP (2.0 mg/l), after 20 days.

C. Direct shoot regeneration of *A. galanga* rhizome bud on MS + KN (1.0 mg/l), after 40 days of culture.

D. Direct shoot regeneration of *K. rotunda* rhizome bud on MS + KN (2.0 mg/l), after 40 days.

PLATE – 3

Plate 4: Multiple shoot induction from rhizome buds of *A. galanga* and *K. rotunda* on MS + cytokinin + auxin medium

A. Multiple shoot induction from rhizome bud of *A. galanga* on MS + BAP (1.0 mg/l) + IAA (0.5 mg/l). One prominant shoot encircled by eight smaller shoots. After 15 days of subculture

B. Multiple shoot induction from rhizome bud of *A. galanga* on MS + BAP (1.0 mg/l) + IAA (0.5 mg/l), after 40 days of subculture. Three prominant shoots and 4 smaller ones are visible.

C. Direct multiple shoots and roots induction from rhizome bud of *K. rotunda* on MS + BAP (2.0 mg/l) + IAA (0.5 mg/l), after 60 days of culture.

D. Multiple shoot induction from rhizome bud of *A. galanga* on MS + KN (1.0 mg/l) + IAA (0.5 mg/l)

E. Multiple shoot induction from rhizome bud of *K. rotunda* on MS + KN (2.0 mg/l) + IAA (0.5 mg/l). Radiating hairy roots are also seen.

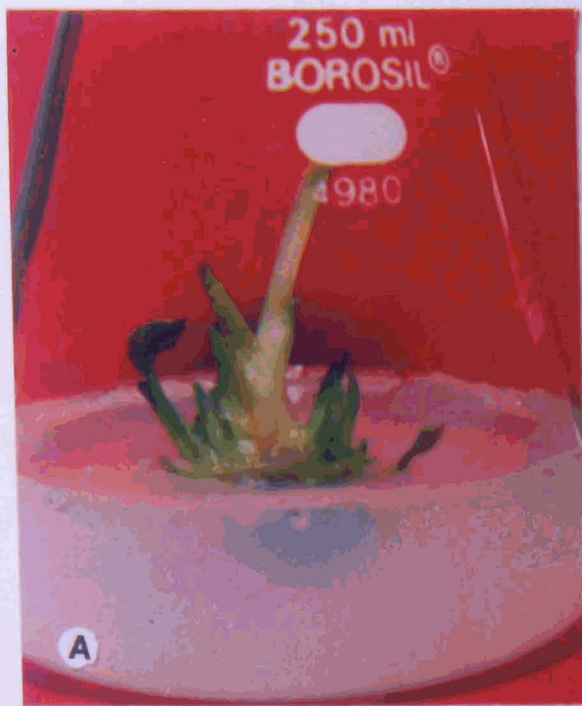

PLATE – 4

Plate 5: In-vitro root development from *in vitro* obtained shoots of *A. galanga* and *K. rotunda* on MS + auxin medium.

A. *In vitro* shoots of *A. galanga* producing roots on MS + NAA (2.5 mg/l) medium, after 20 days of subculture.

B. *In vitro* shoot of *K. rotunda* producing roots on ½ MS + NAA (2.0 mg/l). Over a dozen roots are being developed. After 20 days of subculture.

C. *In vitro* shoots of *A. galanga* produced roots on hormone free medium (after 60 days).

PLATE – 5

Plate 6: Hardening and field transfer of *A. galanga* and *K. rotunda,* plantlets obtained through direct multiple shoot induction.

A. Plantlet of *A. galanga* on hardening (after 10 days of transfer)

B. Hardened plantlet of *A. galanga* in garden pot (after 2 weeks of transfer)

C. Plantlet of *K. rotunda* on hardening (after 10 days of transfer)

D. Hardened plantlet of *K. rotunda* in garden pot. (after 2 weeks of transfer).

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PLATE – 6

Plate 7: Development of multiple shoots of *A. galanga* and *K. rotunda* on MS medium having various concentrations of sucrose (1-5) while all other ingredients in the MS medium retained unaltered. Hormone concentration 1.0 mg/l BAP + 0.5 mg/l IAA for *A. galanga* and 2.0 mg/l BAP + 0.5 mg/l IAA for *K.rotunda*

A. *A. galanga* showing three swollen spouts on 5% sucrose medium.

B. *K. rotunda* showing one swollen prominent shoot with smaller ones emerging out from the base on 5% sucrose medium

C. *A. galanga* showing two long slender shoots on the medium having 1% sucrose.

D. *K. rotunda* showing one long and slender shoot on 1% sucrose medium.

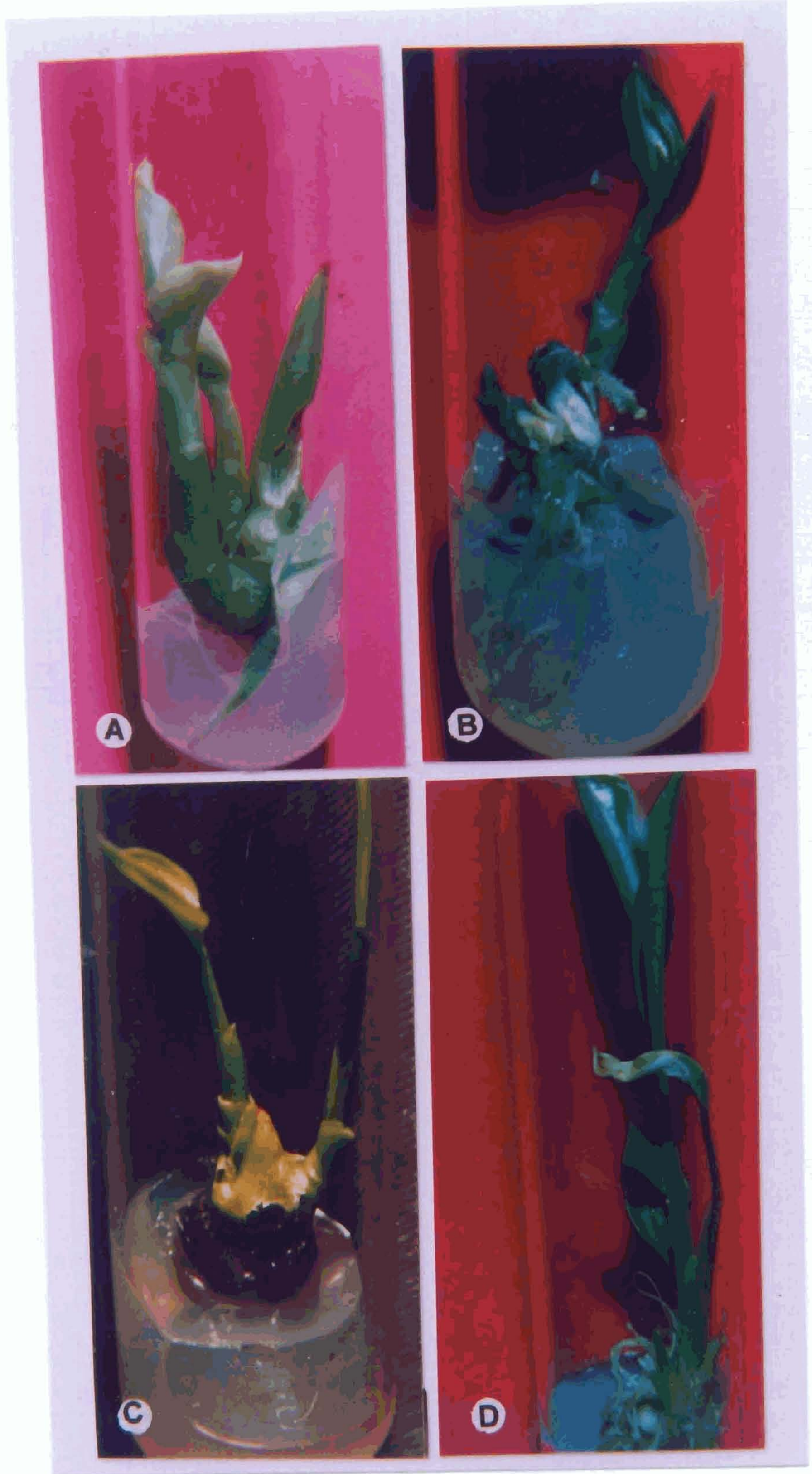

PLATE – 7

Plate 8: Non-embryogenic callus proliferation from the rhizome discs of *A. glanga* and *K. rotunda* on MS + auxin, medium

A. Callus induction from rhizome discs of *A. galanga* on MS + 3.0 mg/l NAA. The water soaked friable callus is getting brownish for dying.

B. Callus induction from rhizome discs of *K. rotunda* on MS + 2.5 mg/l NAA.. On prolonged incubation simultaneous regenaration and dying are visible.

C. Callus induction from rhizome discs of *A. galanga* on MS + 2.5 mg/l 2,4-D -Chrlorophyll development was very poor.

D. Callus induction from rhizome discs of *K. rotunda* on MS + 2.5 mg/l 2, 4-D. Chlorophyll development was very poor and hairy root development was also visible.

86A

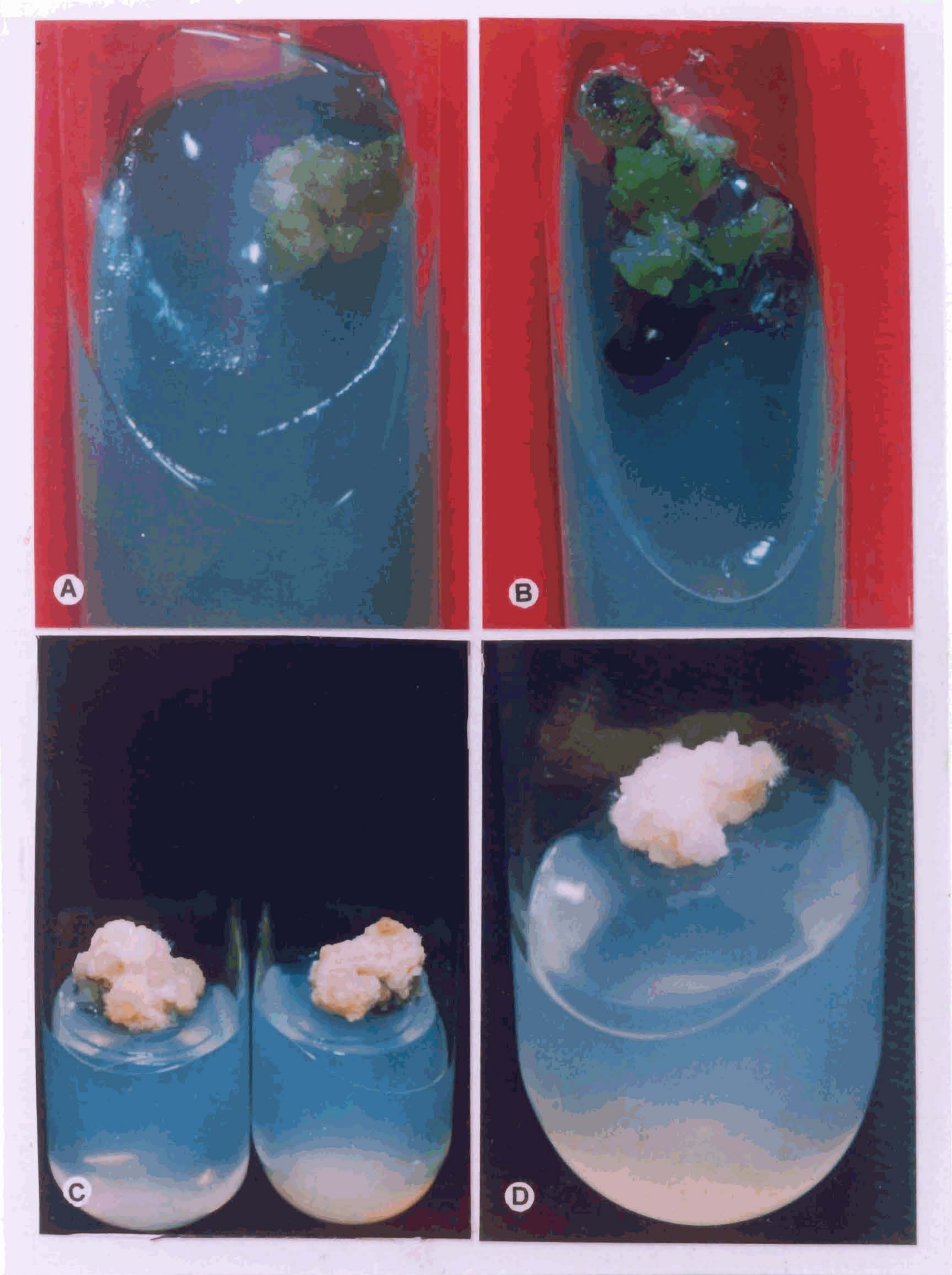

PLATE – 8

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Plate 9: Morphological variations of different calli, obtained when rhizome discs of *A. galanga* and *K. rotunda* were grown on MS medium, in which auxin and cytokinins were coupled.

