

CELL TISSUE AND ORGAN CULTURE IN SOME IMPORTANT MEDICINAL PLANTS

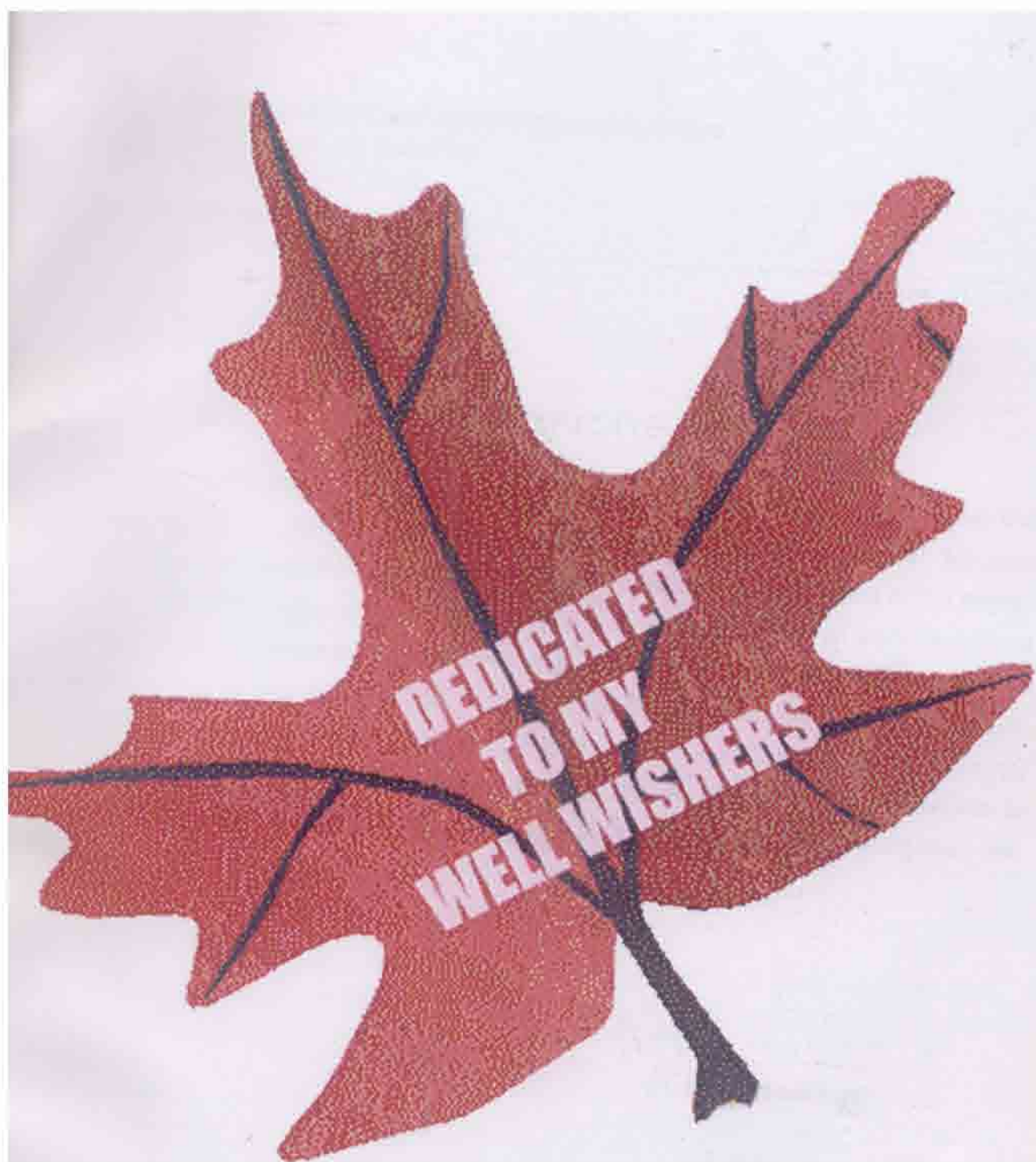
*A Thesis
Submitted to the
Bidhan Chandra Krishi Viswavidyalaya
For the award of the Degree of Doctor of philosophy
In
Genetics*

By

Moumita Chakraborty



**DEPARTMENT OF GENETICS
FACULTY OF AGRICULTURE
BIDHAN CHANDRA KRISHI VISWAVIDYALAYA
MOHANPUR, NADIA
WEST BENGAL**





Bidhan Chandra Krishi Viswavidyalaya

Department of Genetics

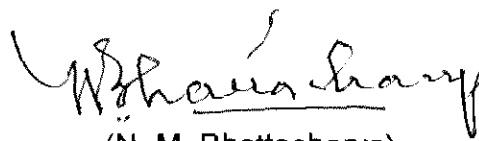
Mohanpur, Nadia, West Bengal

Prof. N. M. Bhattacharya

Date 08. 03. 2007

CERTIFICATE

This is to certify that the work recorded in this thesis entitled "Cell, Tissue and Organ culture in some important medicinal plants" submitted by Ms. Moumita Chakraborty to the Bidhan Chandra Krishi Viswavidyalaya, for the award of the degree of Doctor of Philosophy in Genetics, is faithful and bona fide research work carried out by her under my supervision and guidance. The thesis is, in my opinion, worthy of consideration for the award of the degree of Doctor of Philosophy in accordance with the regulations of the Viswavidyalaya. The results embodied in this thesis have not been submitted to any other University or Institution for the award of any degree or diploma. The assistance and help received during the course of investigation and source of literature have been fully acknowledged.


(N. M. Bhattacharya)

Acknowledgement

In this auspicious moment, I would like to convey my thanks to those without whom my work would have remained goal-less. Primarily, I pay tribute and my kind homage to the 'Almighty'.

I appraise my deep sense of gratitude and indebtedness to my cogent, perspicacious and reverend chairman of my Research Advisory Committee Prof. N. M. Bhattacharya, Department of Genetics, Faculty of Agriculture, Bidhan Chandra Krishi Viswavidyalaya, for his unequivocal motivation, benevolent guidance, affectionate encouragement, valuable discussions and constant supervision during the entire period of the present investigation, which helped me to execute the whole programme of my dissertation. His indefatigable spirit shall always be my panacea.

It is a gratifying moment to convey my sincere gratitude and heartfelt thanks to Prof. S. K. Ghose, Head, Department of Genetics, for his valuable suggestions, co-operation and constructive ideas throughout the pursuit of my present work. His tireless help and suggestions during the entire course of this work as member of the Research Advisory Committee are also duly acknowledged.

I offer my special thanks to Prof. H. K. Sarkar, Ex-Head, Department of Genetics for his constant support, encouragement and suggestions. My sincere thanks are due to all my teachers, with special mention of Prof. P. K. Das, Prof. B. G. Sasmal, Prof. K. Pradhan, Prof. (Ms.) F. Hossain, Prof. S. Bandyopadhyaya, Dr. P. K. Mondal and Dr. S. Bhattacharya. I also duly acknowledge the support and help extended by the non-teaching staff members of the Department.

It is a great pleasure to record my thanks to Prof. P. K. Bandyopadhyaya, Head, Department of Plant Physiology for extending his thoughtful and unhesitating suggestions as member of my Research Advisory Committee.

I would also like to offer my appreciation and respectful thanks to Dr. N. Mandal, Reader & Head Department of Bio-technology, Instrumentation and Environmental Science for his constant support and helpful suggestions as member of the Research Advisory Committee and also providing me the opportunity to carry out this work in his laboratory.

I remain grateful to Dr. D. Majumdar, Reader & Head, Department of Agricultural Statistics for his help and guidance in computing the data for statistical analyses.

Sincere thanks are conveyed to Prof. Anjan Bhattacharya, Officer in Charge, central sector scheme on Development of Medicinal and Aromatic Plants (GOI) for

allowing me to get plant materials as and when required for the present work from the Medicinal Plant Garden and his appropriate unreserved help during my entire study.

I am extremely grateful to Honorable Vice-chancellor, the Registrar, the Dean, PG Studies and the Dean, Faculty of agriculture, Bidhan Chandra Krishi Viswavidyalaya, for giving me the opportunity for carrying out my dissertation smoothly. The facilities provided by the Central Library and ARIS cell are also gratefully acknowledged.

I am thank full to Biswanath Da , Harti Da, Krishna Da, Alok Da, Anuradha Di and other staff of the laboratory of Biotechnology, Instrumentation and Environmental Science, B.C.K.V. for their endless help in field as well as in the laboratory work to complete my research study.

I remain grateful to Prof. (Ms.) Shampa Das, PMCG, Bose Institute, Kolkata for her affectionate help and cooperation towards availing Library and technical facilities in Bose Institute.

A chalice of thanks to Sudipto, Nassim, Avijit, Tirthankar, Anamika, Mr. Krishnendu Chattopadhyay, Mr. Srijib Panda, Mr. Sutanu Sarkar who have helped a lot and encouraged me in every other way during the course of investigation.

I convey my sincere thanks to Madhumita, Suparna, Jayeeta, Samapika, whose company kept me lively through out the tenure of my research work.

I express my profound thanks to my loving juniors Suparna, Sukonya, Anchala, Nandini for their immense help.

A word of appreciation far my special friends Arpita and Sreela for their encouragement and constant inspiration which helped me to proceed to the completion of my work.

Words are not sufficient for me to express my indebtedness and owe my unlimited gratitude to my beloved parents for their most affectionate encouragement, selfless devotion, endurance and blessing throughout my carrier.

I am really grateful and never would I forget to extend my sincere and lovable thanks to my husband Mr. Turbasu Dutta and all the family members for their encouragement and endless support.

I cannot forget to express my deep sense of love and affection to my didiibhai, dada and sister.

*Date: the 7th day of March, 2007
BCKV, Mohanpur.*

*Moumita Chakraborty
(Moumita Chakraborty)*

APPROVAL OF EXAMINERS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN GENETICS

We the undersigned, having being satisfied with the performance of Ms. Moumita Chakraborty, in the *vive-voice* examination, conducted today the 27th Nov., 2007, recommended that the thesis be accepted for the award of degree.