A. Embryogenic callus induction from rhizome discs of *A. galanga* on MS + 2.0 mg/l 2,4-D + 0.5 mg/1. BAP.

(the callus is compact and nodular with creamy white and glossy nature).

B. Embryogenic callus induction from rhizome discs of *K. rotunda* on MS + 2.5 mg/l 2,4-D + 0.5 mg/l BAP. (the callus is creamy white and glossy nature).

C. Rhizome discs of *A. galanga* producing callus on MS + 3.0 mg/l NAA + 0.5 mg/l KN. (after 60 days incubation). A number of tiny shoot primordia are visible on the callus.

D. Healthy shoots are being emerged out of the caulogenic callus of *A. galanga*. (after 15 days subculture when MS medium was supplemented with 3.0 mg/l BAP + 0.25 mg/l. NAA)

E. Development of callus from *A. galanga* rhizome disc on MS medium containing 3.0 mg/l NAA + 0.5 mg/l BAP. (prominent root with very little glossy white callus could be seen).

F. Development of callus from rhizome disc of *K. rotunda* on MS + 2.5 mg/l NAA + 0.5 mg/l BAP. (callus development very little with prominent thread like roots from above and swollen roots below.)

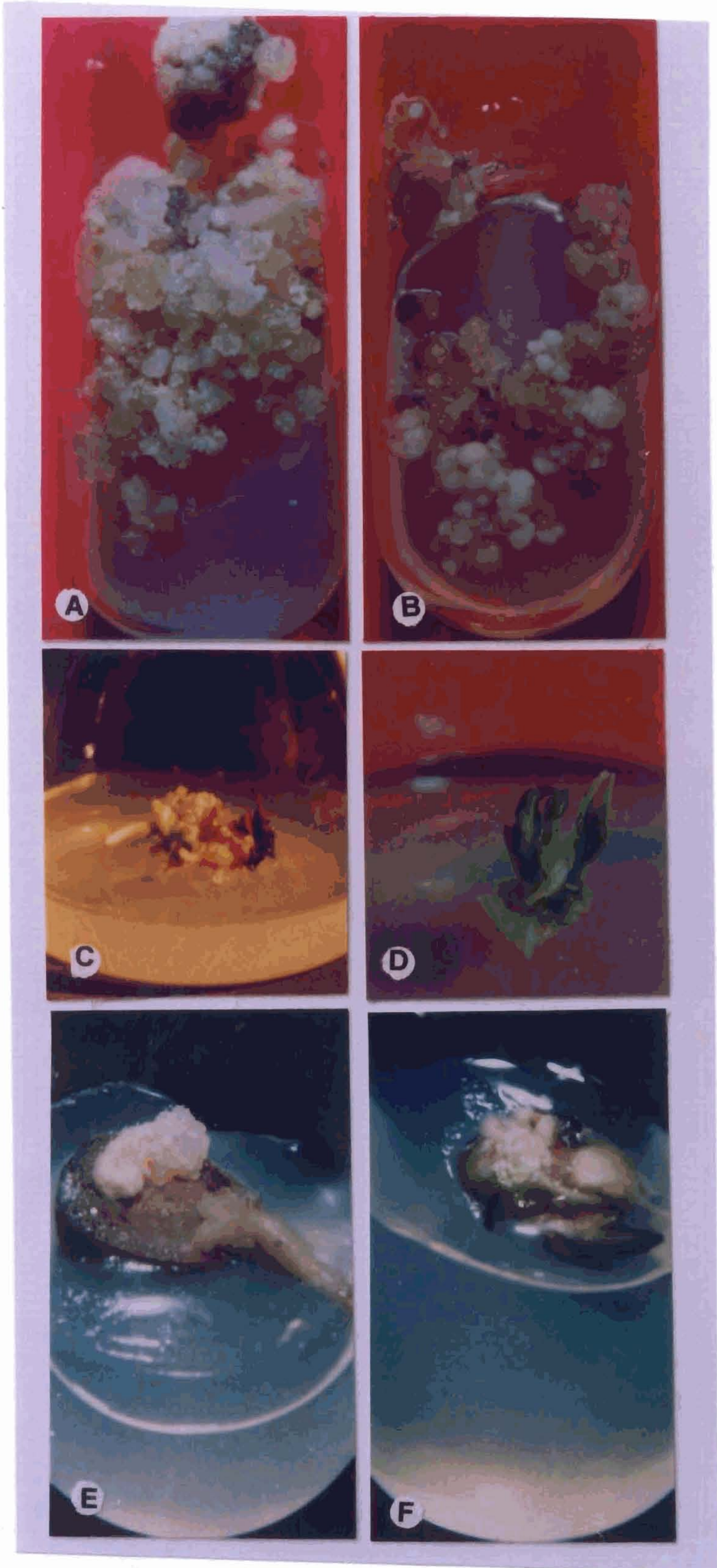

PLATE – 9

Plate 10: Caulogenesis of *A.galanga* callus and its root induction from shoots on MS + hormone medium.

A. About 15 shoots being developed from a single culture on MS + 5.0 mg/l BAP.

B. Simultaneous development of shoot and roots from the *A. galanga* callus in the presence of 2.0 mg/l. GA3 + 0.25 mg/l NAA + 5.0 mg/l BAP.

C. Simultaneous regeneration of shoots and roots on hormone free MS medium, on prolonged culture.

D. Root induction from *in vitro* shoots on MS + 1.5 mg/l NAA.

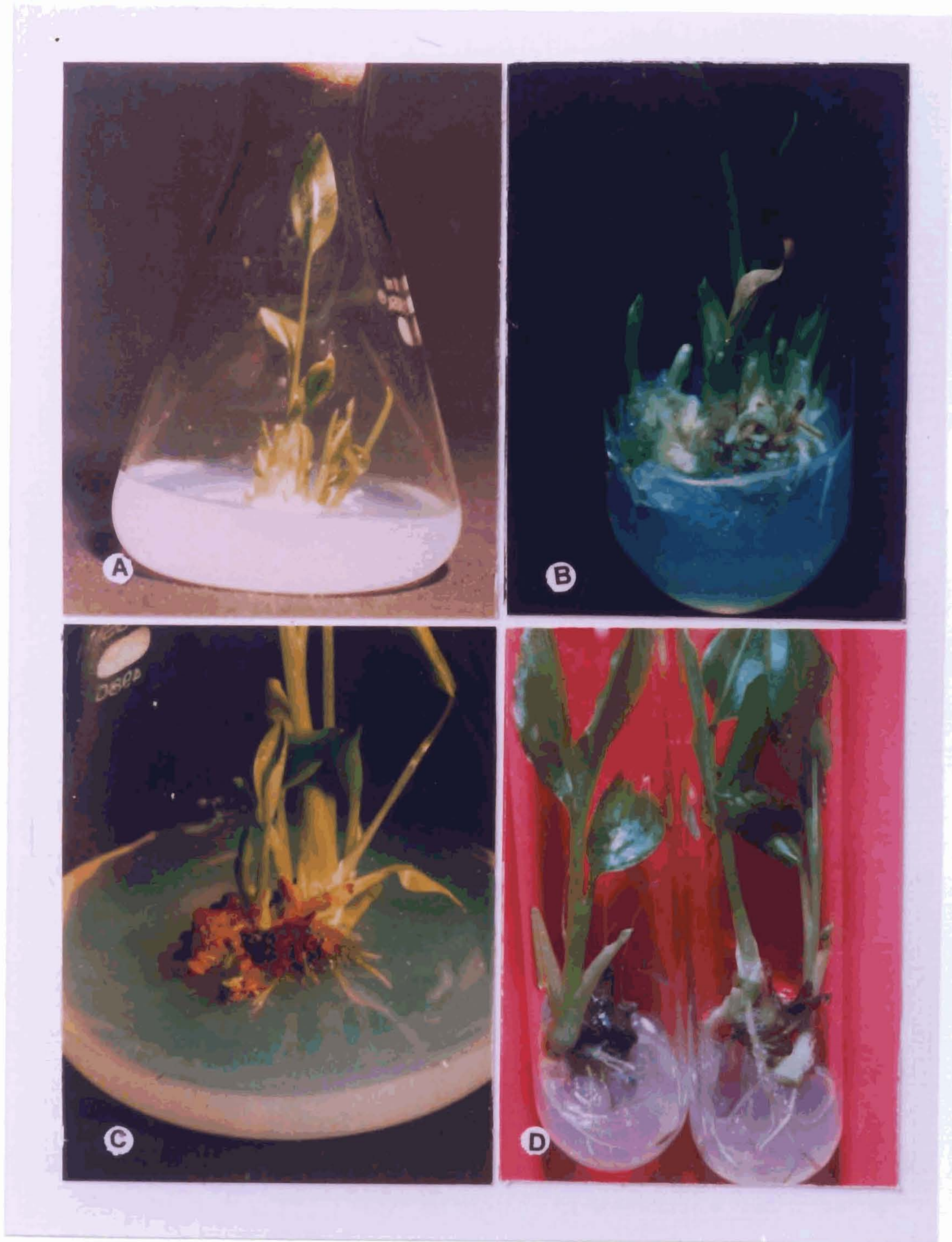

PLATE – 10

Plate 11: Hardening and field establishment of plantlets of *A. galanga,* obtained through callus culture.

A. Indirectly obtained plantlets on hardening on the next day of pot transfer.

B. Above hardened plantlet (plate 11 A) being established in garden pot, after two weeks of transplantation.

PLATE – 11

Plate 12: Regeneration from embryogenic callus of *A. galanga* and *K. rotunda* on MS medium.

A. Amorphous bulbil like embryos of *A. galanga* initiate regeneration on MS + 0.25 mg/l 2,4-D + 3.0 mg/l BAP.

B. On subculture, somatic embryos of *A. galanga* (Plate 12:A), being developed to produce plantlets on MS + 5.0 mg/l BAP.

C. Development of a single plantlets from the above culture (plate 12. B) on hormone free MS medium.

D. Bulbil-like somatic embryos of *K. rotunda* initiated regeneration on MS + 0.25 mg/l 2,4-D + 3.0 mg/l BAP.

a = independent somatic embryo, b = independent embryo shows both shoot and root poles; c = a clump of embryos.