Name	Designation	Signature
<u>Subhas Kumar Hazm</u>	External Examiner	<u>Subhas Kumar Hazm</u> 27/11/2007
Prof. N.M. Bhattacharya	Chairman (Advisory Committee)	<u>W. Bhattacharya</u> 27.11.2007
Prof. S.K. Ghose	Member (Advisory Committee)	
Dr. Nirmal Mandal	Member (Advisory Committee)	
prof. P.K. Bandopadhyaya	Member (Advisory Committee)	<u>P.K. Bandopadhyaya</u> 27.11.07
<u>Prof. H.K. Saha</u>	Departmental nominee	<u>Prof. H.K. Saha</u> 27.11.2007
.....	Head	

Content

Chapter	Page
I. INTRODUCTION	1-5
II. REVIEW OF LITERATURE	6-24
III. MATERIALS AND METHODS	25-42
3.1. Experimental Materials	
3.1.1 Cultivars/Clones used	25-27
3.1.2 Source of the material	
3.2. Methods	
3.2.1 Mode of culture	28
3.2.2 Media constituents	28-31
3.2.2.1 Inorganic Salts	28
3.2.2.2 Vitamins	29
3.2.2.3 Growth Regulators	30
3.2.2.4 Amino Acids	31
3.2.2.5 Carbon Source	31
3.2.2.6 Inorganic Supplements	31
3.2.3 Composition of various standard media used and their stock preparation.	32-37
3.2.3.1 Preparation of stock solution	35-37
3.2.4 Preparation of MS semi- solid media	38
3.2.5 Preparation of N ₆ semi- solid media	39
3.2.6 Preparation of B ₅ semi- solid media	40
3.2.7 Preparation of liquid media	41

3.2.8	Sterilization of media and preparation of slants & flats	41
3.2.9	Composition of Antioxi (Murashige and Skoog 1962)	41
3.2.10	Aseptic preparation of plant material for <i>in vitro</i> culture	41
3.2.11	Inoculation of the explants on the medium	42
3.2.12	Physical conditions provided for culture	42
3.2.13	Statistical analysis of data	42

IV. MICROPROPAGATION IN SAFED MUSLI **(*Chlorophytum bovilianum*)** 43-90

4.1	Initiation of culture and <i>in vitro</i> response of rhizome sections of Safed Musli (<i>Chlorophytum borivilianum</i>) to different nutritional combinations	43-52
4.1.1	Rationale	43
4.1.2	Methodology	45
4.1.3	Result and Discussion	46
4.2	Study on the morphogenetic response of preformed microshoots regenerated from rhizome explants of Safed Musli (<i>Chlorophytum borivilianum</i>)	53-58
4.2.1	Rationale	53
4.2.2	Methodology	53
4.2.3	Result and Discussion	54
4.3	Morphogenetic response of embryoids of <i>Chlorophytum borivilianum</i> following their culture on B₅ medium supplemented with varying IBA - BA combination.	59-65

4.3.1	Rationale	59
4.3.2	Methodology	59
4.3.3	Result and Discussion	60
4.4	Indirect regeneration of microplants from intervening granular calluses of <i>Chlorophytum borivillianum</i>	66-71
4.4.1	Rationale	66
4.4.2	Methodology	66
4.4.3	Result and Discussion	67
4.5	Effect of Gibberellic acid (GA₃) on the production and growth of <i>Chlorophytum</i> microshoots cultured <i>in vitro</i>	72-78
4.5.1	Rationale	72
4.5.2	Methodology	72
4.5.3	Result and Discussion	73
4.6	Multiple shooting in germinating shoot base in <i>Chlorophytum borivillianum</i>	79-84
4.6.1	Rationale	79
4.6.2	Methodology	79
4.6.3	Result and Discussion	80
4.7	Modifications in media constituents to scale-up <i>in vitro</i> production of multiple shoots in Safed Musli (<i>Chlorophytum borivillianum</i>)	85-90
4.7.1	Rationale	85
4.7.2	Methodology	85
4.7.3	Result and Discussion	86

V.	Micropropagation in Ekangi (<i>Kaempferia galanga</i>)	91-103
5.1	Initiation of culture and <i>in vitro</i> response of rhizome sections of Ekangi (<i>Kaempferia galanga</i> L.) to different nutritional combinations	91-96
5.1.1	Rationale	91
5.1.2	Methodology	92
5.1.3	Result and Discussion	93
5.2	Effect of different BA-NAA combinations on <i>in vitro</i> morphogenetic response of the isolated microshoots and adventitious shoot bud production in <i>Kaempferia galanga</i> L.	97-103
5.2.1	Rationale	97
5.2.2	Methodology	97
5.2.3	Result and Discussion	98
VI.	Micro-cloning in Kaculus (<i>Atalantia malaberica</i>)	104-127
6.1	Initiation of culture and <i>in vitro</i> response of seed embryo with cotyledon explants of <i>Atalantia malaberica</i> to different nutritional combinations	104-111
6.1.1	Rationale	104
6.1.2	Methodology	105
6.1.3	Result and Discussion	106

6.2	Multiple shoot culture from nodal segments of young <i>Atalantia malabérica</i> plants	112-120
6.2.1	Rationale	112
6.2.2	Methodology	112
6.2.3	Result and Discussion	113
6.3	Anther culture studies in <i>Atalantia malabérica</i>	121-127
6.3.1	Rationale	121
6.3.2	Methodology	121
6.3.3	Result and Discussion	122
VII.	Summary And Conclusion	128-140
	Bibliography	i-ix

Lists of Tables

Lists	Pages
3.2.1 constituents of inorganic salts	29
3.2.2 constituents of vitamins	30
3.2.3 List of growth regulators used	30
3.2.4 Inorganic macro nutrients present in the three media used in the present study	32
3.2.5 Inorganic micro nutrients present in the three media used in the present study	33
3.2.6 Organic micro nutrients present in the three media used in the present study	34
3.2.7 Carbon source and gelling agents used	34
3.2.8 Preparation of stock solution for MS medium	35
3.2.9 Preparation of stock solution for B ₅ medium	36
3.2.10 Preparation of stock solution for N ₆ medium	37
4.1.1 Relative response of various sterilization treatments followed to obtain clean rhizome cultures of <i>Chlorophytum borivilianum</i>	50
4.1.2 Response of rhizome sections of <i>Chlorophytum borivilianum</i> supplemented with different combinations of growth regulators after 6 weeks of culture.	51
4.1.3 Response of rhizome sections of <i>Chlorophytum borivilianum</i> to B ₅ medium supplemented with different auxin – cytokinin combinations after 6 weeks of culture	52

4.2.1	Morphogenetic response of isolated microshoots of <i>Chlorophytum borivillianum</i> regenerated directly on the rhizome section after six weeks of their transfer to MS medium supplemented with different hormone combinations	57
4.2.2	Homogeneous subsets of Morphogenetic response of height of isolated microshoots of <i>Chlorophytum borivillianum</i> regenerated directly on the rhizome section	58
4.2.3	Homogeneous subset of Morphogenetic response of no of leaves <i>Chlorophytum borivillianum</i> regenerated directly on the rhizome section	58
4.3.1	Morphogenetic response of embryoids of <i>Chlorophytum borivillianum</i> following their culture on B ₅ medium supplemented with varying IBA - BA combinations as observed following 4 weeks of culture Homogeneous subsets of morphogenetic response of embryoids of <i>Chlorophytum borivillianum</i>	64
4.3.2	Homogeneous subsets of morphogenic response of embryoids of <i>Chlorophytum borivillianum</i>	65
4.4.1	Response of friable granular callus of <i>Chlorophytum borivillianum</i> subcultured on MS medium supplemented with varying concentrations of IBA-BA and IBA-KIN combinations after six weeks of culture.	70
4.4.2	Homogeneous sub sets of the response of friable granular callus of <i>Chlorophytum borivillianum</i> after 6 weeks of culture	71
4.5.1	Effect of Gibberallic acid (GA) on the production and growth of <i>Chlorophytum</i> microshoots cultured <i>in vitro</i> after 8 weeks of culture	76

4.5.2	Homogeneous sub sets of the effect of gibberellic acid on no of microshoot production of <i>Chlorophytum borivillianum</i> after 8 weeks of culture	77
4.5.3	Homogeneous sub sets of the effect of gibberellic acid on number of large shoot production in <i>Chlorophytum borivillianum</i>	78
4.5.4	Homogeneous sub sets of the effect of gibberellic acid on no of leaves of <i>Chlorophytum borivillianum</i> after 8 weeks of culture	78
4.6.1	Multiple shooting in young shoot base culture of Safed Musli (<i>Chlorophytumborivillianum</i>) after 4 weeks of inoculation of decapitated shoot bases in MS medium	83
4.6.2	Homogeneous sub sets multiple shooting in young shoot base culture of Safed Musli (<i>Chlorophytum borivillianum</i>) after 4 weeks of inoculation of decapitated shoot base	84
4.7.1	Effect of inorganic and organic constituents of MS medium in the production of multiple shoots.	90
4.7.2	Homogeneous subsets of effect of inorganic and organic constituents of MS medium	90
5.1.1	Initial response of rhizome section of <i>Kaempferia galanga</i> <i>in vitro</i> in MS medium supplemented with different growth regulator combinations	96
5.2.1	Effect of different BA-NAA combination in the MS medium in the production of multiple shoots on rhizome sections <i>in vitro</i>	102
5.2.2	Homogeneous sub sets of the effect of different BA-NAA combination in the MS medium in the production of adventitious buds on rhizome sections <i>in vitro</i>	103

6.1.1	<i>In vitro</i> response of seed embryo with cotyledon explants (EWC) of <i>Atalantia malabérica</i> to various growth regulator combinations cultured on MS medium	109
6.1.2	Homogeneous subsets of <i>in vitro</i> response of EWC explants of <i>Atalantia malabérica</i> to various growth regulator combinations cultured in MS medium	110
6.1.3	Isolated microshoots cultured on MS medium supplemented with various growth regulators combinations	111
6.2.1	<i>In vitro</i> response of nodal explants of <i>Atalantia malabérica</i> in MS medium supplemented with different combinations of growth regulators as recorded after 10 weeks of culture	118
6.2.2	Homogeneous subsets for <i>in vitro</i> response of nodal explants of <i>Atalantia</i> in MS medium supplemented with different combinations of growth regulators	119
6.2.3	<i>In vitro</i> culture of isolated micro shoots of nodal region on various nutritional combinations after 6-8 weeks of culture	120
6.3.1	Response of <i>Atalantia malabérica</i> anthers cultured in N ₆ medium supplemented with various growth regulator combinations	126
6.3.2	Subculture of anther derived callus with low level of Auxin-Cytokinin combinations both in agar gelled and without agar (liquid) ½MS medium	127

List of Plates

- 3.1 Safed Musli (*Chlorophytum borivillianum*)
- 3.2 Ekangi (*Kaempferia galanga*)
- 3.3 Kacilus (*Atalantia malaberica*)
- 4.1 Response of rhizome sections of *Chlorophytum borivillianum* to MS / B₅ medium supplemented with different combinations of growth regulators
- 4.2 Regeneration *in vitro* through multiple shooting in the rhizome sections of Safed Musli (*Chlorophytum borivillianum*)
- 4.3 Regeneration *in vitro* through direct embryogenesis in the rhizome sections of Safed Musli (*Chlorophytum borivillianum*)
- 4.4 Indirect regeneration of microplants from intervening rhizome calluses of *Chlorophytum borivillianum* in MS medium
- 4.6 Multiple shooting in shoot apex culture of Safed Musli (*Chlorophytum borivillianum*)
- 5.1 Regeneration of whole plants of Ekangi (*Kaempferia galanga*) *in vitro* from rhizome sections
- 5.2 Regeneration of whole plants of Ekangi (*Kaempferia galanga*) *in vitro* from rhizome sections
- 6.1 Seed embryo culture for micropropagation in Kacilus (*Atalantia malaberica*)
- 6.2 Multiple shoot culture of *Atalantia malaberica* from nodal sections *in vitro*
- 6.3 Anther culture in Kacilus (*Atalantia malaberica*)

Abbreviations used

MT	Murashig and Tucker
MS	Murashig and Skoog
B ₅	Gamborg
N ₆	Nitsch and Nitsch
mg l ⁻¹	Miligram per Litre
g l ⁻¹	Gram per Litre
Sp.	Species
NAA	1- Naphthalene acetic acid
BAP	6-Benzyl amino purine
IBA	Indole butyric acid
BA	6- Benzyl adenine
GA ₃	Gibberellic acid
2,4-D	2,4- Dichloro phenoxy acetic acid
IAA	Indole acetic acid
Kin	Kinetin
2ip	2-Isopentanyl adenine
° C	Degree centigrade
RH	Relative Humidity
pH	Hydrogen ion concentration
yrs	Years
hrs	Hours
lx	Lux
%	Percentage
mm	Millimeter
ml	Millilitre
cm	Centimeter
μM	Micromole
mM	Millimole
v/v	Volume per volume
NaCl	Sodium chloride
ppm	Parts per million
CWE	Cotyledon with embryo
CWOE	Cotyledon without embryo
cv	Cultivar
EDTA	Ethylene diamine tetra acetic acid
F	Variance ratio
SE _m	Standard error
min.	Minute
var.	Variety
w	Weight

Chapter I

Introduction

Plants are sources of important therapeutic aid for alleviating human and animal ailments in the traditional system of medicine for time immemorial and have such an important historical and traditional role in healing that they are treated as a special category i.e., those particularly valued for their medicinal qualities are called medicinal plants.

Man's curiosity in medicinal plants is as old as human civilization. Almost every civilization has a history of medicinal plant use. Approximately 80% of the people in the world's developing countries rely on traditional medicine for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts (Constable, 1990). Interest in phytomedicine has exploded during the last two decades, and about 500 different plant species are used as key ingredients, and many are still being collected from the wild (Mendelsohn and Balick, 1994). The resurgence of public interest in plant-based medicines coupled with rapid expansion of pharmaceutical industries have necessitated an increased demand for medicinal plants, leading to over-exploitation that threatens the survival of many rare species. Also, many medicinal plant species are disappearing at an alarming rate due to rapid agricultural and urban development, uncontrolled deforestation and indiscriminate collection.

In India the heritage of medicinal plants use has an ancient history. The epic of Ramayana witnessed *Sanjivani buti* as life saving drug. The earliest mention of the medicinal use of plants has been found in "Rig Veda", which was written between 4000 and 1600 B.C. In the "*Atharva*

Veda", we find a more varied use of drugs. It is in the "*Ayurveda*", which is considered as an "*Upa Veda*" that definite properties of drugs and their uses have been given in great detail. "*Charak Samhita*" records the use of over 340 drugs of vegetable origin. "*Susruta Samhita*" also dealt with plants related to medicine. In this context it is also notable that *Roulvolfia*, which has now attained world wide popularity, finds mention in ancient Hindu scriptures as well as in the monumental work of Charka where the plant was mentioned as useful antidote for snake bites and insect stings (Agarwal, 1997).

It is estimated that around 70,000 plant species, from lichens to flowering trees, have been used at one time or other for medicinal purposes. As per WHO, about 20,000 plants are being used in one or the other form in traditional system of medicine throughout the world, especially in the developing countries (Singh and Goutam, 1997; Srivastava and Raina 1997). In India, more than 7,500 medicinal plants have been recorded for their use by different ethnic communities for human and veterinary health care across the various eco-systems from Ladakh in the Trans-Himalayas to the southern coastal tip of Kanyakumari and from the deserts of Rajasthan and Kutch to the hills of North-East.

Due to growing awareness about side effects and complications of chemical and synthetic medicines, cosmetics and health supplements, usage of herbal products has gained importance both in the Eastern and Western worlds. In India, according to recent estimates, more than 80% of raw materials for drugs used in Indian system of medicine and homoeopathy are based on plant derived products (Agarwal, 1997).

Therefore, studies in medicinal plant species featured prominently amongst the thrust areas of research in the recent times. Although the

plant species from which medicinal products can be derived, have been the subject of research for the plant scientists engaged in the tissue culture studies for over three decades, but a renewed interest in this field has been a recent trend in the light of growing importance of herbal medicines.

Combinations of *in vitro* propagation techniques and cryopreservation may help in conservation of biodiversity of locally used medicinal plants. Several medicinal plant species have been successfully cryopreserved (Bajaj, 1995). *In vitro* cell and tissue culture methodology is envisaged as a mean for germplasm conservation to ensure the survival of endangered plant species, rapid mass propagation for large-scale revegetation, and for genetic manipulation studies (Chandel and Sharma, 1993).

Using tissue culture technology, a huge number of endangered medicinal plants can be regenerated and conserved, therefore, the domain of cell, tissue and organ culture has gained renewed importance in the recent times. By now over 60 medicinal plant species have been undertaken for *in vitro* studies with varied objectives. Most of these studies either intended towards rapid cloning through micro-propagation and *in vitro* conservation, or towards extensive experimentation aimed at trying to produce and extract active medicinal principles from cell cultures. Micropropagation of medicinal plants have been extensively reviewed by George and Sherington (1984), Kumar *et al.* (1996) and Nalawade *et al.* (2003), while *in vitro* conservation studies have been summarily reviewed by Chandel (1996). Studies relevant to *in vitro* production of active principles have been reviewed by Alferman & Reinhard (1980), Fujiwara (1982), Kurz (1989) and Charlwood & Rodes (1990).

Plant cell and tissue culture is looked upon as a technique that holds great promise for a lucrative future towards crop improvement, both in

agriculture and horticulture. Plant tissue culture, the most successful aspect of plant biotechnology, besides offering many advantages for research in molecular biology, bio-chemistry, and plant developmental biology has also opened vast scope for crop improvement. The present role of tissue culture can be seen in production of specific pathogen free plants, isolation of variants, propagation of genetically manipulated superior clones and *ex situ* and/or *in situ* conservation of valuable germplasm, and at the same time rapid micropropagation, for having more homogeneous plant material that has long been practically exigent, to produce bulk planting material in a short period for large scale farming.

The use of cell and tissue cultures in medicinal plant breeding and genetics is relatively a newer method. Using this method there can be three fold achievements: to arrive at a higher degree of homogeneity in plant material, or conversely to raise heterogeneity in heredity, and last but not least to induce variability. Thus cell, tissue and organ cultures provide us the most potent alternative because they can also produce plants of haploid state through anther and microspore culture.

The clonal propagation of plants *in vitro* has become the first major practical application for the plant tissue culture. During the past 2 – 3 decades a sizeable specialized micropropagation industry has developed for this purpose, particularly in the USA, Europe and Australia, each producing above 30 million ornamental plants annually by tissue culture (George and Sherrington, 1984). Although the techniques are frequently not competitive with traditional propagation methods, yet that has no way discouraged tissue culture workers.

It is worth mentioning that the know how of *in vitro* regeneration of plants in a wide range of plant species is always important, even if it has no commercial value. The knowledge of *in vitro* plant regeneration through cell,

tissue and organ culture has a pivotal role in exploiting the advantages of the modern researches in molecular biology and genetic engineering for possible production of genetically modified plants. At the same time advanced biotechnological methods of culturing plant cells and tissues, should provide new means of cryoconserving and rapidly propagating valuable, rare, and endangered medicinal plants.

In the above back drop a program of work was undertaken to study comprehensively the cell, tissue and organ culture aspects in three important medicinal plants namely, *Chlorophytum borivillianum* (Safed Musli), *Kaempferia galanga* (Ekangi) and *Atalantia malaberrica* (Kaculus), appropriately described in detail elsewhere (in Chapter III under materials). Though the studies widely involved cultural and nutritional management for successful initiation and maintenance of cultures in these medplant species for micropropagation but precisely progressed in the following two major directions:

- i) Studies aimed at developing *in vitro* plant production system having its application not only for rapid cloning but also for future biotechnology and genetic engineering research.
- ii) Studies aimed at developing technical protocol for microspore / anther culture *in vitro* that can possibly provide a potential tool for manipulating cells at haploid state wherever needed and and their sub sequent regeneration.

Chapter II

Review of Literature

A review of tissue culture studies in *Chlorophytum boriuilianum*, *Kaempferia galanga* and other such rhizome bearing species revealed that these studies may be categorized based on the plant part or the explant that has been used. Hence, it is thought that reviewing the past literature especially relevant towards *in vitro* plant regeneration may conveniently be done on the basis of explants used by the earlier workers.

Rhizome

Chlorophytum boriuilianum usually propagated through rhizome. The review also suggested that, many works have been done using the rhizome section as explant.

Ferwerda (1994) regenerated adventitious shoots from the buds of rhizomes of *Languas galanga*. Green house grown shoots were excised and propagated clonally on a medium with MS salts, vitamins, 2% sucrose and BA 2 mg l⁻¹. They obtained the increased rate of production per 4 weeks circle.

Dogra *et al.* (1994) reported about the *In vitro* propagation of *Zingiber officinal*. They conducted the experiment successfully on MS medium. Even the root was also induced on the same basal MS medium. Multiple shoots were produced on MS media supplemented with 2.5 mg l⁻¹ BA and 0.5 mg l⁻¹ NAA from the rhizome bud of Ginger.

Shirin *et al.* (2000) worked upon *Kaempferia galanga* in *in vitro* condition for large scale propagation using rhizomes as explants. They have reported about the plantlet production on 0.75 × MS medium

supplemented with 12 μM BA, 3 μM NAA and 3% sucrose. Interestingly *Kaempferia galanga* developed 10.1 fold more number of plantlets on MS medium supplemented with 12 μM BA. Comparative study revealed that among the 3 carbon sources, sucrose was proved to be the best one in producing the plantlets from the rhizome section of *Kaempferia galanga*.

Raju *et al.* (2005) propagated microshoots of *curcuma caesia* and *Curcuma zedoaria* using rhizome buds as explants. They used MS basal medium supplemented with 4 mg l^{-1} BA and 7.5 mg l^{-1} NAA for *curcuma caesia*. The treatment can induce 3.5 fold increase of production. MS Basal medium supplemented with BA 1 mg l^{-1} and NAA 0.5 mg l^{-1} can induce 4.5 fold increase of production of *C. zedoaria*. Best result for root induction in case of both plants was obtained in the medium supplemented with IAA 0.5 mg l^{-1} .

Hybrid lilies specially Eastern or Asian type were also responsive for *in vitro* micropropagation using the rhizome as explant. They had established a protocol for the production of 106-109 plants from a single explant per year. Micropropagation was successfully established in case of *Lilium hansonii*, *L. brownii*, *L. washingtonianum* and *L. sheriffiae* (Rumynin and sklyusarenko, 1989).

Meenakshi *et al.* (2001) cultured rhizome of turmeric cv. Cuddapah in *in vitro* condition on the MS medium supplemented with 0.8, 1.0 and 2.0 mg l^{-1} IBA, 1.0 mg l^{-1} BAP, 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} GA₃. Best response of shoot elongation was obtained from the MS medium supplemented with 1.0 mg l^{-1} BAP, 1.0 mg l^{-1} GA₃ and 0.1 mg l^{-1} NAA. It was also observed that, this medium combination was also responsive for the production of longest shoots and leaves. The shoots supplemented with 0.3 mg l^{-1} NAA developed healthy roots.

Mogor *et al.* (2001) worked up on the clonal propagation of *curcuma longa*. They reported that MS medium supplemented with 1 or 2 mg l⁻¹ BA was best responsive for shoot and bud induction of the plant.