E. A cluster of shoots and bipolar embryos are being developed from the clump of embryos (plate 12 D) when sub cultured (after 40 days) on MS containing 6.0 mg/l BAP.

F. From the above culture (plate 12 E) two bipolar embryos separated for photographing (individual shoot and roots are evident).

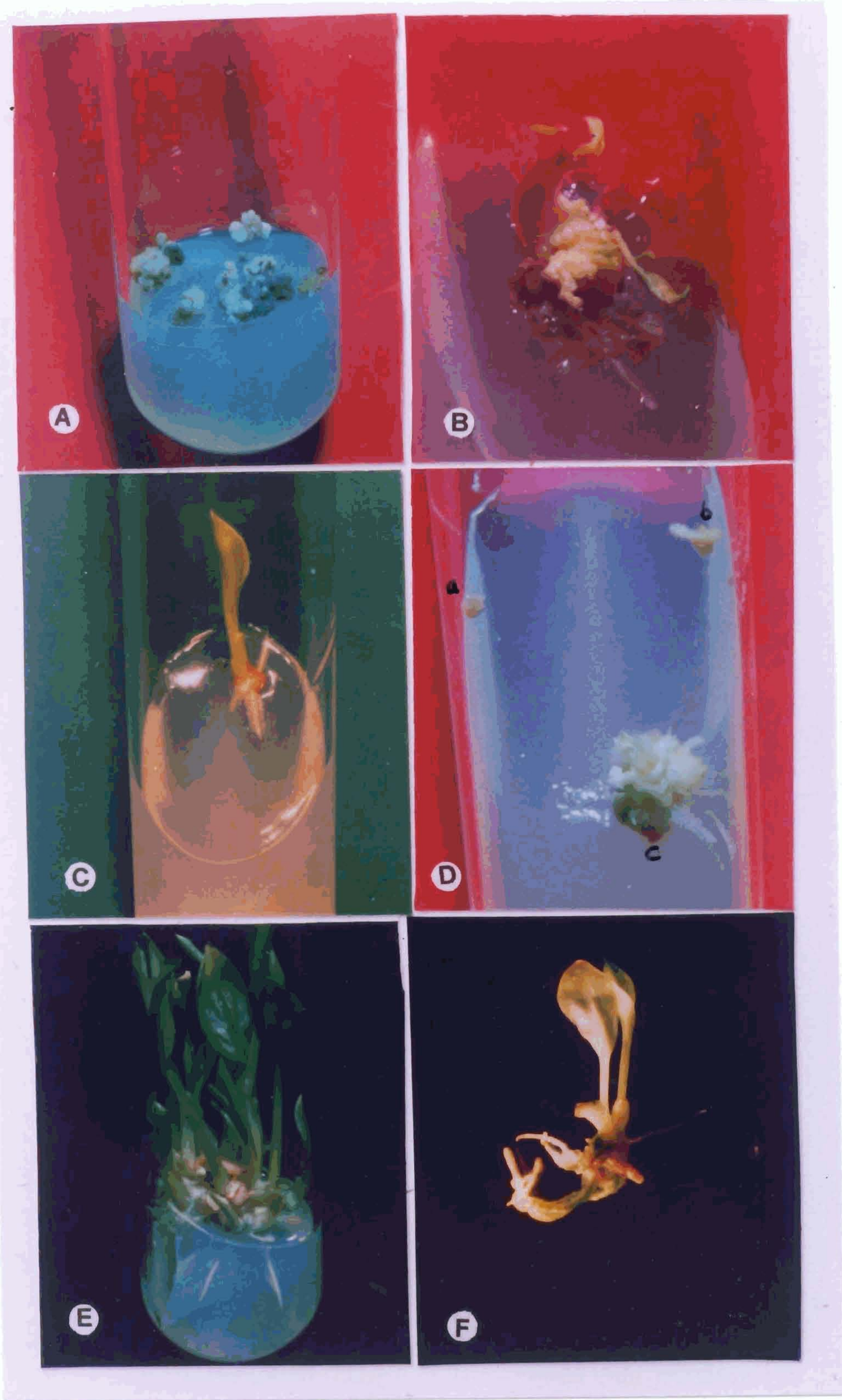

PLATE – 12

Plate 13: Different developmental stages of somatic embryos of *A. galanga* and *K. rotunda*.

A. Various stages of somatic embryos of *A. galanga* . Developmental stages of root and shoot poles are clear, in petri-plate.

B. Isolated bipolar embryos of *A. galanga* shows clear root and shoot primordium.

C. Isolated embryos of *K. rotunda* showing various stages of development, on glass plate.

D. Plantlet of *A. galanga,* obtained by somatic embryo development on hormone-free MS medium.

E. Two healthy plantlets of *K. rotunda* obtained by somatic embryo development on hormone free MS medium. Prominent anastamosed roots also visible.

PLATE – 13

Plate 14: Hardening and field transfer of *A. galanga* and *K. rotunda* plantlets obtained *via* regeneration of somatic embryos.

A. *A. galanga* plantlets on hardening after 30 days of transplantation.

B. *A. galanga* plantlets (plate 14A above) established on plastic mug, after 20 days transfer.

C. *K. rotunda* plantlet on hardening, after 30 days of transplantation.

D. *K. rotunda* plantlet established on plastic mug. (after 20 days of transplantation).

PLATE – 14

Plate 15: Hardening and field transfer of *K. rotunda* plantlets obtained by indirect shoot regeneration.

A. Plantlet on hardening, after 15 days of transplantation.

B. Plantlet on garden pot, after two weeks of transplantation of the above (Plate 15A).

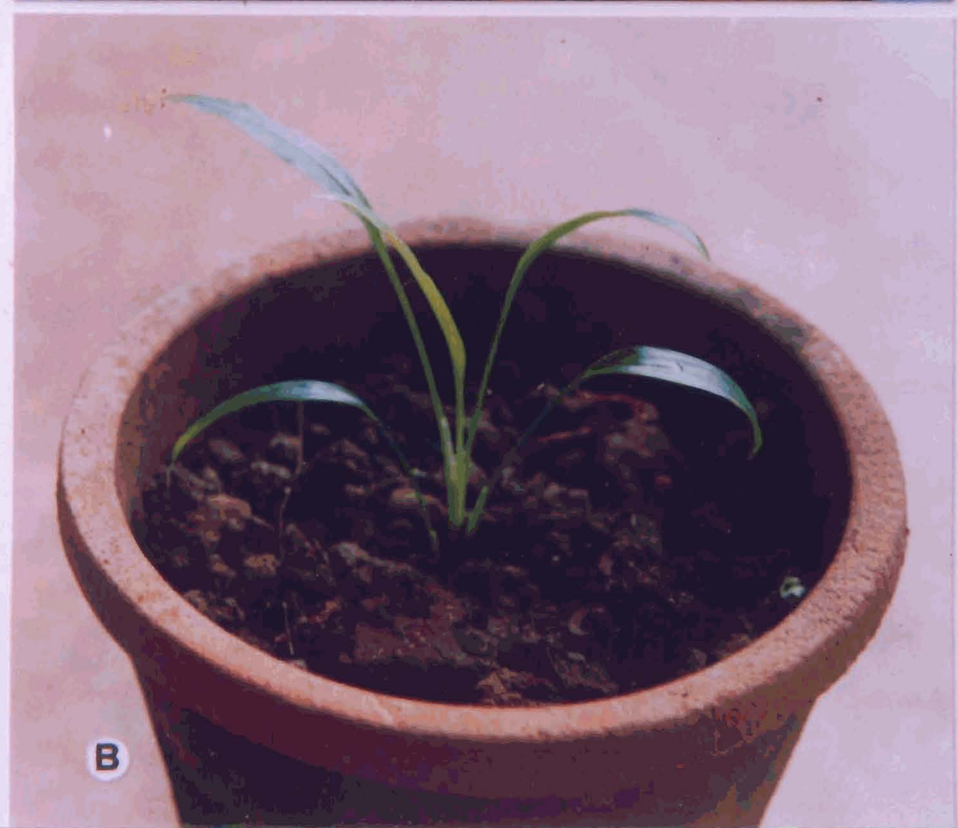

PLATE – 15

Plate 16: Photographs of microtome sections of the embryogenic callus of *K. rotunda* showing different developmental stages.

A. Globular embryos, protruding from the callus (a, b, c).

B. Longitudinal section of the developing embryo, showing both shoot and root pole (rp. & sp) respectively.

C. Developing shoot apex (sa) directly from the callus, encircled by leaf primordia (lp).

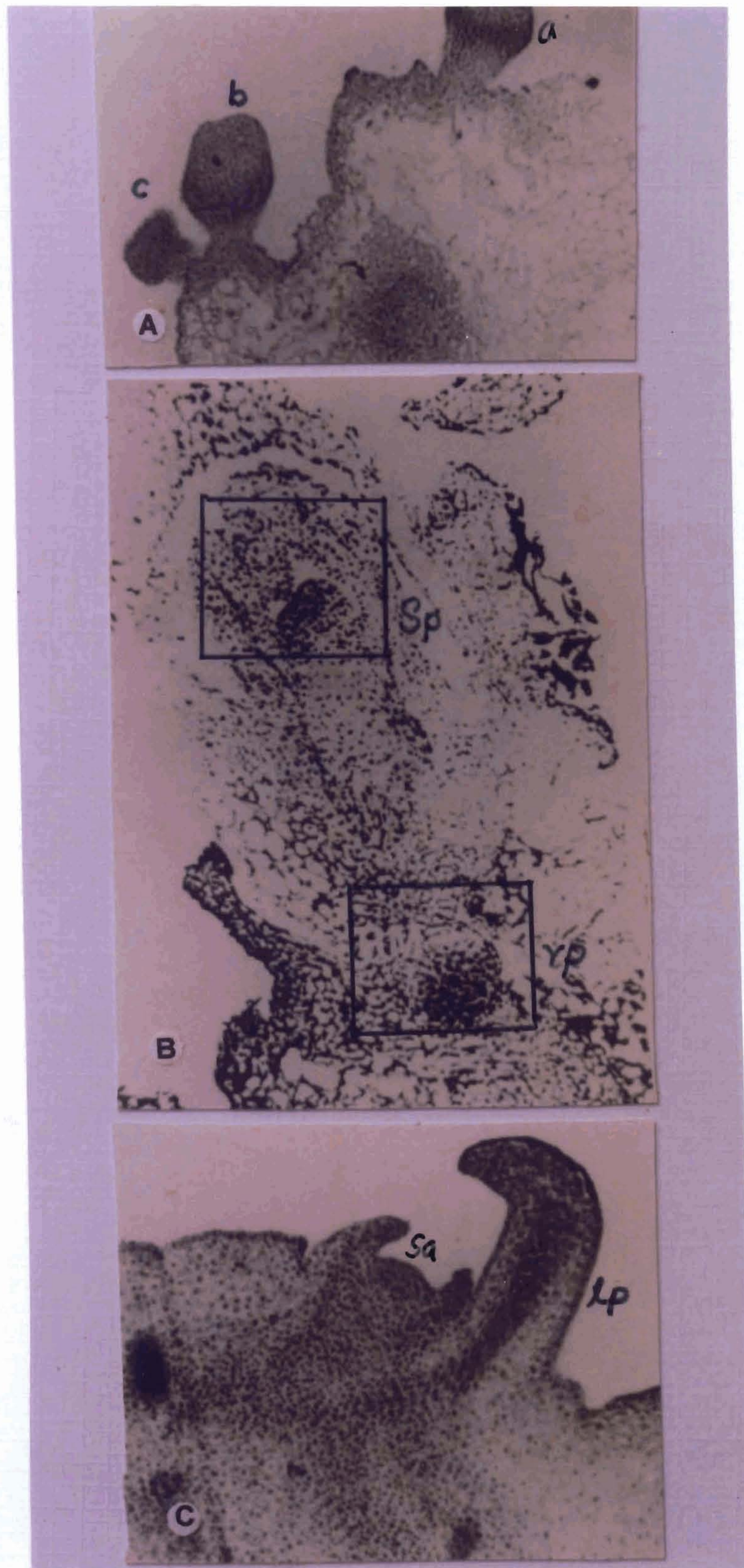

PLATE – 16

Plate 17: Encapsulation studies of the somatic embryos of *A. galanga* and *K. rotunda* in calcium alginate beads.