Similarly, Sunitibala *et al.* (2001) established a protocol for the induction of callus and direct multiplication of *Curcuma longa*. They used rhizome buds, and shoot tips for the *in vitro* culture. reported that, best response of clonal propagation of *Curcuma longa* Linn. was in the MS medium supplemented with NAA 1 mg l⁻¹ and Kin 1 mg l⁻¹ or NAA mg l⁻¹ and BAP 2 mg l⁻¹. They also obtained optimum rate of callus in the medium supplemented with 2.5-3.0 mg l⁻¹ from the rhizome explant. The plantlets could be successfully induced from the callus which was rooted and transplanted to the soil. Interestingly they have also induced rhizome in *in vitro* condition using the rhizome as explant in the MS medium containing different concentration of sucrose and NAA 0.7 mg l⁻¹ and Kin 1 mg l⁻¹ at 27± 2°C under an 8 hrs photoperiods.

Babu *et al.*(1993) obtained plantlets from the rhizome part of the cardamom, ginger, turmeric, black pepper and the tree species cinnamon, clone and nut meg.

Vincent *et al.* (1992) developed embryogenic callus in MS medium supplemented with 0.5 mg l⁻¹ BA and 1.0 mg l⁻¹ 2,4-D using the rhizome section with vegetative buds of *Kaempferia galanga*. The protocol indicated that, the embryogenic callus produced small globular embryoids on the peripheral layers in the MS medium supplemented with 0.1 mg l⁻¹ BA and 1.0 mg l⁻¹ NAA which ultimately produced plantlets in the growth regulator free MS medium.

Anand *et al.* (1997) regenerated multiple shoots from the rhizome explant of *Kaempferia rotunda*. They have developed a protocol for the production of an average of 7 shoots from single

explant. The workers identified that MS medium supplemented with BA (2.0 mg l^{-1}) and IAA (0.5 mg l^{-1}) were ideal for both shoot and root induction.

Shoot base

Pudake and Dhumale (2003), developed an *in vitro* protocol of *Chlorophytum borivilianum*. Using the shoot base and stem disc as explant huge number of microshoots were developed on MS medium fortified with 2 mg l^{-1} BA. The newly formed microshoots developed rooting on the medium accompanied with IBA 3 mg l^{-1} . Upto 90% plantlets were successfully transplanted to the field.

Dave *et al.* (2004) reported about the *in vitro* propagation of *Chlorophytun borivilianum* using alginate coated shoot bud. The response of *in vitro* production was remarkably influenced by storage conditions, gel matrix media and period of storage using the same supplementary hormone (5 mg l^{-1} BAP). Long storage of encapsulated shoot bud like more than 30 days has a negative influence on the regenerative capacity of the coated shoot bud. They also noticed that, alginate matrix with sucrose and MS salts (ALMSS) produced better result compared to that with MS salt alone or without sucrose or MS salts. However, in an average of different effect the shoot bud produced normal shoot on SM and multiplied at a rate of 2.5 fold per culture of 21 days each. The success rate of rooting was 70%.

Gaikwad *et al.* (2003) conducted a comparative study on the *in vitro* response of different explants like seedling, roots, stem disc and leaf explant in nine different MS media composition. Best rooting medium was M7 where as M3 medium was best for shoot multiplication and callus induction. The work indicated that the best response was obtained from stem disc which was at par with the response of basal half of leaf. But the poorest response was obtained from the root explant.

Teng, W.L. (1997) reproduced nodule from the chapped shoot base of pineapples (cv. Variegatus). The nodule was formed on modified half strength MS medium supplemented with NAA (2.69 – 5.37 μ M) and BA (4.44 μ M). The nodules were multiplied in the same medium using the chapped pieces of 1-3 mm of previously formed nodule. But in case of pineapple microshoots were formed from the nodule, not from the shoot base. Moreover, the regenerative capacity of nodule in the medium supplemented with NAA at 0.54 μ M and BA at 0.44 – 8.88 μ M was highest than direct caulogenesis or indirect organogenesis. This medium was also responsive for root induction. Usually many shoots were regenerated roots in the same medium after 10 weeks of culture.

Hong *et al.* (1995) induced *in vitro* somatic embryogenesis from the shoot base culture of the seed ling of garden leek (*Allium porrum*). The shoot base explant produced different types of response in different media composition. Direct shoot multiplication was obtained in the MS medium with 17.6 mM BA shoot base regenerated nodular callus using 9 mM 2, 4-D. This nodular callus was responsible for the induction of embryogenic callus and ultimate embryogenesis following the media composition 9 mM 2, 4-D + 7.6 mM abscisic acid and 9.8 mM 2ip respectively. They have also reported that 2,4-D and light condition was essential for nodular callus induction and somatic embryogenesis.

Purohit *et al.* (1994) developed a protocol of clonal multiplication of the endangered sp. *Chlorophytum borilivianum* using the shoot base as the explant in the MS medium. The shoots multiplied 4 fold within 3 weeks on the MS medium supplemented with 22.2 μ M BA. The rooting was successful in the medium $\frac{3}{4}$ strength inorganic and organic constituents of MS medium and 9.8 μ M IBA.

Rani *et al.* (1999) reproduced a huge amount of callus on MS medium supplemented with 2, 4-D (2 mg l^{-1}) and Kin (0.2 mg l^{-1}). The callus was differentiated into microshoot in MS medium supplemented with BA (2 mg l^{-1}). Rooting was induced best in MS medium supplemented with IBA (2 mg l^{-1}) alone, or with IBA (2 mg l^{-1}) and IAA (2 mg l^{-1}). Plantlets were acclimatized in the culture room and successfully transplanted to the soil.

Kang *et al.* (2004) developed adventitious shoots from the rhizome part of *Scopolia parviflora* Nakai (Solanaceae). They have worked with B₅ medium. The workers obtained best response (about 12 shoots per explant) on half strength B₅ liquid medium containing 3% (w/v) sucrose and $5.77 \text{ } \mu\text{M}$ GA₃ within 12 weeks of culture. Shoot elongation and root induction from multiple shoots occurred on growth regulator free half strength B₅ solid medium.

One of the latest developments of multiple shoots from the *Curcuma zedoaria* Roscoe was reported by Nguyen *et al.* (2005). Rhizome sprout culture can induce multiple shoots in basal MS medium containing 20 g l^{-1} sucrose, 20 (v/v) coconut was and $0.5 - 5.0 \text{ mg l}^{-1}$ BA. The maximum averages of 5.6 numbers of microshoots per explant were obtained in the MS medium supplemented with 20 (v/v) cw and 2 mg l^{-1} NAA. More than 95% of the rooted plants were ultimately established supplemented with 1.0 mg l^{-1} BA and NAA 0.5 mg l^{-1} using rhizome explant of *Alpinia* 80% of the microshoots produced roots on MS medium containing 2.5 mg l^{-1} NAA.

The aseptic culture in *in vitro* condition of the *Kaempferia galanga* produced high frequency organogenesis and multiple shoots using the rhizome explant. The plant gave response in the MS medium with various concentrations of NAA, IAA, BA, 2, 4-D and kinetin ranging from 0.5 to 2.5. mg l^{-1} Maximum amount of multiple shoots and high frequency of organogenesis was obtained in the MS medium supplemented with 0.5 mg l^{-1} IAA and 2.5 mg l^{-1} BA. Reportedly

rooting was induced in the medium containing 0.5 mg l^{-1} IAA and 2.0 mg l^{-1} BA. (Swapna *et al.*, 2004).

Harikrishnan *et al.* (1997) reported about the *in vitro* positive response of the rhizome buds of *Acorus calamus*. The response was obtained in the media containing different type of cytokinins and auxins with different combinations. The plant produced average 5 numbers of shoots from each rhizome part using 0.5 mg l^{-1} BAP and 0.05 mg l^{-1} NAA in the MS medium. Interestingly, the rates of regeneration of these microshoots were same. MS solid media supplemented with 1.5 mg l^{-1} NAA and 0.2 % activated Charcoal resulted in 4-6 roots/ shoot.

Hui *et al.* (1996) established a protocol of the production of buds of *Glehnia littoralis* from the rhizome cuttings. The buds multiplied on MS medium containing KT (kinetin), BA and IAA. The plantlets rooted on half-strength MS medium supplemented with IBA and active carbon. They have also observed an influence of temperature and NaCl concentration on the regeneration stage.

Harikrishnan *et al.* (1997) worked on the tissue culture of *Acorus calamus* (sweet flag) using the young rhizome buds. They obtained maximum number of shoots (4–6) on MS medium supplemented with BA (0.5 mg l^{-1}) and NAA (0.5 mg l^{-1}). Sub culturing of the *in vitro* regenerated multiple shoots produced 625 shoots within 100 days. The newly produced shoots were rooted on MS medium supplemented with 1.5 mg l^{-1} NAA and 0.2% activated charcoal and they were successfully established in the soil.

Jose *et al.* (2002) developed single step *in vitro* protocol for rapid propagation of *Kaempferia galanga* using rhizome as explant. A combination of 4 mg l^{-1} BA and 1 mg l^{-1} kin induced the highest number of shoot buds (30). But 20 shoot buds were obtained from each explant on MS medium fortified with only 4 mg l^{-1} BA after 90

days of culture but only 10 no of microshoots were induced in the medium containing 3 mg l^{-1} Kin. But the best response was obtained in the MS medium fortified with 4 mg l^{-1} BA, 1 mg l^{-1} Kin and 1 mg l^{-1} NAA (55 shoots). Profuse root formation was achieved along with shoot bud development in all culture.

The rhizome section of *Curcuma aromatica* responded significantly in *in vitro* condition for callus induction and shoot regeneration. A combination of 1.5 mg l^{-1} 2, 4-D, 1 mg l^{-1} NAA and 0.5 mg l^{-1} BAP was highly responsive for callus growth from young rhizome. The callus shifted to organogenesis in the medium containing GA_3 (0.5 mg l^{-1}) and IAA (0.5 mg l^{-1}) and BAP (1 mg l^{-1}). But best result was obtained in the medium containing 0.5 mg l^{-1} IAA and 1.5 mg l^{-1} BAP (Anand *et al.*, 1997).

Lakshmi and Mythili (2003) also reported about the embryogenic callus formation from the rhizome bud explants of *Kaempferia galanga*. They produced callus on MS medium supplemented with 1 mg l^{-1} , 2, 4-D and 0.5 mg l^{-1} BAP. In another combination of hormones (0.5 mg l^{-1} , 2, 4-D and 0.2 mg l^{-1} BAP and 16.336 mg l^{-1} tryptophan) the somatic embryos were formed. Final plantlets were formed in the MS medium supplemented with 3 mg/l and 4 mg/l BAP. Rooted plantlets were obtained within 3 weeks of culture. They also encapsulated somatic embryos with calcium alginate coating for the production of synthetic seeds.

Corm

Berua *et al.* (2003) developed a process of rapid shoot proliferation using the axillary bud along with a piece of corm tissue of gladiolus cv. American beauty. In case of axillary bud along with the corm, the organogenic response like higher number of multiple buds/explant and longer shoots were obtained with BA, 2, 3 and 4 mg l^{-1} and NAA 0, 0.5 and 1 mg l^{-1} . In case of both single elongated bud

and bisected elongated buds mass, the total number of shoots as well as shoot length, after 3 sub culturing, were maximum, in the medium supplemented with BA 3 mg l⁻¹.

Kim *et al.* (1988) reproduced microshoots from callus derived from corm explant of gladiolus. The corm of Firebrand and Topar formed callus on MS solid medium containing 10 ppm 2, 4-D. But best response was obtained on MS medium containing 1 ppm kinetin, 2 – 3% sucrose and 0.5 – 1.0% agar at pH 5.7. But in contrast, a cytokinin free medium was more successful for the callus older than 6 months.

Zakutskaya and Murin (1990) worked with gladiolus for *in vitro* micropropagation using various parts of corm bud. The best result was obtained with halves corm bud placed in such way so that stem primordium resides upward. Maximum number of microshoots were obtained on MS medium supplemented with 3 mg l⁻¹ BA and 0.5 mg l⁻¹ NAA.

Appleton *et al.* (1995) reproduced shoots and callus using corm of *Hypoxis angustifolia*. But direct and indirect plantlet production was achieved by separating all meristematic tissue and undifferentiated callus at each subculture. The maximum response of direct microshoot production were obtained on MS medium fortified with 2 mg l⁻¹ BA, but indirect regeneration were obtained on MS medium with 0.5 mg l⁻¹ BA. Alternatively another way of micropropagation were obtained where corms were produced followed by roots each without any growth regulator. Then the plant lets were hardened and transplanted to the pot normally.

Chug and Goh (1994) worked with *Colocasia esculenta* var. *esculenta* for *in vitro* microshoot production. They developed a protocol of production of well developed shoots and roots from 80% corm axillary bud and shoot axillary bud on MS medium supplemented with 20 g sucrose and solidified with 2.4 g gelrite / lit (BM medium).

Average of 81 shoots per mini corm after 8 weeks of culture were obtained on BM medium supplemented with 50 mM BA. Plantlets were ready for patting after 4 weeks of whole bud culture.

The corm axillary buds of gladiolus Cv. Friendship responded promisingly on MS medium containing 3.0 mg l⁻¹ BA and 0.5 mg l⁻¹ NAA. The responsive elongated buds were transferred to a new combination of 2.0 mg l⁻¹ BA and 0.5 mg l⁻¹ NAA for the rapid production and multiplication of microshoots. Simultaneously, these corms also produced larger and early tubers on MS medium fortified with 5% sucrose, 0.5 mg l⁻¹ NAA and 5.0 mg l⁻¹ triadimefon. But most large corms were obtained on MS liquid medium with 5% sucrose and 3.0 mg l⁻¹ triadimefon. (Hussain *et al.*, 1997)

Iphigenia indica an alternative source of colchicines was highly responsive for *in vitro* micropropagation. The plant lets were produced from both the explants whole corms and corm buds. Comparative analysis also revealed the fact that whole corms produced very small micro corms which ultimately regenerated the microshoots whereas corm buds initiated a huge number of microshoots in presence of NAA and BAP., It was also reported that highest response in corm and bud multiplication obtained in MS basal medium supplemented with 2.69 M NAA and 8.88 M BAP. The rooting medium was MS medium fortified with 5.38 M NAA and 4.92 M IBA. Within 2 weeks in culture (Mukhopadhyay *et al.*, (2002).

Negash *et al.* (2000) conducted a comparative study on the effect of corm, leaf tissue and zygotic embryos of three clones of *Ensete ventricosum*. Multiple shoots from both corm and embryo explants were obtained using the same medium supplemented with 10 M or 20 M BAP. Rooting was obtained using medium with 5M IBA, 1 M BAP and 1 g l⁻¹ activated charcoal.

Ndong et al. (2006) produced an efficient protocol for direct and indirect *in vitro* regeneration of *Hypoxis hemerocallidea landrace Gaza* using the corm explant. According to this protocol direct regeneration of microshoots were obtained on basal medium supplemented with 3.0 mg l⁻¹ Kinetin. On the other hand calluses were regenerated on the medium with high level of kinetin 3 mg l⁻¹ and NAA 1 mg l⁻¹. But shoots were induced from the callus mass on the MS medium containing BA (1.5 mg l⁻¹) and NAA (0.5 mg l⁻¹). The shoot regeneration frequency was 100% and 30 – 35 shoots / explant was obtained.

Dinkelman and Staden (1988) reported about micropropagation of *Ixia maculata* following the indirect process of regeneration. Callus production was induced from corm tissue on MS medium with 1 mg l⁻¹ BA. After 120 days of callus formation, the microshoots were regenerated on the same medium. A remarkable number of microshoots were regenerated after subculturing on MS medium with BA 1 mg l⁻¹ and NAA 0.5 mg l⁻¹. The plantlets with 3 – 5 leaves were transferred to the pots. Containing 2 : 1 mixture of peat and sand.

Stefaniak, B. (1994) developed somatic embryos from the corm explant of *Gladiolus hortulans*. The corm slices and other explants regenerated somatic embryos and friable embryogenic callus on 2 mg l⁻¹ 2, 4-D in case of Blue Isle, Jennylee, and Risa Supreme and on 10 mg l⁻¹ 2, 4-D in case of Peter Pears. Somatic embryos regenerated plantlets on hormone free MS medium. Comparative study also revealed that, the response of corm slices was better than any other explant.

Afza et al. (1996) developed a protocol of formation of adventitious buds and somatic embryos from corm tissue. The callus derived from the explant was cultured on MS basal medium supplemented with BA or Zip. Interestingly, elongation of somatic embryos was also achieved on the same medium and rooting was induced on half strength MS basal medium supplemented with IBA.

But, remarkably there was no phenotypic difference observed among more than 200 potted regenerates.

Kim and Lee (1993) worked with eleven cultivar of gladiolus to study the response of the different cultivar using corm tissue as explant. The callus was induced on MS medium with 10 mg l^{-1} 2, 4-D for all cultivar. But the response varied from 21.5 to 100% in 26 days depending on different cultivar. However, uniform callus could only be maintained and proliferated with Cv. Topar. The callus obtained from other cultivars was mixed with somatic embryos or organs. Optimum concentration of 2, 4-D in the first and second subcultures were 0 – 5 and $0.1 - 1 \text{ mg l}^{-1}$ respectively.

Bulb

Cook *et al.* (1988) worked on the tissue culture of a medicinal plant *Bowica volubilis* which is used as heart stimulant. According to their finding shoot proliferation (about 3.50 after 3 months of culture) was promoted in MS medium supplemented with 1 mg l^{-1} BA and 1 mg l^{-1} NAA. The stock culture was maintained in the same medium. But the shoots were sub cultured in the growth regulator free medium. Bulbs produced within a year after transplanting the plants in the soil.

Drewes *et al.* (1993) reported that, bulb was formed on bulb scale explants using MS medium supplemented with BA and NAA. A combination of 2 mg l^{-1} BA and 2 mg l^{-1} NAA induced the best response with an average of 14.1 shoots per explant. 0.5 mg l^{-1} and 1.0 mg l^{-1} NAA was ideal for root induction.

Heansch (1996) cultured the bulb scale of 23 different genotypes of liliun hybrids on MS medium supplemented with $4 \mu\text{M}$ 2, 4-D for obtaining the somatic embryos. Among all the genotypes 4 genotypes responded to the treatment showing the emergence of somatic embryos. Comparative study revealed that the best response of

formation of somatic embryos was obtained with 2, 4-D than the other growth regulators.

Wawrosch *et al.* (2001) established a protocol for *Lilium nepalense*. They regenerated axillary shoots from longitudinally split shoot halves on the MS medium supplemented with cytokinin, kinetin and auxin. It was observed that more than 7 shoots were obtained from one explant in a 4 week culture period. The plantlets were successfully established in soil after root formation.

Lim *et al.* (1996) reported about the direct organogenesis and somatic embryogenesis of *Allium victorialis* var. *platyphyllum*. They produced direct shoots of *Allium victorialis* var. *platyphyllum* using bulb explants in the MS medium supplemented with NAA 0.2 mg l⁻¹ and zeatin 2.0 mg l⁻¹. They also obtained somatic embryos on MS medium supplemented with NAA at 0.2 mg l⁻¹ BAP at 0.2 mg l⁻¹ and picloram at 1.0 after 4 – 5 weeks of culture using bulb as explant.

Tuber

Many of the medicinal plants are vegetatively propagated in the in situ condition. Wu *et al.* (1999) reported, about the micropropagation of *Zantedeschia aethiopica* using the tuber as explant. The cultivar produced microshoots and callus on MS medium with 1.0 – 2.0 mg l⁻¹ BA and 0.1 mg l⁻¹ NAA. The basic medium supplemented with 0.5-1.0 mg l⁻¹ BA and 0.1-0.5 mg l⁻¹ were favorable for induction of adventitious shoots and their growth. But less than 0.2 mg l⁻¹ BA improved the growth of adventitious shoots but reduced the amount of callus. Observation indicated that, higher concentration of BA were desirable for shoot induction and callus formation where as low concentration of BA was responsible for the growth of the shoot.

Anjum and Hakoomat (2004) reproduced microshoots from the tuber explant of 4 potato genotype among which two were wild species

and rest two were the cultivar of *Solanum tuberosum* (Desiree and Maris Piper). The response of tuber explant was highest of the medium of Ahloowalia in comparison with Iapichino *et al.* and Jarret *et al.* on the other hand, regeneration frequency percentage and number of microshoots were highest on the medium of Iapichino *et al.* and in Maris Piper.

Li *et al.* (2002) established a protocol for *in vitro* plant regeneration of a traditional Chinese medicinal plant *Stachys sieboldii* using the tuber as explant. It was noticed that 60.4% tuber explant reproduced 9.1 shoots/explant using MS medium in combination with 1.0 mg l^{-1} thidiazuron after 4 weeks of incubation. The regeneration capacity was enhanced upto 33.5 shoots/ explant after another 4 weeks of incubation on the hormone free MS medium. The result of sub culture was very much similar to the initial culture. Rooting was achieved up to satisfactory level on both MS and half MS medium.

Esna and Villiers (1998) developed shoots, roots and callus from the tuber discs of potato cv. Desiree in the MS medium containing BAP and 2,4-D. They regenerated callus in MS medium along with 1 mg l^{-1} BAP + 0.5 mg l^{-1} 2, 4-D. Similarly shoots were directly formed from the same explants on MS medium with only 1 mg l^{-1} BAP. Even nodules were also formed on the surface of the explant from the same explant after 4 weeks of culture which ultimately produced 6 shoots/nodule. After 9 weeks roots developed in the MS medium without any growth regulators.

Sharma *et al.* (2003) reported about the clonal propagation of *Gloriosa superba* and *Gloriosa rothschildiana*. The *in vitro* comparative study was planned using tuber as the explant on MS medium with different concentration of BA (50, 100, 150 ppm), IBA (100, 250 and 500 ppm) and Ethrel (250, 500 and 1000 ppm). Best result for micro tuber production was obtained with Ethrel at 1000 ppm. *In vitro* shoot multiplication was obtained in the MS medium supplemented with BA

(10 ppm) + NAA (1 ppm) and GA₃ (5 ppm) using tuber tip as explant. The plantlets were rooted on the MS medium supplemented with IBA (0.8 ppm).