A. *A. galanga* embryos entrapped in calcium alginate beads.

B. Synseeds of *K. rotunda*- encircled bead shows sign of germination.

C. Synseeds of *A. galanga* showing sign of regeneration after 15 days of incubation on hormone-free MS medium.

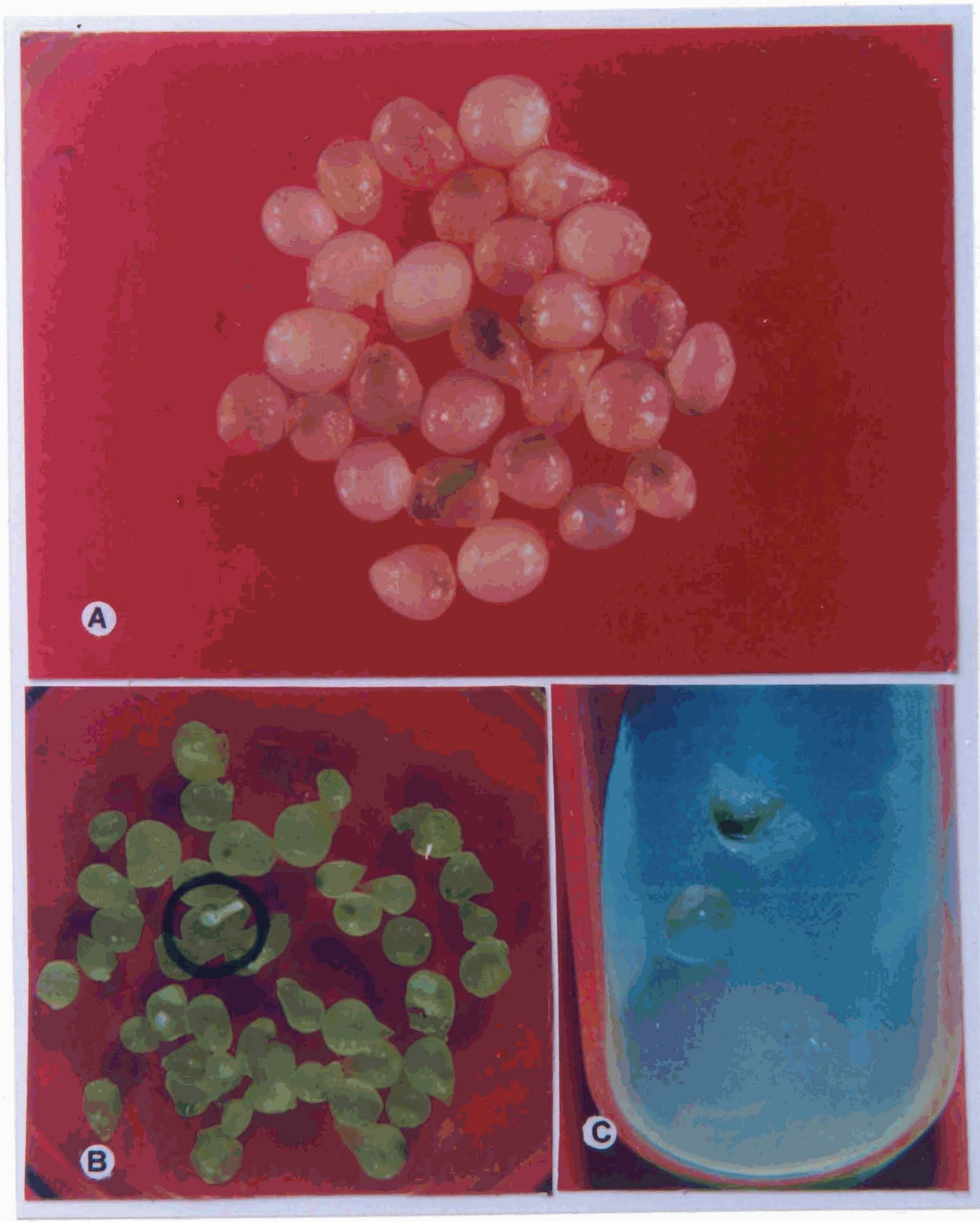

PLATE – 17

CHAPTER V

DISCUSSION

Rapid improvements have been made for the preservation of MAPs from its unbridled and widespread destruction, by tissue culture practices. Two South Indian medicinal plants *viz. Alpinia galanga* (Linn.) Willd. and *Kaempferia rotunda* Linn. have been selected for the present study. The experiments focussed mainly upon the development of an economic and reproducible strategy for the micropropagation of these traditionally used plants and reincarnate them to their wild natural habitats.

For convenience, the discussion part is framed up in such a way that the major outcomes of the study should be highlighted individually.

V.1. ***Selection of Explants:*** It is important to select an explant that should give maximum number of shoots or callus within a short time. Different researchers used different explants for their study. Literature survey revealed that meristem, shoot tips, axillary buds; nodes, rhizome buds, vegetative buds, ovary tissues, anther, root tips, leaf discs inflorescence *etc.* (Tab: I) are being used more frequently. For convenience, most important works reported in *Zingiberaceous* members using different explants have been summarised in the Table (19).

Table 19. A profile of important works using different explants in *Zingiberaceae*

	Plant source	Explant used	Reference
1.	*Alpinia*		
	A. calcarata	Rhizome buds	Agretious *et al.* (1996)
	A. galanga	Rhizome sprout buds	Anand and Hariharan (1997)
	A. purpurata	Inflorescence buds	Chang and Criley (1993)
			Illg and Faria (1995)
2.	*Amomum*		
	A. subulatum	Rhizome buds	Sajina *et al.* (1997)
3.	*Costus*		
	C. speciosus	Rhizome tissues	Jain and Chaturvedi (1985)
4.	*Curcuma*		
	C. amada	Rhizome buds	Barthakur and Bordoloi (1992)
	C. aromatica	Rhizome buds	Anand and Hariharan (1999)
	C. longa	Vegetative buds	Nadgauda *et al.* (1978)
	C. longa	Rhizome buds	Balachandran *et al.* (1990)
	C. species	Rhizome tissues	Yasuda *et al.* (1998)
5.	*Elettaria*		
	E. cardamomum	Immature panicles	Kumar *et al.* (1985)
	E. cardamomum	Shoot buds	Vatsya *et al.* (1987)
	E. cardamomum	Rhizome buds	Priyadarshan *et al.* (1988)
	E. cardamomum	Vegetative sprout buds	Nadgauda *et al.* (1983)
6.	*Kaempferia*		
	K. galanga	Vegetative buds	Vincent *et al.* (1991, 1992)
	K. rotunda	Rhizome buds	Anand *et al.* (1997)

Contd....

7.	*Zingiber*		
	Z. officinale	Rhizome buds	Balachandran *et al.* (1990)
	Z. officinale	Rhizome buds	Hosoki and Sagawa (1977)
	Z. officinale	Shoot tips	Pillai and Kumar (1992)
	Z. officinale	Shoot tips	Ilahi and Jabeen (1987)
	Z. officinale	Leaf discs	Kackar *et al.* (1993)
	Z. officinal	Leaf discs	Nirmal Babu *et al.* (1992)
	Z. officinal	Anther	Ramachandran and Chandrasekharan Nair (1992)
	Z. officinale	Anther	Samsudeen *et al.* (1996)
	Z. officinal	Ovary tissue	Nirmal Babu *et al.* (1996)
	Z. officinale	Shoot meristem	Bhagyalakshimi and Singh (1988)
	Z. officinale	Shoot lips or buds	Sharma *et al.* (1994)

From this above table, it is clear that most of the workers used rhizome buds or vegetative buds for their tissue culture works. Out of different explants - like rhizome sprout buds, sprout discs, leaf discs, root tips, flower buds, *etc.* have been tried in the present study, sprout buds and sprout discs have shown maximum response (Tab: 3). Hence, the sprout buds selected for direct multiple shoots production, and sprout discs used for the induction of callus and indirect shoot multiplication in both species (Tab: 3., Fig: 1 & Fig: 2, A, B).

The maximum response of the sprout buds or discs reported in the present study might be due to the presence of apical and cambial meristems inside the explant tissues, as opined by Murashige (1974); Ammirato (1983); Bajaj (1986) and Gamborg and Phillips (1996)

V.2. *Size of the Explants:*

Size of the explant was important factor in *in vitro* studies. The explants which have size or cell counts lower to a critical size may not respond to the culture at all (Narayanaswamy, 1994: Gamborg & Phillips, 1996]. This critical value is not fixed, and should be varied for different plant species. In fact, callusing is not normally depend on the size or shape of the initial explants (Narayanaswamy, 1994). However, the use of direct and large sized (1.0 –2.0 cm.) explants had higher survival and growth rates than the smaller ones, for the tissue culture of meristem and shoot tips (Hu & Wang, 1983).

Of the different sizes tried here, explants of ~1.0 cm^3 were most suitable for the culture initiation of *A. galanga* and *K. rotunda* (Plate I A, B & Fig: I). When explants of size above 1.0 cm used for the culture, contamination rate was high, owing to large number of soil microflora, and those with below 1.0 cm, showed very low response and gradually explants shrivelled off on the supporting medium.

The earlier reports in *Zingiberaceae* members proved that medium size explants (0.5 - 2.0 cm) were better than those of the larger or smaller size. Nirmal Babu *et al.* (1992, 1996) in *Zingiber*, Vincent *et al.* (1992) in *Kaempferia* and Agretious *et al.* (1996) in *Alpinia*, used explants of size 1.0 cm for the culture initiation. These findings are in agreement with the present work also. However, Yasuda *et al.* (1998) in *Curcuma*, and Illg and Faria (1995) in *Alpinia* have used explants of size below 1.0 cm. In short, the critical size of the explant would vary with the plant species used for the culture experiments.

V.3. *Age of the Explants:*

Age of the explants play a major role in the tissue culture experiments (Murashige, 1974; Ammirato, 1983; Gamborg & Phillips, 1996). Earlier reports about the *Zingiberaceous* members did not clearly explain the age of the explants they used and nobody had conducted precise study on explants with different age. In *A. galanga,* explants collected from 2 months old rhizome was better, while explants collected from 3 months old rhizome was better for *K. rotunda* (Fig: 3) (Anand & Hariharan, 1997; Anand *et al.,* 1997). This higher response rate of the explants during this stage may be due to the increased physiological activity of the rhizome, which is being transferred to the growth regions like buds and meristems, during acclimatisation of the rhizomes on the sand beds.

V. 4. *Surface Sterilisation:*

Normally, plants growing in the field or in a glass house would be affected with micro-organisms and surface contaminants. Hence, excised plant parts should be sterilised or disinfected by biochemical means, before their further trimming to smaller explants for inoculation purpose (Narayanaswammy, 1994; Murashige, 1974; Gamborg & Phillips, 1996).