Embryo

A very few work was done on the tissue culture or microcloning of the different species of *atalantia*. It is note worthy no work on tissue culture with *Atlantia malaferica* was performed. Very few works with the species or family was enlisted below:

Ling *et al*(1997) were the earlier workers who had done their study with the immature seeds of 8 citrus relatives including *Aegle marmelos*, *Atalantia ceylanica*, *Citropsis gabunensis*, *Clausena excavate*, *Glycosmis pentaphyla*, *Microcitrus australasica*, *Murraya paniculata* and *Severinia buxifolia*. They got success with using the MT medium combined with 0.05 mg l⁻¹ 2,4-D, 0.05 mg l⁻¹ BA and 400 mg l⁻¹ malt extract. The caulogenesis was induced from the somatic embryos. Interestingly, after at least six successive sub cultures, the callus became regenerative and this phenomenon was independent of any kind of growth regulators. But somatic embryogenesis was obtained from this callus with MT medium containing 5% lactose.

Another finding obtained by Jumin (1995) has resemblance with the work of Ling *et al*(1997). He induced embryogenic callus in the MT medium combined with 5.0 mg BA, 2.5mg 2,4-D and 600 mg malt extract/litre with 5% sucrose.

Ling *et al*.(1990) used unfertilized and under developed seeds of *Citrus unshiu* for regeneration. They used MT medium for induction of embryogenic callus of citrus. The callus was induced from the hypocotyl region of the embryo. The somatic embryos were formed from this callus using the same medium containing 50gl⁻¹ lactose in the absence of any growth regulators. But for the formation of

plantlets, these embryos were needed to be cultured on the medium with sucrose and GA₃. Another advanced study revealed that the protoplasts isolated from this callus produced somatic embryos through colony formation. Entire plants were subsequently regenerated.

A comparative study was done by Teo *et al* (1988) on differential response of different types of explants like cotyledonary tissues, smaller embryos, nucellus and leaf segments of *Citrus microcarpa*, *C. limon*, *C. sinensis* and *Fortunella polyandra*. These findings revealed that, roots and callus were frequent in case of all explants but in different combinations of growth regulators. As for example, whole embryo of *C. microcarpa* germinated on basal medium alone, but callus was induced in the medium with NAA. Shoot buds developed on roots in 3 months old cultures of *C. grandis*, *C. limon*, and *C. microcarpa*. But the response of nucellus and leaf segments was not satisfactory.

Another comparative study of differential response of different explants was done by Chang *et al* (1992). Huang and Xiong (1992) reported about a tufted shoot production and embryoid formation from juvenile embryos of *C. reticulata*. They worked with MT medium. Initial response was obtained in the medium supplemented with 0.5 mg l⁻¹ BA or 0.2 mg l⁻¹ B.A + 1.5 mg l⁻¹, 2,4-D mg l⁻¹. They compared the response of different explants of *Pinellia ternate* with Citrus. They reported that bulbils showed the highest regenerative ability, followed by leaf blade and petiole. But in case of *Anoectochilus formosanus*, *Rehmannia glutinosa* and *Platycodon grandiflorus*, shoot tips and lateral buds were superior to other organs in terms of differentiation.

Parthasarathy and Nagaraju (1994) produced nucellar seedlings from the nucellar embryos of mandarin cultivars Khasi and Santra and orange cv. Musambi in the MS medium supplemented with NAA

0.2 mg l⁻¹ and malt extract (500 mg l⁻¹). The better observations were found using the malt extract.

Another work with nucellar embryo of *Citrus Sinesis* (c.v Mosambi) was done by Das *et al.* (2000). They have standardized that, three to four nucellar embryos and a zygotic embryo could be excised form a single mature seed and successfully regenerated as healthy plants in basal MS medium supplemental with NAA (1 mg l⁻¹) or 2,4-D (1 mg l⁻¹) and GA₃ (1 mg l⁻¹). Five to six shoots were obtained from collar region of both category of embryos in MS medium supplemented with BAP (1 mg l⁻¹) within 60 days of inoculation. The numbers of plantlets were almost doubled after transfer in the same medium and cultured for another 30 days. Moreover, higher dose of BAP resulted in initiation of callus directly from embryos. The regenerated shoots were rooted in MS medium supplemented with either NAA (0.75 mg l⁻¹) or NAA (0.5 mg l⁻¹) and IBA (2 mg l⁻¹) combination.

Karwa *et al.* (2004) studied the response of nucellar embryos of mature *Citrus sinensis* seeds. Nucellar embryos were initially responded in the MS medium supplemented with BAP at different concentration and NAA (at 0.54-05.37 µM l⁻¹). After achieving 2-4 cm height of the micro shoots, they were isolated and transferred to MS medium containing various IBA combinations (0.98-4.9 µM l⁻¹) for root induction. The best result of microshoot initiation was obtained in the MS medium supplemented with 4.44 µM l⁻¹ of BAP and 2.69 µM l⁻¹ of NAA.

Grosser *et al.* (1998) produced somatic hybrids by protoplast fusion. Remarkably the somatic hybrids of sweet orange- lemon parentage were consistently resistant to phytophthora. On the other hand, hybrids of *Fortunella*, *Citropsis*, *Atalantia*, *Microcitrus* and *citrus ichangensis* parentage were resistant to *Radopholus citroplillus*.

study revealed that, one type of callus was compact and differentiated normally, where as, other type had a few haploid cells and little differentiation. After the plantlet formation, they observed a number of plants (42.2 %) were chimeras of haploid and diploid cells. But maximum number of plants were diploid in nature followed by haploid (33.8 %) and polyploidy (4.6 %). The study also observed distinct phenomenon that the flower mostly in uninucleate stage were induced calluses very fast.

Reforgiato worked on *in vitro* citrus production as well as embryo and nucellus culture in the year 1998. Hidaka and Omura (1989) reported that, plantlets were originated from the microspores of *Poncirus trifoliata* and *Citrus aurantium* in the MS medium. Three types of microspores were developed, among which only C type was multinucleate and further developed into microspore embryoids showing the abnormal nuclear division. The calluses from the inside of the anthers originated from both anther wall and microspores.

Germana (2003) induced callus from the anther of *Citrus aurantium* and *Citrus reticulata*. The scientist cultured the anther of uninucleate stage after a chilling treatment and cultured in the different media. The direct and indirect somatic embryogenesis were induced after 3-4 months of culture. The embryogenic ability was maintained for more than 3 yrs subculturing the embryos in the fresh media at a regular interval. Well developed somatic embryos vigorously germinated on the said medium (at 80—89%).

Figure 3.1



Safed Musli (*Chlorophytum borivillianum*): a, plant materials utilized for the present study being maintained in the Medicinal Plant Garden of the university; b, plant at young stage; c, plant at flowering stage; d, roots at harvest time.

B. Common Name	: Ekangi / Chandramul
Scientific Name	: <i>Kaempferia galanga</i>
Family	: <i>Gingiberaceae</i>
Active Principle	: 2.5 to 4% essential oil, whose main components are ethyl cinnamate (25%), ethyl- <i>p</i> -methoxycinnamate (30%) and <i>p</i> -methoxycinnamic acid, Resimic acid
Plant part used for medicinal purpose	: Rhizomes, Roots and leaves
Medicinal value of the plant	: i) Rhizomes used in dyspepsia, leprosy, nausea, Malaria fever, asthma and bronchitis. ii) Roots are used as expectorant, digestive and antihelminthic. iii) Rhizome used as flavoring agent in South East Asia and China. iv) It also possesses tonic and antibacterial qualities. v) The rhizomes were also reported to play cytotoxic properties.

The plant *K. galanga*, (Ekangi) is native to South India, but now mainly cultivated in South East Asia and China. *K. galanga*, is an almost stemless plant that develops its few short-lived leaves and the flower at ground level. Rhizome resembles ginger in shape in that the subunits are flat (elliptical cross-section), but it is much smaller in size. It has a dark reddish-brown skin, and the soft interior is nearly white.

Figure 3.2



Ekangi (*Kaempferia galanga*): a, Plants being maintained in the Medicinal Plant Garden of the university; b, a young plant; c, plant at flowering stage; d, the rhizome.

C. Common Name	: Kacilus
Scientific Name	: <i>Atalantia malaberica</i>
Family	: Rutaceae
Active Principle	: Essential oil,
Plant part used for medicinal purpose	: Fruits and leaves
Medicinal value of the plant	: i) Oil from berries used externally in Chronic rheumatism and paralysis. ii) Leaves are used in snake bite.

This is a wild woody tree brought under plantation in the recent times. Interestingly, in the state of Orissa, the stem is used as the walking stick to treat lumbago (Mohanty, 2005). Manimaran *et al.* (2003). Species is largely propagated by seeds or by vegetative means. Seed propagated population is highly heterogeneous, while clonal propagation is extremely slow.

3.1.1 Cultivars or clone used:

All the three medicinal plant species used for the present study are local land races. Collected and maintained either in the University Medicinal Plant Garden or in the Farmer's field. Not yet identified and catalogued.

3.1.2 Source of materials:

Rhizomes with shoot buds of Safed Musli and Ekangi were collected from the green house of Medicinal Plant Garden of the university being maintained under a Govt. of India Project on Medicinal Plants.

The different explants of Kacilus that included nodal sections, seed embryo and flower buds were collected from 5-7 years old mature plants maintained in the farmer's field.

Figure 3.3



Kaculus (*Atalantia malabérica*): **a**, young plant being maintained in the Medicinal Plant Garden of the university; **b**, a flower; **c**, flowers with very young fruits; **d**, young fruits (zoomed in)

3.2 Methods

3.2.1 Mode of culture:

Standard techniques have been used for growing plant cells and tissues in *in vitro* condition. These techniques are mostly based on culture of such cells or tissues in liquid medium or semi solid medium i.e. agar gelled. The studies reported in the following sections are experiments generally conducted on agar gelled semi solid medium. Only for anther culture studies liquid medium devoid of agar has been used.

3.2.2 Media constituents:

All tissue culture media principally consist of inorganic salts (macro and micro elements), a carbon source (usually sucrose), vitamins, amino acids and growth regulators as well as various organic supplements.

3.2.2.1 Inorganic Salts:

Inorganic salts (macro and micro elements) which were used at various concentrations for the preparations of different media compositions are listed below:

Table 3.2.1 constituents of inorganic salts

Category	Salts	Grade	Make
Major Salts	NH_4NO_3	AR	E. Merck
	KNO_3	GR	E. Merck
	$\text{CaCl}_2 \cdot \text{H}_2\text{O}$	AR	SRL
	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	Pure	E. Merck
	KH_2PO_4	Pure	E. Merck
	$\text{NH}_4\text{H}_2\text{PO}_4$	Pure	E. Merck
	$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	Pure	E. Merck
	$(\text{NH}_4)_2\text{SO}_4$	Pure	E. Merck
	$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	Pure	E. Merck
Minor Salts	H_3BO_3	Pure	SRL
	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	AR	E. Merck
	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	GR	E. Merck
	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	Pure	E. Merck
	KI	AR	SRL
	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	AR	SRL
	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	AR	E. Merck
	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	Pure	E. Merck
	$\text{Na}_2\text{ EDTA}$	GR	E. Merck
	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	AR	LOBA

All the inorganic salts except iron sources were weighed out on an electronic analytical balance as per requirement and dissolved separately in double distilled water.