Selection of the sterilising agent depends mainly upon the factors like, nature of explants collected (underground or not), extend of surface microflora and the sensitivity of the explant tissues to various sterilising agents, optimum treatment time and concentration of sterilising agents *etc.* and should be determined by trial and error

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methods. The protocol used for the surface sterilisation was described in Tab: 4 and Figs: 4 to 7. However, once contamination free culture obtained, that could be used for further culture studies.

The earlier reports about *Zingiberaceous* members have adopted a general procedure for the surface sterilisation, which involved washing of explants with tap water, washing with detergents, treatment with 70% alcohol, treatment with mercuric chloride solution, rinsing with double distilled water, and finally ready to inoculation. The treatment time vary with different species used for the culture (Hosoki & Sagawa, 1977; Nadgauda *et al.*, 1983; Malamug *et al.*, 1991; Sakamura & Suga, 1989). However, the results obtained here show slight divergence from this main pattern, *ie.* during final inoculation of fresh explants incorporation of minute quantity of antibiotic, *viz.*, erythromycin increased the survival rate of explant by resisting the growth of inborn microbial contaminants (Fig: 6). Hence, the experiment with *A. galanga* and *K. rotunda* involved the following steps; washing of explants with 2% Dithane -M 45 and, followed by tap water, blotting with blotting paper, washing with 5% extran for 30 minutes; treatment with 0.1% $HgCl_2$ solution for *A. galanga* for 10 minutes and 0.15% $HgCl_2$ for *K. rotunda* for 15 minutes, treating with 70% alcohol about 5-10 seconds, rinsing with double distilled water (about 3 times) and final inoculation to medium having erythromycin (Figs: 4-7 & Tab: 4).

V. 5. *Direct Multiple Shoot Regeneration:*

Direct multiple shoot regeneration (DMSR) aims at the production of disease-free plants, breeding true, on large scale in limited time. DMSR is relatively a new technology, and application of innovative methods have served to overcome barriers to progress in multiplication of elite species and their improvement further. (Narayanaswamy, 1994; George & Sherrington, 1984).

It is evident that DMSR has successfully been accomplished using various explant sources - like rhizome sprouts, nodes, axillary buds leaf discs, anther inflorescence, *etc.* (Tab: 1). Prominent works showing DMSR of rhizome buds of *Zingiberaceae* are in vogue *viz: A. subulatum* (Sajina *et. al.*, 1997); *A. calcarata* (Agretious *et al.*, 1996); *C. speciosus* (Jain & Chaturvedi, 1985); *C. amada* (Barthakur & Bordoloi, 1992); *E. cardamonum* (Priyadarsan *et al.*, 1988, Vatsya *et al.*,1987); *K. galanga* (Vincent *et al.*, 1992) and *Z. officinale* (Nirmal Babu *et al.*, 1996; Sharma & Singh, 1997).

Illg and Faria (1995) reported 5-10 multiple shoots from inflorescence buds of *A. purpurata,* when cultured on MS medium supplemented with 2.5 mg/l BA and 0.9mg/l IAA or NAA. In *K. galanga,* rhizome buds produced 8-10, shoots when cultured on MS medium having BAP + KN (Vincent *et al.*, 1992).

During the DMSR studies, sprout buds of *A. galanga* and *K. rotunda* , responded variously to the medium with or without growth hormones (Anand & Hariharan, 1997; Anand *et al.*, 1997). Explants on hormone free medium showed a delayed response and slow growth

(Fig: 8). Excised sprout buds should not have sufficient endogenous cytokinins to support growth and development as on an artificial medium, as suggested by Hu and Wang (1983). Therefore, an exogenous application of hormones has been recommended (Murashige, 1974), addition of cytokinins stimulated the development of multiple shoots from both the materials (Tabs: 5, 6 & Fig: 3 A - D).

V. 5.1. ***Effect of Cytokinins:***

Being effective plant growth regulators, cytokinins induce cell division and activate the dormant buds of the explants to enlarge and grow (Sachs & Thimann, 1964). As explained in Tabs: 5, 6 and Plate 3 A - C, BAP played an important role in this study. In *A.galanga,* medium with 1.0 mg/l BAP produced 3-4 shoots per culture, while the same result was obtained in *K.rotunda* in the presence of higher concentration of BAP (2.0mg/l.) (Tabs: 5, 6 & Plate 3 A,B). The superior effect of BAP emphasized earlier in many *Zingiberaceous* medicinal plants such as *Zingiber* and *Curcuma,(*Balachandran *et al.,* 1990). In their study, buds produced 3 to 4 shoots on MS medium supplemented with BAP (3.0 mg/l). The stimulatory effect of BAP was also reported by the studies on other medicinal plants - like *Ocimum sps* (Pattnaik and Chand, 1996); *Piper sps* (Bhat *et al.,* 1995); and in *Alpinia* (Chang & Criley, 1993).

Higher concentration of BAP (above 2.0 mg/l) showed decreased frequency of shoot induction in both *A. galanga* and *K. rotunda* (Tabs: 5, 6). This elevated concentration of BAP might have imparted a toxic effect on to the explants by retarding cell division. Werner and Boe (1980) noticed abnormal growth/dying of cells and

tissues, when explants cultured in the presence of BAP, above its optimum concentration. Investigators like McComb and Newton (1981); Mathew (1993); and Sahoo and Chand (1998) also substantiated the arguments of Werner and Boe (1980).

Kinetin is an important cytokinin, having the ability to induce multiple shoots from expalnts. Nadgauda *et al.* (1980) produced healthy shoots in ginger, when cultivated on MS supplemented with 0.1 mg/l KN. In *H. indicus*, axillary buds produced an average of 8.2 shoots, when MS medium containing 0.2 mg/l KN + 0.1mg/l NAA (Pattnaik & Chand, 1996). Contrary to this, in the present study, KN showed a decreased effect in both *A. galanga* and *K. rotunda* with regard to the induction of multiple shoots (Tabs: 5, 6 & Plate 3 C, D). Agretious *et al.* (1996) also reported, the ineffectiveness of KN for the induction of multiple shoots from rhizome buds of *A. calcarata*. In their experiment MS + 0.5 - 3 mg/l KN produced 2 or 3 shoots per culture. In *A. subulatum*, rhizome buds showed superior shoot bud induction, when it cultured on MS + BAP medium (Sajina *et al.* 1997). The ineffectiveness of KN in the present study might be due to the specificity of explant to certain growth regulators. Hu and Wang (1983) reported that most of the micropropagation reports (68%) amplify the superior effect of BAP, with comparatively very low percentage (22%) to the promotive effect of KN on micropropagation.

Coconut water (CW) also showed a tendency to induce multiple shoot buds from the expalnts to some extend. Naseem and Jha (1997) found that CW, coupled with KN can induce multiple shoots by the thalamus culture of *C. gynandra*. Shoot induction was reported by Eck

and Kitto (1992) from the leaf explants of peppermint on MS medium, supplemented with CW (250 mg/l). In fact, experiments with *A. galanga* and *K. rotunda,* using CW reflected its retarding effect on shoot induction percentage (Fig: 9).

In comparison, the individual application of BAP was better than KN and CW (Tabs: 5, 6; Plate 3 A -D; & Fig: 9).

V 5.2. *Synergistic Effect of BAP + KN:*

Combined effect of two cytokinins (BAP + KN) increased the shoot induction in many plants. Vincent *et al.* (1992) and Agretious *et al.* (1996) obtained increased number of shoot induction in *K. galanga* and *A. calcarata,* respectively, when MS medium fortified with BAP + KN was used. Based on this report, combined effect of BAP + KN was also conducted on *A. galanga* and *K. rotunda.* Surprisingly in *A. galanga,* that effect produced negative result, while in *K. rotunda* showed a little enhanced performance in shoot production (Tabs: 5, 6 & Fig: 10).

In comparison, this foregoing discussion clearly state that the individual application of BAP would be satisfactory than other cytokinins/its combinations for the induction of multiple shoots in *A. galanga* and *K. rotunda* .

V. 5.3 *Interaction of Cytokinin/Auxin:*

Auxins are a group of plant growth regulators which generally produce adventitious roots from shoots. Higher concentration of auxin may induce callus from explants. Unlike, the occurrence of absolute concentration of the plant growth regulators, it is the balance

between auxin and cytokinin that regulate the growth and differentiation (Evans *et al.*, 1983; Debergh & Zimmerman, 1991; George, 1993a).

In the present study, coupling of auxin (below 1.0 mg/l) along with cytokinin (BAP) induced more number of shoots, than without auxin in the medium. Hu and Wang (1983) suggested that, though exogenous auxin did not generally promotes axillary shoot proliferation, culture growth could be improved by its presence. One of the possible roles of auxin in the shoot multiplication stage is to nullify the suppressive effect of high cytokinin in the medium, when they present together. Many reports are available, in which auxin-cytokinin combinations favoured shoot multiplication. For instance, Priyadarshan *et al.* (1988) succeeded in the clonal propagation of different cultivars of cardamom on MS medium, fortified with IAA and BAP. Similarly, *C. amada* rhizome buds produced 7-12 shoots and roots simultaneously when MS medium fortified with 0.5 mg/l NAA + 4.0 mg/l BAP (Barthakur & Bordoloi, 1992). More number of shoots and roots were induced with *A. subulatum*, when BAP+IAA were added to the MS medium (Sajina *et al.*, 1997). In 1993, Samarajeewa *et al.* (1993) reported adventitious shoot induction in *G. superba*, a member of *Liliaceae*, on MS medium containing 0.05 mg/l BAP + 0.01-0.05 mg/l, IBA, IAA or NAA..

Auxin - cytokinin combination also favoured multiple shoot induction both in *A. galanga* and *K. rotunda*. In *A. galanga*, an average number of 6.9 shoots were produced on MS + 1.0 mg/l BAP + 0.5 mg/l IAA, and 8.0 shoots were obtained with *K. rotunda*, on MS

fortified with 2.0 mg/l BAP + 0.5 mg/l IAA (Tabs: 7, 8, & Plate 4 A,B,C). These results are in consonance with the reports of Agretious *et al.* (1996) in *A. calcarata* and Harikrishan and Hariharan (1996) in *P. rosea*. They found that the coupling effect of IAA + BAP produced more number of shoots. Synergistic effects of other auxins and BAP on promoting shoot multiplication of medicinal plant species have well documented, such as: BAP + IBA in *P. nigrum* (Philip *et al.*, 1992); *G. jasminoides* (George *et al.*, 1993); BAP + NAA in *G. officinalis* (Mercier *et al.*, 1992); *T. indica* (Sharma & Chandel, 1992) and *A. purpurata* (Illg & Faria, 1995). Likewise, earlier works in ginger also proved the beneficial activity of BAP - NAA combination for multiple shoot induction (Sakamura *et al.*, 1986, Chalwood *et al.*, 1988; Sakamura & Suga, 1989). However, other auxins like IBA, NAA along with KN did not result in promising results, when used in *A. galanga* and *K. rotunda* (Tabs: 2, 8).

In comparison to the medium with cytokinin coupled with auxin, the shoot induction frequency was less in medium containing cytokinin alone (Tabs: 5,6,7,8. & Plates 3 A-D, 4 A-C). In fact, this frequency of regeneration was dependent purely upon the concentration of the hormones employed. The effective concentration of auxin-cytokinin may vary from one species to another, and even in cultures derived from different varieties of plants. Effective treatment may also depend on the nature and origin of explants, its endogenous hormone contents and the conditions provided for the *in vitro* culture (Varghese & Kaur, 1988). The cytokinin content of the explant may vary with different species, and may be different from the cytokinin

present in the medium. Though the precise role of cytokinin in earlier studies was focused on its possible improvement in controlling shoot growth, many workers have reported on the control of *in vitro* morphogenesis by interaction between auxin and cytokinin concentration on the medium (Evans *et al.*, 1983; Debergh & Zimmerman, 1991; George, 1993a).