3.2.2.2 Vitamins:

Following vitamins were used as per requirement at various concentrations:

Table 3.2.2 constituents of vitamins

Vitamins	Make
myo-inositol	SRL
Thiamine HCL	Sigma,USA
Pyridoxin HCL	SRL
Nicotinic acid	Sigma, USA

3.2.2.3 Growth Regulators:

The growth hormones which were used at various concentrations during the course of the experiments are listed below:

Table 3.2.3 List of growth regulators used

Growth regulators	Name	Make
Auxins	2,4 Dichlorophenoxy acetic acid (2,4 D)	E. Marck
	Indole-3-acetic acid (IAA)	E. Marck
	1-Naptheline acetic acid (NAA)	Sigma, USA
Cytokinins	Indole-3-Butyric acid (IBA)	Sigma, USA
	Kinetin (kin)	Sigma, USA
	N6-Benzyl adenine (BA)	Sigma, USA
Others	Gibberallic Acid	Sigma , USA

For preparing the solutions, individual auxins were weighed and dissolved separately in a small volume of ethanol and later diluted with double distilled water to get the desired concentrations. Individual cytokinins were weighed separately and dissolved separately in 1N NaOH and gradually diluted with double distilled water to get the desired concentrations.

3.2.2.4 Amino Acids:

L-glutamin (E. Merck) and Glycine (E. Merck) were used at various concentrations to supplement the nutrient medium. L-Glutamin was dissolved in distilled water and used at desired concentrations.

3.2.2.5 Carbon Source:

Sucrose (LOBA) was the only source of carbon in all experiments conducted. Sucrose was directly added (w/v) to the nutrient solution at desired concentrations. Stock solutions were prepared for the major salts, minor salts, vitamins and growth regulators which were stored under refrigerated conditions (4°C) and used within a short period. Other media components were prepared freshly for use.

3.2.2.6 Inorganic Supplements:

Two types of inorganic supplements were used to enrich the original nutrient media composition. Required amount of Ammonium sulphate and Calcium nitrate were weighed out and were dissolved in double distilled water and prepared freshly prior to use.

3.2.3 Composition of various standard media used and their stock preparation:

For conducting the various experiments, MS (Murashige and Skoog (1962), B5 (Gamborg *et al.*, 1968) and N₆ (Nitsch and Nitsch, 1951) media compositions were used. Composition of each medium is detailed in the subsequent tables (Table 3.2.4 - 3.2.6)

Table 3.2.4 Inorganic macro nutrients present in the three media used in the present study

Constituents	Murashige and Skoog (1962) (mg l ⁻¹)	Nitsch and Nitsch (1951) (mg l ⁻¹)	Gamborg(B ₅) (1968) (mg l ⁻¹)
(NH ₄) ₂ SO ₄	-	-	134
MgSO ₄ . 7H ₂ O	370	85	500
Na ₂ SO ₄	-	-	-
MgCl ₂ . 6 H ₂ O	-	-	-
KCl	-	-	150
CaCl ₂ . 2H ₂ O	440	166	-
NaNO ₃	-	-	-
KNO ₃	1900	950	3000
Ca(NO ₃) ₂ . 4H ₂ O	-	-	-
NH ₄ NO ₃	1650	720	-
Na H ₂ PO ₄ . H ₂ O	-	-	150
NH ₄ H ₂ PO ₄	-	-	-
KH ₂ PO ₄	170	68	-

Table 3.2.5 Inorganic micronutrients present in the three media used in the present study

Constituents	Murashige and Skoog (1962) (mg l ⁻¹)	Nitsch and Nitsch (1964) (mg l ⁻¹)	Gamborg(B ₅) (1968) (mg l ⁻¹)
FeSO ₄ 7H ₂ O	27.85	27.85	-
Na ₂ EDTA	37.25	37.25	-
MnSO ₄ 4H ₂ O	22.3	25.0	10.0
ZnSO ₄ 7H ₂ O	8.6	10.0	2.0
CuSO ₄ 5H ₂ O	0.025	0.025	0.025
Fe ₂ (SO ₄) ₃	-	-	0.025
CoCl ₂ 6H ₂ O	0.025	-	-
AlCl ₃	0.025	-	-
KI	0.83	-	0.75
H ₃ BO ₃	6.2	10.0	3.0
Na ₂ MoO ₄ 2H ₂ O	0.025	0.25	0.25
Fe (as Sequestrene)	-	-	28.0
MnSO ₄ H ₂ O	-	-	-

Table 3.2.6 Organic micronutrients present in the three media used in the present study

Constituents	Murashige and Skoog (1962) (mg l ⁻¹)	Nitsch and Nitsch (1964) (mg l ⁻¹)	Gamborg(B ₅) (1968) (mg l ⁻¹)
Sucrose	30 g l ⁻¹	20 g l ⁻¹	20 g l ⁻¹
Myo-inocitol	100 mg l ⁻¹	-	100
Nicotinic acid	0.5 mg l ⁻¹	-	1
Pyridoxine HCL	0.5 mg l ⁻¹	-	-
Thiamine HCL	0.1 mg l ⁻¹	1	10
Glycine	2 mg l ⁻¹	-	-

Table 3.2.7 Carbon source and gelling agents used

Constituents	Murashige and Skoog (1962) (g l ⁻¹)	Nitsch and Nitsch (1964) (g l ⁻¹)	Gamborg(B ₅) (1968) (g l ⁻¹)
Sucrose	30.0	30.0	30.0
Agar powder	8.0	8.0	8.0

3.2.3.1 Preparation of stock solution:

For the preparation of MS, B₅ and N₆ medium the stock solutions for different organic, inorganic and vitamins were prepared and kept into the refrigerator. The chemicals required for 1000 ml stock solution was given in the Table (3.2.7). Table (3.2.8) enlisted the amount of stock solution required for one liter MS medium.

Table 3.2.8 Preparation of stock solution for MS medium

Solution	Content	g l ⁻¹
Nitrate Solution (MS I)	NH ₄ NO ₃	41.25
	KNO ₃	47.50
	MgSO ₄ . 7H ₂ O	3.7
Sulphate solution (MS II)	MnSO ₄ . 4 H ₂ O	2.23
	ZnSO ₄ . 7 H ₂ O	10.58
	CuSO ₄ . 5H ₂ O	0.025
Chloride solution (MS III)	CaCl ₂ . 2H ₂ O	44.0
	CoCl ₂ . 6H ₂ O	0.025
	KI	0.88
Micronutrient solution (MS IV)	H ₃ BO ₃	0.62
	Na ₂ MoO ₄ 2H ₂ O	0.25
	KH ₂ PO ₄	17.0
Sodium EDTA solution (MS V)	FeSO ₄ . 7H ₂ O	2.785
	Na ₂ EDTA. 2H ₂ O	3.725
Amino acid (MS VI)	Glycine	0.25

Table 3.2.9 Preparation of stock solution for B₅ medium:

Solution	Content	mg/l
Sulphate solution (Stock I)	MgSO ₄ , 7H ₂ O	25000
	MnSO ₄ , 4 H ₂ O	500
	ZnSO ₄ , 7 H ₂ O	100
	CuSO ₄ , 5H ₂ O	1.25
	(NH ₄) ₂ SO ₄	6700
Chloride solution (Stock II)	CaCl ₂ , 2H ₂ O	7500
	CoCl ₂ , 6H ₂ O	1.25
	KI	0.88

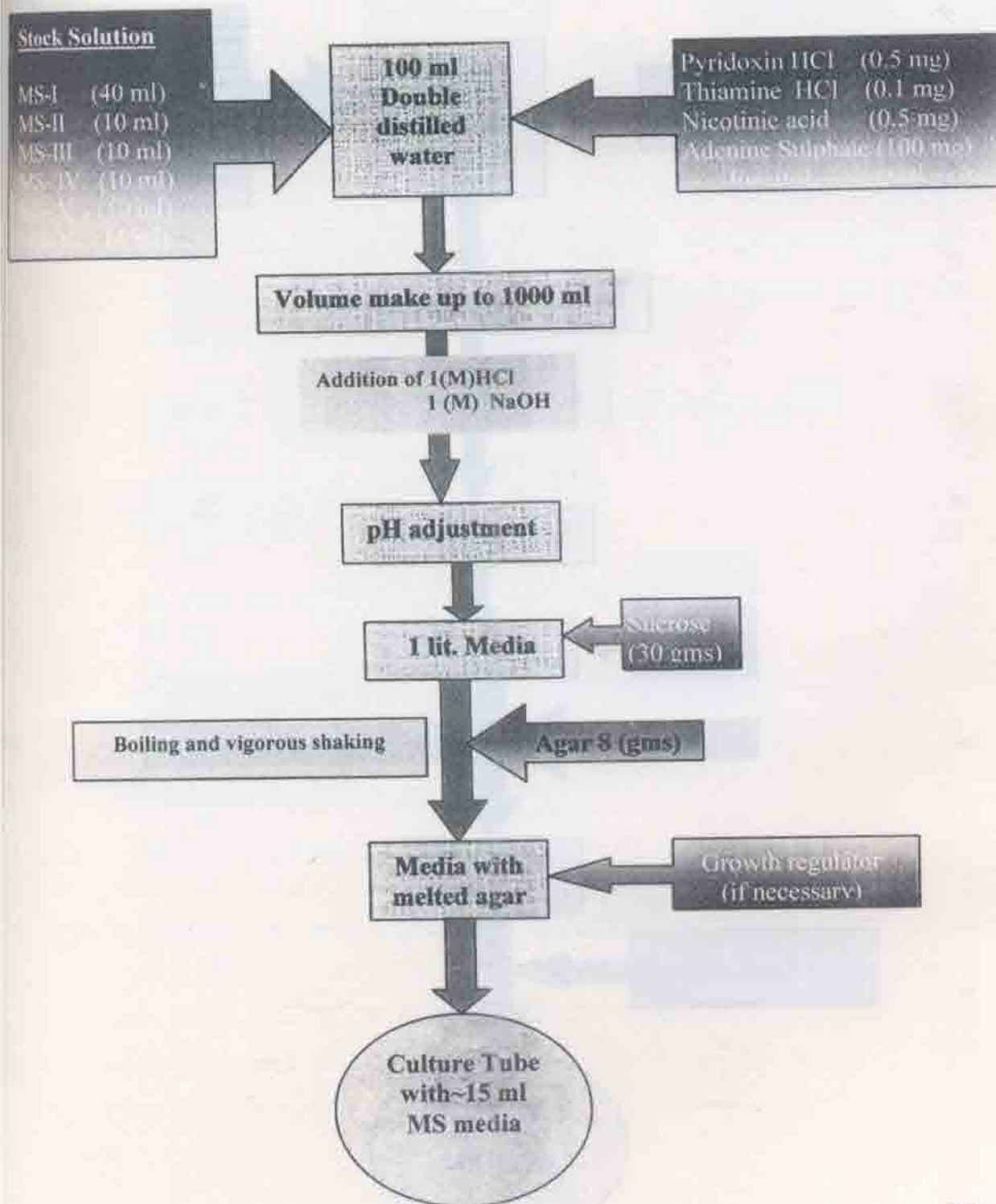
For the preparation of 1000 ml B₅ medium stock I, stock II and stock III solution were added at the rate of 10 ml each. 3 gm of KNO₃ was added directly to the preparation. Other constituents were added as per the standard of the particular media.