On the basis of above discussion and observations, it could be concluded that the optimum hormonal supplement for the induction of direct multiple shoots for *A. galanga* should be 1.0 mg/l BAP + 0.5 mg/l IAA, and for *K.* rotunda, it is being 2.0 mg/l BAP + 0.5 mg/l IAA.

V. 6. In Vitro *Rooting of Shoots:*

Rooting of shoots is the critical step in the *in vitro* production of complete plantlets, and their subsequent maintenance. The concentration of growth regulators and its contents in the medium plays a vital role in the rooting process. It is generally accepted that auxin imparts a pivotal role in the induction of adventitious roots during the clonal propagation of vegetative shoots. Skoog and Miller (1957) suggested that the determination of root initiation is generally depend on the auxin or auxin/cytokinin ratio in the medium. High concentration of auxin also induces roots or callus from different explants (Skoog & Miller, 1957; Hu & Wang, 1983; George, 1993a).

Numerous studies indicated that NAA is the most effective hormone for root initiation (Hu & Wang, 1983). For the rooting of *Zingiberaceous* members, the auxin NAA was more preferable than

others (IBA or IAA) (Nirmal Babu *et al.*, 1996; Sajina *et al.*, 1997). In 1993, Kackar *et al.*, found that, ginger plantlets derived from callus produced roots on MS medium supplemented with 3 mg/l NAA. In another experiment, ginger plantlets derived from leaf callus, produced roots on MS containing 1.0 mg/l NAA (Nirmal Babu *et al.*, 1992). Ginger plantlets, derived from immature inflorescence, produced extensive roots, when it cultured on MS medium fortified with 1.0 mg/l NAA (Nirmal Babu *et al.*, 1992b).

In *A. galanga* and *K. rotunda*, experiments proved that NAA was effective than IBA (Tabs: 9, 10 & Plate 5A, B). At 2.5 mg/l levels of NAA on MS full strength medium, *A. galanga* produced 10.8 roots per culture (Plate 5 A & Tab: 9). However, *K rotunda* shoot produced roots on ½ MS medium supplemented with 2.0 mg/l NAA, and produced nearly a dozen of roots/culture (Tab: 10 & Plate 5 B). The combined effect of NAA and IBA found to be less effective in *A. galanga* for rooting (Tab: 9).

For *A. galanga*, 2.5 mg/l NAA was optimum for maximum root induction (Tab: 9 & Plate 5 A). Illg & Faria (1995) found that 1.0 mg/l NAA along with MS medium was suitable for the induction of roots from *in vitro* raised shoots of *A. purpurata*. This is in agreement with the results obtained in *A. galanga* and *K. rotunda*. The superior effect of NAA for rooting was also explained on medicinal plants like *G. jasminoides* (George *et al.*, 1993), *C. ternatea* (Kumar *et al.*, 1993) and in *L. ovallei* (Lu *et al.*, 1995).

When the concentration of NAA increased from 2.5 mg/l to 3.0 mg/l, a slight callusing was also noticed in association with rooting

(Tabs: 9,10). The increased concentration of auxin in the medium showed a tendency to produce callus. This findings is in corroboration with the views of Hu and Wang (1983); Evans *et al.* (1983) and George (1993a). They also suggested that increased concentration of auxin in the medium lead to the production of callus from the explants. In contrast, to this research, Jain and Chaturvedi (1985) explained that the medium fortified with 3.0 mg/l IAA produced roots from *in vitro* raised shoots of *C. speciosus*, a *Zingiberaceous* member. Sharma and Chandel (1992) and Kukreja (1998) also explained the ineffectiveness of NAA and superior role of IAA in *T. indica* and *G. glabra*, respectively for rooting.

Regenerated shoots of *K. rotunda*, produced roots in shoot inducing medium containing 2.0 mg/l BAP + 0.5 mg/l IAA (Plate 4 C, & Tab: 6). This result is also in agreement with the findings of Rao *et al.* (1982); Ilahi and Jabeen (1987) and Balachandran *et al.* (1990) in ginger, Vincent *et al.* (1992) in *K. galanga*, Agretious *et al.* (1996) in *A. calcarata* and Sajina *et al.* (1997) in *A. subulatum*. All these reports proved the induction of roots in shoot inducing medium. This phenomenon was due to the root inducing factors which are intrinsic in the rhizomes of these plants, as such effect was reported only in rhizomatous species (Agretious *et al.*, 1996).

The experiment with *A. galanga* and *K. rotunda* proved that IBA was less effective for the induction of roots compared to NAA (Tabs: 9, 10). However, earlier reports in medicinal plants proved that IBA promotes rooting, *viz.*, in *P. rosea* (Harikrishnan and Hariharan 1996);

C. borivilianum (Purohit *et al.*, 1994) and in *G. elegans* (Komalavalli and Rao, 1997).

As shown in *K. rotunda,* basal medium had some effect on rooting from *in vitro* raised shoots. Earlier reports imply that basal medium with ½ MS and 2.0 mg/l NAA produced more number of roots (Tab: 10 & Plate 5 B). However, in *Z. officinale,* Nadgauda *et al.* (1980) used low salt medium (WH) for the induction of roots from *in vitro* raised plantlets.

In conclusion, this part of work proved that ½ MS with 2.0 mg/l NAA was suitable for *in vitro* rooting of *K. rotunda* and full strength MS with 2.5 mg/l NAA was better for rooting of *A. galanga* (Tabs: 9, 10 & Plate 5, A B).

V. 7. *Effect of Sucrose Concentration:*

Carbohydrate source in the medium play a vital role for the direct multiple shoot regeneration. For the tissue culture works, predominantly used carbohydrate source is sucrose (Murashige & Skoog, 1962; Hu & Wang, 1983). Carbohydrates like glucose and fructose also produced satisfactory results in plants (Ammirato, 1983; George & Sherrington, 1984).

As explained in Figs: 13, 14 and Plates 3 A - D., 4 A - C, 7 A-D, the regeneration capacity of *A. galanga* and *K. rotunda* vary with different percentage of sucrose used in the medium. This aspect proved that the optimum concentration of sucrose for the direct multiple shoot induction in both *A. galanga* and *K. rotunda* was 3% (Tabs: 5,6,7,8 & Plates 3 A-D., 4 A-C). When the sucrose level was

altered (1-5%) and tested for the multiple shoot induction from the explants of *A. galanga* and *K. rotunda,* it was found that sucrose level below or above 3% was not so good for both *A. galanga* and *K. rotunda* (Plate 7 A-D & Figs: 13,14).

Earlier reports on medicinal plants such as *R. micrantha* (Sudha & Seeni, 1996); *G. elegens* (Komallavalli & Rao, 1998) and *T. procumbense* (Sahoo & Chand, 1998) proved that 3% sucrose was optimum for the direct multiple shoot regeneration. However, in *A. purpurata,* (Chang & Criley 1993) and *Z. officinale* (Hosoki & Sagawa, 1977), it was found that 2% sucrose was best for the clonal multiplication of these plants.

According to Molnar (1988), optimum sucrose level should dependent upon the other amendments made to the basic medium formula. Sucrose plays an important role in differentiation of xylem and Phloem. According to Murashige and Nakano (1968), shoot morphogenesis is an energy required process, in which starch acts as a direct cellular energy reserve, needed for the morphogenisis. However, in the present study, the reduced effect of explants on lower levels of sucrose (1%) might be due to insufficiency of sucrose for starch synthesis and at a higher level (5%), it could be attributed to the inability of the explants to utilise the sucrose. However, as reflected by this forgoing discussion and observations, 3% sucrose in MS basal medium was more beneficial than a lower or higher percentage for the direct multiple shoot regeneration, in both *A. galanga* and *K. rotunda* (Figs: 13,14 & Plate 4 A-C, 7 A-D).

V. 8. *Effect of Different Nutrient Medium:*

Many medicinal plants grown on wide range of media formulations such as B5, MS, and SH, and thus appeared to be rather specific. However, many species like *A. calcarata* Agretious *et al.* (1996) and *Z. officinale* (Nadgauda *et al.,* 1980) showed a considerable quantity of growth variation, when cultured on different nutrient media. Earlier studies with *Zingiberaceous* members revealed that MS medium was more preferred to other media like B5, SH and WH (Kumar *et al.,* 1985; Balachandran *et al.,* 1990; Chang & Criley, 1993; Vincent *et al.,* 1991, 1992; Agretious *et al.,* 1996).

This general view was found to be applicable in the case of *A. galanga* and *K. rotunda* also (Figs: 11, 12 & Plate 4 A-C). Roy and Pal (1991) found that SH medium was better for the clonal propagation of *Costus speciosus.* Besides, Barthakur and Bordoloi (1992) used B5 medium for the clonal propagation of *Curcuma amada.* In fact, the WH medium showed least response in *A. galanga* and *K. rotunda* (Fig. 11, 12). This observation is in corroboration with the results of Agretious *et al.* (1996). They found that WH medium showed less effective, while experimenting with *A. calcarata,* because macro-nutrients (comparatively less in WH medium) are contributing the major role in the induction of multiple shoots (Agretious *et al.,* 1996). Thus, major reduction in the level of nutrients would adversely effect the multiple shoot induction from explants of *A. galanga* and *K. rotunda.* The major difference between B5, SH or WH with MS is the lower levels of macro nutrients like calcium, potassium and less NH salts in B5, SH or WH than in MS medium (Tab: 2).This piece of work proved that MS

medium without any alteration was superior to B5, SH, or WH media, for the induction of multiple shoots in both the plants.

Towards conclusion of the foregoing discussion, the successful protocol developed here for the DMSR of *A. galanga* and *K. rotunda* include the following stages; collection of explants of about 1.0 cm size, washing of explants with water containing 0.2% Dithane M - 45 and 5% detergents about 30 minutes, treatment with 0.1 - 0.15% fungicide about 15 minutes, treatment with 70% alcohol and final inoculation to medium containing antibiotic erythromycin and BAP + IAA, sub-culture of rooting of *in vitro* shoots; hardening and planting out.

V. 9. Indirect Shoot Multiplication:

V. 9.1. ***Callus induction:*** According to Greshoff (1978), induction of callus involves: initiation of cell division, continued proliferation of cells and subsequent structural and physiological differentiation. Callus formation in *A. galanga* and *K. rotunda* was influenced by the interaction of auxin and cytokinins (Tabs: 12, 13 & Plate 9 A-F). Low concentration of cytokinin along with high concentration of auxin induced sufficient quantity of callus from rhizome sprout discs of *A. galanga* and *K. rotunda* (Tabs: 12,13 & Plate 9 A-F). Auxin alone could induce only very little callus (Plate 8 A-D & Tabs: 12, 13).

The importance of auxin/cytokinin and their balance in the medium for callus induction were explained by Skoog and Miller (1957); Halperin (1986) and George (1993a). George (1993a) opined that auxin alone could induce callus in cultures of monocotyledons.

Similar effect was obtained by Nirmal Babu *et al.* (1996) and Kackar *et al.* (1993), while working with ginger.

Contrary to this in the present study, presence of NAA or 2, 4 -D alone in the medium produced water soaked callus (Plate 8 A-D & Tabs: 12, 13). The friable compact calli was produced on MS medium fortified with 2, 4 - D and BAP (Tabs: 12, 13 & Plate 9 A-F). It was in corroboration with the results of Vincent *et al.* (1992) in *K. galanga,* when MS medium was supplemented with 2,4-D + BAP. Likewise, 2,4-D – BAP combination induced callus in *C. fenestratum* cultures (Nair *et al.,* 1992). According to Hunault *et al.* (1989), cytokinin tends to increase the compactness and green colour of callus. In earlier report of Kirkham and Holder (1981), revealed that kinetin increased compactness of the callus which made them less liable to take up water from surrounding medium. The BAP could have exerted the same effect on to *A. galanga* and *K. rotunda* . However, Samsudeen (1996) found that 2,4-D -KN combination could produce friable compact callus in ginger. In another experiment with ovary tissues of ginger, Nirmal Babu *et al.* (1996) explained that the 2,4-D -BAP could induce friable and compact embryogenic calli.

In another combination of 3.0 mg/l NAA and 0.5 mg/l KN, *A. galanga* produced caulogenic callus (Tab: 12 & Plate 9, C-D). This observation was in consonance with the results of Kim *et al.* (1994), wherein they could induce caulogenic callus on MS medium containing 1.0 mg/l NAA + 0.1 mg/l KN. Remashree *et al.* (1994) observed callusing from *A. bracteolata* nodal explants, when it cultured on MS + KN + NAA + IAA.

From the above discussions, it is clear that a combination of 2,4-D (2.0 mg/l or 2.5 mg/l) and BAP (0.5 mg/l) on MS medium produced embryogenic callus from the explants of *A. galanga* and *K. rotunda*. From this discussion it is clear that the hormone 2,4-D is an essential factor for the induction of friable compact calli.

V. 9.2. ***Regeneration From Callus:*** According to Skoog and Miller (1957) and Narayanaswamy (1994), the auxin/cytokinin balance in the medium regulates the organogenesis from the callus. Narayanaswamy (1994) stated that it was possible to initiate direct organogenesis and embryogenesis of culture of responsive cell, by varying their chemical and physiological environments. Organogenesis and plant regeneration have been reported in many *Zingiberaceous* plants like cardamom from seedling callus (Rao *et al.*, 1992); from shoot bud callus of ginger (Nadgauda *et al.*, 1980; Malamug *et al.*, 1991; Ishida & Adachi, 1997) and from rhizome bud callus of *Curcuma* (Yasuda *et al.*, 1988; Anand & Hariharan, 1999).

As shown on Plate 9,C-D, 10 A-C and Tab: 14, calli transferred from high auxin- low cytokinin medium to low auxin- high cytokinin medium, resulted shoot organogenesis from the callus. Later, this was transferred to hormone free medium produced roots. This result is in consonance with the results of Sarasan *et al.*, (1994) and Sehgal and Abbas (1994). According to them, removal of auxin, along with the increase in the level of cytokinin in the medium was necessary to induce shoot organogenesis from callus cultures of *H. indicus* and *T. ammi*, respectively.

Addition of GA_3 to the multiplication medium resulted increase number of shoot induction from the callus (Plate 10 B & Tab: 14). The role of gibberellic acid with respect to shoot bud induction in medicinal plant species remains controversial. However, GA_3 - BAP coupling along with weak auxin (IAA) improved the shoot bud induction in C. *aromatica* (Anand & Hariharan, 1999). Similar reports are available on *G. elegans* by Komalavalli and Rao (1997); *Phytoloca dodecandra* by Demake and Hughes (1990). Very recently, Sahoo and Chand (1998) also reported the coupling of BAP-GA_3 for increasing the shoot bud regeneration in *Tridax procumbense.* However, according to Kartha *et al.* (1974) and Coumans Gilles *et al.* (1981), GA_3 showed inhibitory role of shoot induction in soybean and sugerbeet, respectively.

Though, GA_3 proved to have inhibitory effect in many plants, it also showed promotive effect in many medicinal plants (Komalavalli & Rao, 1997; Demake & Hughes, 1990; Sahoo & Chand, 1998). According to these present studies, addition of 2.0 mg/l GA_3 along with NAA and BAP produced increased number of shoot regeneration from *A. galanga* callus (Plate 10 B & Tab: 14).

V. 10. *Embryogenesis:*

Following the pioneering observations of Steward *et al.* (1958) and Reinert (1959) in carrot tissues, somatic embryogenesis had been reported in many plants species (Ammirato, 1971; Bapat & Rao, 1979; Kim *et al.*, 1994; Delbreil and Jullien, 1994; Rout & Das, 1994). Sharp *et al.* (1980) described the two routes for somatic embryogenesis, this may be either direct (without callus formation) or indirect (through

callus formation), where the callus are predetermined for embryogenesis.

As explained in *A. galanga* and *K. rotunda,* embryogenesis and plantlet formation were obtained when the callus transferred from high auxin-low cytokinin medium to low auxin-high cytokinin medium (Tabs: 12,13,15,14). Embryogenesis is being controlled by a delicate balance of growth regulators, especially 2,4-D was shown to have an effect in the early events of inducing somatic embryos (Ammirato, 1983; Nirmal Babu *et al.,* 1992,1996).

According to Mouras and Lutz (1980), the choice of medium also affects the embryogenic potential of the calli. In *A. galanga* and *K. rotunda,* embryogenesis was obtained on MS medium. Evans *et al.* (1981) reported that MS medium was suitable for the high frequency of embryogenesis. In *Zingiberaceae,* Nirmal Babu *et al.* (1996) and Kackar *et al.* (1993) also proved this, while working with ginger callus. Kim *et al.* (1994), and Gosh and Sen (1991) also found that MS medium was more preferable to other media for embryogenesis in *Q. acustisima* and *A. cooperi,* respectively. However, in *C. arietinum* direct embryogenesis was obtained on B5 medium supplemented with BA + 2,4 - D (Ramana *et al.* 1996).

In the present studies gradual elimination of 2,4 - D was necessary for the embryogenesis in both *A. galanga* and *K. rotunda.* It was found that initial embryogenic competence achieved because of unorganized growth of the callus in the presence of 2, 4 - D. It is necessary to remove 2, 4 - D from the medium or reduce its concentration to a considerable extend, when the callus achieved

embryogenic competence. This observation was in agreement with the reports of Vincent *et al.* (1992); Nirmal Babu *et al.* (1992, 1996) and Sarasan *et al.* (1994). The role of 2, 4 - D in somatic embryogenesis has been reported in many medicinal plants like *V zizanioides* (Muccierelli *et al.*, 1993); *Foeniculum vulgare,* (Mathew, 1993) and in Sugarcane, (Manickavasagam & Ganapathi 1998).

As explained in Plates 12, 13,14 and Tables: 12,13,15,16,17, calli of *A. galanga* and *K. rotunda* produced somatic embryoides, when such calli were transferred to a medium supplemented with 0.25 mg/l 2,4 - D + 3.0 mg/l BAP or KN. Later, the embryoides transferred to a medium containing cytokinin only, and subsequent transfer to a hormone free medium resulted the formation of full-fledged plantlets (Plates 12,13,14).

Different from *A. galanga,* when the *K. rotunda* callus originated on MS medium supplemented with 2.5 mg/l 2,4-D + 0.5 mg/l BAP transferred to a medium having 0.25 mg/l 2,4 - D + 3.0 mg/l BAP, resulted embryogenesis and caulogenesis simultaneously (Tab:16 & Plate 12 D,E). When this was sub-cultured on to a medium having 6.0 mg/l BAP, resulted in the maturation and conversion of somatic embryos into plantlets and simultaneous development of shoots from the callus. This pattern of callus growth was noticed by Samsudeen (1996), while working with ginger callus. This result is in agreement with the view of Narayanaswamy (1994). To him, callus originated in high auxin-low cytokinin medium produces, organogenesis or embryogenesis, when it transferred to a medium having low auxin-high cytokinin or auxin free medium. This view has been strengthened

by the reports of Subhadra *et al.* (1995) in *T. eastivum* and Stefaniak *et al.* (1994) in *Gladiolus hort* and Nataraja and Neelambika (1996) in *Punica granatum*.

The efficiency of embryogenesis was very less in both the species under this investigation (Tabs: 15 & 16). Mostly, the embryo failed to develop further. The maturation of embryo should be controlled by the auxin-cytokinin balance, and the concentration of protein present in the culture medium (Altman *et al.,* 1990). As revealed by the present experiments, less frequency of embryogenesis may be due to the hormonal imbalance and less amount of protein in the medium, which may cause the decreased mitotic division during the development of embryo.

Nirmal Babu *et al.* (1996) developed a procedure for embryogenesis in ginger which involved: callus induction in 2,4-D enriched medium; gradual elimination of 2,4-D by repeated subculture; and final subculture to hormone free medium. The protocol in the present work involved - induction of embryogenic callus on high auxin-low cytokinin medium; transfer of this callus to low auxin - high cytokinin medium; subculture to the medium containing cytokinin alone; and subsequently to hormone free medium, finally hardening and field transfer to pots (Tabs: 12,13,15,16,17 & Plate 12,13,14).

V. 11. ***Deflasking and Field Establishment:***

Murashige (1974) suggested that the successful rooting and transfer of plantlets from auxinic cultures to soil is one of the most

difficult and neglected technical problems of *in vitro* propagation research. Generally, plantlets from sterile cultures are transferred to sterile or non-sterile medium (soil). Desiccation due to the absence of waxy cuticle or open stomata, are some major problems when introducing the plantlets into new environments (Brainerd & Fuchigami, 1982; Donnelly & Vidaver, 1984).

Plantlets developed *in vitro* are adopted to the environmental conditions, prevailed which are *in vitro*. They have to be readopted to *ex vitro* condition, prior to their successful establishment to outside. In *in vitro* propagation research, many plantlets do not acclimatize easily (Murashige, 1974; Ammirato, 1983; Donnelly & Vidaver, 1984). In present study, plantlets produced by direct multiple shoot regeneration were survived in 70% (*A. galanga*) and 80%(*K. rotunda*), respectively (Tab: 11 & Plate 6) (Anand & Hariharan, 1997; Anand *et al.*, 1997). However, 70% of the plantlets from both the plants produced by caulogenesis were survived (Plate 11 A, B & 15 A, B). Again, the survival percentage was highly decreased in the case of plantlets produced by embryogenesis; only 10-20% of survival was noticed (Tab: 17 & Plate 14 A-D). However, there are reports, which showed 80 – 90% of survival of plantlets (Nirmal Babu *et al.*, 1992; 1996.,Balachandran *et al.*, 1990; Sajina *et al.*, 1997). The variation of survival percentage may be due to the difference in the age of plant, time of transfer, number of roots present at the time of transfer, culture conditions, *etc* during deflasking.

V. 12. *Histological Studies:*

Through histological studies, it is possible to understand the origin of embryos whether from single cell or group of cells (Haccius, 1978). In *Zingiberaceae,* only one or two reports were available about the histological studies on callus (Vincent *et al.,* 1992 & Kackar *et al.,* 1993).

As explained in Plate 16 A, rounded structures were protruded from the peripheral regions of the embryogenic callus. This round bodies are surrounded by a prominent epidermal layer proved that as an embryo with globular stage. Same type of embryo formation was observed by Vincent *et al.* (1992) in *K. galanga* callus. From the Plate 16 B, it is understood that the embryo produced both root and shoot poles simultaneously. As explained in the Plate 16 C, the shoot buds are originated from the sub epidermal regions of the callus, and may be due to the seating of meristamatic regions on the sub epidermal regions of the callus.

V. 13 . *Synseeds:*

Synseeds obtained by encapsulating globular embryos or shoot buds were considered as an important application in conservation of *in vitro* raised embryos or propagules. Synseed production was first reported by Redenbaugh *et al.* (1984) in Celery. Subsequently, this method was successfully employed by many workers, as reported in many medicinal plants like, *Zingiber* and cardamom (Ganapathy *et al.,* 1994; Ravidran *et al.,* 1996). Other reports regarding medicinal plants includes, Corrie and Tandon (1993) in C. *giganteum,* Gosh and Sen

(1994) in *Asparagus cooperi* and Nayak *et al.* (1998). in *Spathoglottis plicata*.

Synseeds produced by entrapping globular embryos or shoot tips of both *A. galanga* and *K. rotunda* in 3% sodium alginate gel prepared in MS without calcium chloride (Plate 17 A, B & Tab: 18). Sharma *et al.* (1994) reported the production of disease free encapsulated buds of ginger in 4% sodium alginate. However, in the present study the 3% sodium alginate was more suitable for medium textured bead production out of *A. galanga* and *K. rotunda*. As shown in Plate 17 C, the synseeds of *A. galanga* showed signs of germination within two weeks, when it cultured on hormone free MS medium. The prepared synseeds could be stored in refrigerator upto 30 days without loss of its viability.

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CHAPTER VI

SUMMARY AND CONCLUSION

India with a large reservoir of medicinal and aromatic plants, is a versatile botanical garden of the world. Medicinal plants are extensively being used and over exploited, there by threatening not only their bio-diversity but extinction as well, and indiscriminate human encroachments like overexploitation, deforestation of tropical, coupled with temperate forests on large scale, soaring international trade of raw material of herbal medicines *etc.*, have contributed much to this phenomena.

A balance between ever-increasing demand for herbal medicines and their actual supply show great difference. Except a few cultivated plants, most of the medicinal plants are collected from the wild. Due to such unscrupulous and unscientific collection of medicinal herbs, some of the most valuable plant species are at the verge of extinction. In fact, along with such plants, most important medicinal plants like *Alpinia galanga* and *Kaempferia rotunda* of *Zingiberaceae* have also got place in the list of endangered plants. Considering this aspect in to account, these two medicinal plants of the family *Zingiberaceae,* have been chosen for the present study for their large scale multiplication by means of direct multiple shoot induction, and indirect shoot regeneration through callus induction and organogenesis. Direct multiple shoot induction, callus induction and embryogenesis could be achieved by judicious adjustment of the hormonal balance during the different steps, which involved in the tissue culture protocol. The major objectives of the present study are summarised below:

VI . 1. *Objectives:*

- To select suitable explant (s) of *A. galanga* and *K. rotunda,* for further *in vitro* studies.
- To standardise a reproducible and easy procedure for the sterilisation of the explants for its maximum survival on the culture medium.
- To select suitable standard medium for further culture studies.
- To standardise most suitable cytokinin for the direct shoot multiplication of *A. galanga* and *K. rotunda.*
- To standardise most suitable combinations of auxin-cytokinin to induce maximum number of shoots .
- To standardise the best auxin for the *in vitro* rooting of shoots, obtained by direct or indirect methods.
- To standardise the most suitable concentration of sucrose for the maximum frequency of shoot multiplication.
- To find out the maximum percentage of field establishment rate of the plantlets produced by direct or indirect multiple shoot induction.
- To standardise suitable hormone combinations for the maximum callus induction from sprout discs of *A. galanga* and *K. rutanda* .
- To induce caulogenesis and rhizogenesis from the calli.
- To induce somatic embryogenesis from the calli.
- To standardise the maximum establishment of plantlets on to the field, produced by somatic embryogenesis.
- To study the various stages of developments of somatic embryos by histological methods.

- To standardise best formula for the preparation of calcium alginate beads for synseed production.
- Popularisation of the protocol developed and transfer technology from the lab to field.

VI . 2. *Methods Adopted:*

Major methods adopted for the successful completion of this work are summarised below:

- Explants like sprout bud, sprout discs, flower buds, leaf discs, root tips, *etc.* with different size and age were tested for their best performance on the culture medium.
- For sterilisation, detergents like teepol, soap water, and extran were used with different concentrations and period of treatment.
- For the disinfecting of explants the fungicides - like $HgCl_2$ solution, Dithane M- 45, Ethanol (70%) and antibiotic like erythromycin or ampicillin were used with different concentrations and period of treatment.
- Different nutrient media like, B5, SH, MS and WH were tested for their efficiency for supporting growth and multiplication of the explants.
- Different concentration of sucrose were tested for its effects on direct multiple shoot induction.
- Effects of different cytokinins like, BAP, KN and CW were tested for the induction of multiple shoots.
- Action of cytokinin- auxin coupling was tested for the induction of multiple shoots.

- For the induction of callus, effects of different auxins alone and auxin-cytokinin combinations were tested.
- For the induction of somatic embryos and caulogenesis from the callus, the medium was supplemented with various levels of hormones.
- Effective methods for the successful hardening and field transferring of *in vitro* raised plantlets through both direct and indirect methods were investigated.
- For the histological studies, callus were fixed in FAA and further sectioning and staining were performed.
- Experiments were conducted for the immobilisation of embryoids/buds in calcium alginate beads with suitable texture.

VI. 3. *Main Outcomes of the Present Study:*

Much promising results were obtained in this study, in fact, many of them are new in literature. Noteworthy and reproducible outcomes are being summarised here:

- The size of the explants for the effective direct multiple shoot regeneration from sprout buds was proved as ~ 1.0 cm.
- The suitable age of the explants was found as two months old for *A.galanga* and three months old for *K.rotunda,* after maintaining the rhizomes in trays containing sand bed.
- In both cases, for the surface sterilisation, initial treatment of the explants with 0.2% Dithane M - 45 and 5% extran for 30 minutes followed by 0.1 to 0.15% mercuric chloride solution for 10-15 minutes, 70% ethanol treatment for 15-20 seconds and final inoculation to the medium containing antibiotic erythromycin yielded maximum survival of the explants in the culture medium.

- MS medium supplemented with 3% sucrose was suitable for the direct multiple shoot induction in both the plants.
- Among different media used, MS medium was better to others for multiple shoot induction.
- For multiple shoot induction, different cytokinin-auxin combinations tried, MS medium supplemented with 0.5 mg/l IAA + 1.0 mg/l BAP (for *A.galanga*) and 0.5 mg/l IAA + 2.0 mg/l BAP (for *K. rotunda*) was found to be successful. Within 40 days an average number of 6.9 and 8.0 shoots were produced in *A. galanga* and *K. rotunda*, respectively.
- For *in vitro* rooting, MS full-strength medium supplemented with 2.5 mg/l NAA (*A.galanga*) and ½ MS supplemented with 2.0 mg/l NAA (*K.rotunda*) were found to be optimum. Within 20 days a dozen of roots were produced in both the plants.
- Plantlets produced by direct multiple shoot induction were survived at 80 and 70 percent respectively, in *A. galanga* and *K. rotunda.*
- Among different explants used sprout discs of size ~1.0 cm^3 responded very well and within 10 to 15 days the callus induction was obtained in both the plants.
- O.5 mg/l BAP + 2.0 mg/l 2, 4 - D was optimum for the induction of embryogenic callus from *A. galanga* and that of *K. rotunda* on MS supplemented with 0.5 mg/l BAP and 2.5 mg/l 2,4-D.
- Caulogenic callus obtained from *A.galanga* on MS supplemented with 0.5 mg/l KN + 3.0 mg/l NAA. Within 25 to 30 days moderate callus formation was obtained.
- A combination of 0.25 mg/l NAA + 3.0 mg/l BAP induced shoots from the callus of *A.galanga.*

- Addition of 2.0 mg/l GA_3 into the above medium enhanced the rate of shoot formation in *A. galanga* and resulted an average number of 22.5 shoots per culture within two weeks of incubation.
- 70% of the plantlets produced by the indirect method was survived in the field in both the plants.
- For the maturation and development of plantlets from somatic embryos, complete removal of 2,4-D from the medium was necessary.
- Caulogenesis and embryogenesis occurred simultaneously within 10 days when two months old callus, of *K. rotunda* transferred from a medium having 2.5 mg/l 2, 4 - D + 0.5 mg/l BAP to a medium containing 0.25 mg/l 2, 4 - D + 3.0 mg/l BAP.
- In *K.rotunda* plantlets produced by caulogenesis is showed 70% survival.
- Plantlets produced by somatic embryogenesis was survived 10% (*A.galanga*) and 20% (*K.rotunda*), respectively.
- Globular embryos/shoot buds can be encapsulated in calcium alginate beads.
- Among the different concentrations of sodium alginate used, 3% sodium alginate in MS medium without calcium chloride was better.
- Among the different concentrations of calcium chloride solution used, 100 μM solution was found to be suitable for the medium textured bead formation.
- Entrapped beads showed sign of germination on hormone free MS medium after two weeks of incubation.
- Longitudinal section of the embryo showed the development of shoot and root poles.

- Longitudinal section of the caulogenic callus showed the development of shoot apex.

VI. 4. *Conclusion:*

Based upon the objectives stated elsewhere, a comprehensive attempt has been made to materialise such ideas to be used in recurrent ways. As cited in literature, *in vitro* multiplication of Greater Galongal, *in vitro* prorogation of *K.rotunda,* propagation of medicinal plants, *etc.* are the first ever reports on these medicinal plants. The protocols developed for such commercially feasible procedures could easily be adopted for the large scale multiplication of these plants with a view to sustainable preservation and eco- friendly exploitation for the wellbeing of the society. Moreover, such protocols are very simple to be instructed and practised even for a layman.

VI. 5. *Scope and Limitations:*

Exploitation of secondary metabolites and molecular characterisation of valuable medicinal plants are least exploited thrust areas of active research in Indian scenario. Initiatives have been taken up to proceed further to reach at these goals. Extraction of secondary metabolites in refined and pure form could be possible with this new initiatives, that would pave avenues for open competition with the multi-nationals, who plunder our valuable resources by elicit means, an indiscriminate way. Further, molecular characterisation could be an authoritative document to overcome the bottlenecks of much debated international patent laws and stealing of one' s on natural resources.

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APPENDIX

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1. Anand, P.H.M. and Hariharan, M (1997). *In vitro* multiplication of Greater Galongal [*Alpina galanga* (Linn) willd] - A medicinal plant. *Phytomorphology* **47**: 45-50.

2. Anand, P.H.M, Harikrishnan, K.N., Martin, K.P. and Hariharan, M (1997). *In vitro* propagation of *kaempferia rotunda* Linn. "Indian Crocus" - A medicinal plant. *Phytomorphology* **47** : 281- 286.

3. Harikrishnan, K.N., Martin, K.P., Anand, P.H.M. and Hariharan, M (1997). Micropropagation of sweet flag (*Acorus calamus*). *Journal of Medicinal and Aromatic Plant Science* **19**: 427- 429.

4. Anand, P.H.M. and Hariharan, M (1998). Propagation of medicinal plants. *Science Express*, May 05 : P. 5.

5. Harikrishnan,.K.N., Hariharan.M., Anand, P.H.M. and Martin, K.P (1998). - Multiple shoot regeneration and somatic embrygenesis from *Saraca asoka* (Roxb.) De willde - A medicinal tree legume. Abst. In plant Biotechnology and *in vitro* - Biology in the 21st century. *IX International Congress On Plant Tissue and Cell Culture*, Jerusalem, Israel.

6. Anand, P.H.M. and Thomas, E (1999). Medicinal plants from Laboratary. *Karshika Madyamam*. Jan 12 : P. 8.

7. Anand, P.H.M. and Hariharan, M (1999). *In vitro* plant regeneration from rhyzome-bud derived callus in yellow Zedoary (*Curcuma aromatica salisb)* - A medicinal plant. In: *Plant Tissue Culture and Biotechnology EmergingTrends*, P.B. Kavi kishor (ed.); PP. 187-192. University press (India) Ltd. Hyderabad, India

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