Table 3.2.10 Preparation of stock solution for N₆ medium:

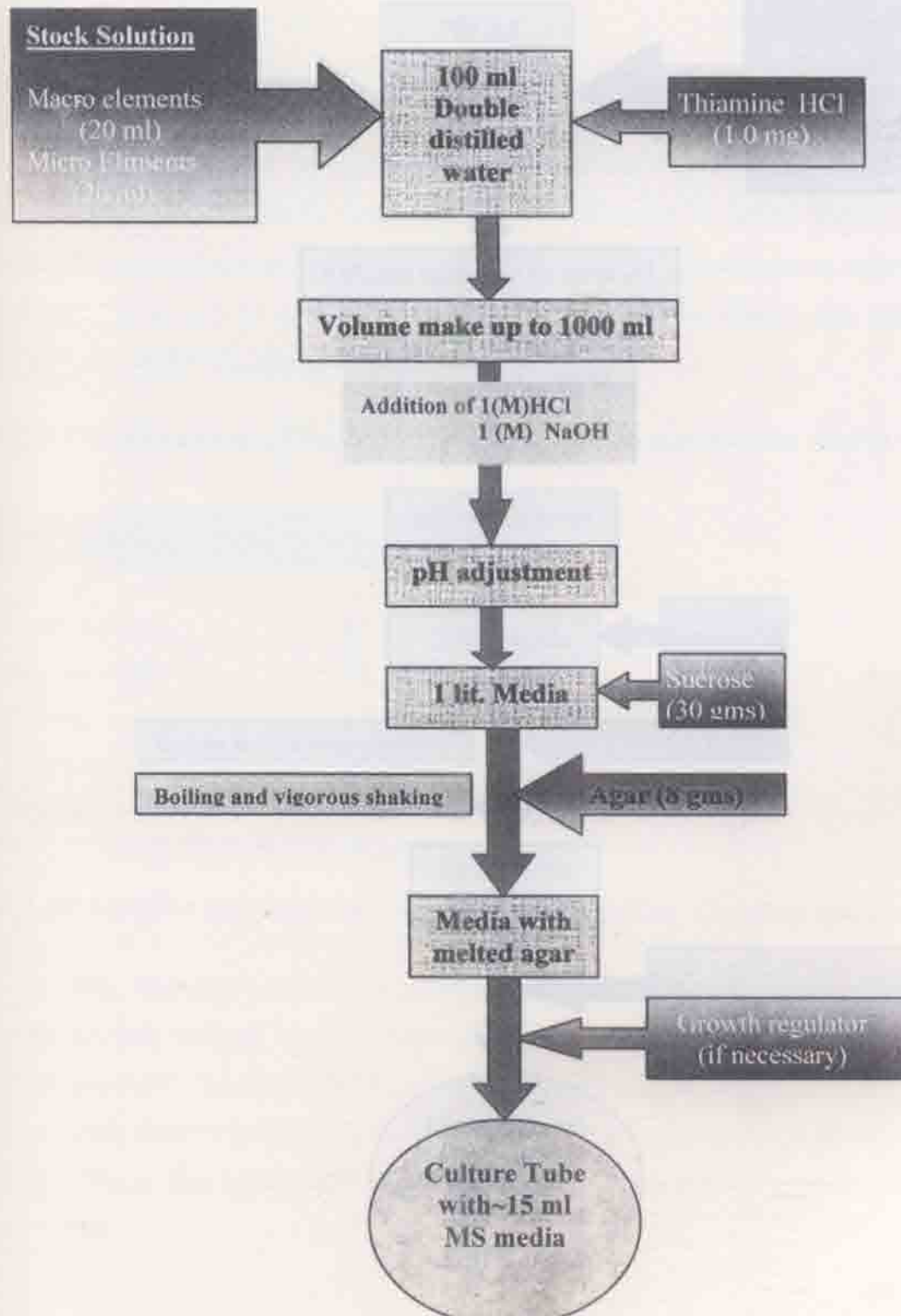
Solution	Content	g l ⁻¹
Macronutrient solution	KNO ₃	35.375
	MgSO ₄ · 7H ₂ O	2.312
	(NH ₄) ₂ SO ₄	5.787
	KH ₂ PO ₄	5
	CaCl ₂ · 2H ₂ O	2.075
Micronutrient solution	H ₃ BO ₃	0.02
	MnSO ₄ · 4 H ₂ O	0.055
	ZnSO ₄ · 7 H ₂ O	0.019
	Glycine	0.025

The macro and micro nutrient solution were present 20 ml each in the 1000 ml media. Other constituents were added as per the standard of the particular media.

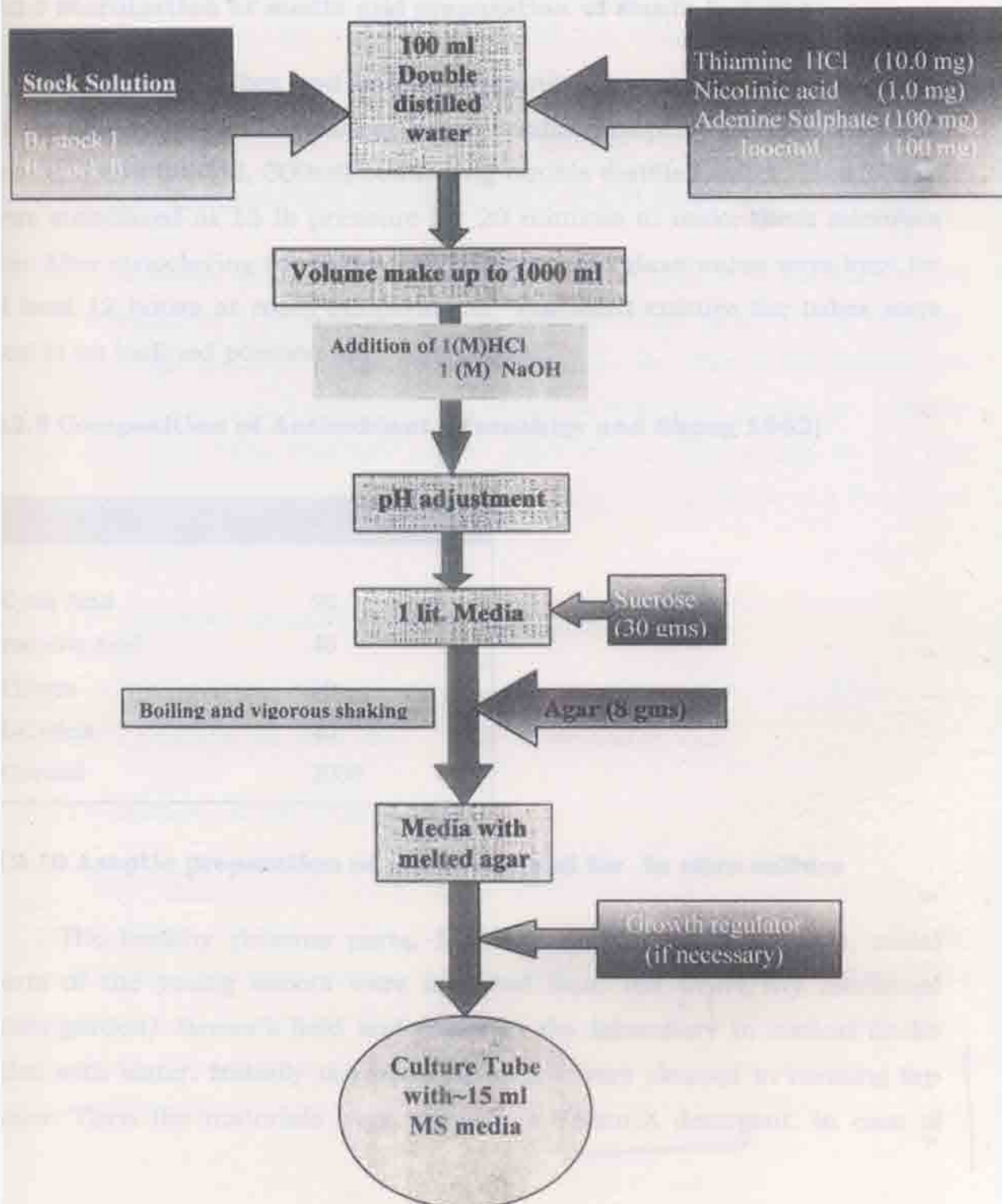
3.2.4 Preparation of MS semi- solid media :



3.2.5 Preparation of N₆ semi- solid media :



3.2.6 Preparation of B₅ semi- solid media :



3.2.7 Preparation of liquid media :

Procedure is exactly the same as that described under preceding section 3.2.4 except adding agar.

3.2.8 Sterilization of media and preparation of slants & flats :

Finally, the tubes and bottles containing agar gelled nutrient media or the conical flasks containing liquid media, scalpels, forceps, scissors, conical flasks (250ml, 500ml) containing double distilled water, Petri plates were autoclaved at 15 lb pressure for 20 minutes to make them microbes free. After autoclaving the tubes, instruments and glass wares were kept for at least 12 hours at room temperature. For slant culture the tubes were kept in an inclined position after autoclaving.

3.2.9 Composition of Antioxidant (Murashige and Skoog 1962)

Constituents	g ^l
Cytric Acid	50
Ascorbic Acid	40
Chlorox	10
L-Cystein	40
Charcoal	2000

3.2.10 Aseptic preparation of plant material for *in vitro* culture

The healthy rhizome parts, flower buds, axillary shoot tips, nodal parts of the young shoots were collected from the University medicinal plant garden/ farmer's field and taken to the laboratory in conical flasks filled with water. Initially the plant materials were cleaned in running tap water. Then the materials were washed in Triton-X detergent. In case of

rhizomes, an additionally the explants prior to surface sterilization, were treated with fungicide Bavistin (1mg l^{-1} of water) for 4-5 hours prior to surface sterilization. Followed by a through wash in double distilled water. In the next step the plant parts were treated with Mercuric chloride solution 0.1% (w/v) for different durations (as per requirement of the explant). The materials were then rinsed 5-6 times with sterile double distilled water and sliced according to the requirement of the culture under the laminar air flow.

3.2.10 Inoculation of the explants on the medium :

The brown paper wrapped, autoclaved, oven dried instruments and glass wares were transferred to the laminar air flow cabinet and both the working place and instruments were exposed to the ultra violet rays for 30-40 minutes. During inoculation, the explants were first placed on a sterile Petri plate and then trimmed as per used with sterile scalpel blades and inoculated into the medium opening the cotton plug in front of a flame and sterile air flow.

3.2.11 Physical conditions provided for culture :

Following inoculation cultures were maintained in the culture room at $25\pm 1^{\circ}\text{C}$, having 65-70% RH.

3.2.12 Statistical analysis of data :

The experimental data were subject to the variance analysis appropriate to the two factor complete randomized block design and the significance of different sources of variations were tested following standard procedures of F-test at 0.05 probability level. Two factor CRD were calculated following variance component analysis technique using SPSS Base 10.0 version software package (Copy write 1997, by SPSS Inc. USA).

Chapter IV

Micropropagation in Safed Musli (*Chlorophytum borivillianum*)

4.1

***In vitro* response of rhizome sections of Safed Musli (*Chlorophytum borivillianum*) to different nutritional combinations**

4.1.1 Rationale

To initiate the present work on the micropropagation in Safed Musli (*Chlorophytum borivillianum*) one of the most critical considerations had been to choose the plant part that was to be taken as explant i.e. the source material for regenerating *in vitro* plantlets. Conventionally Safed Musli is propagated vegetatively and the propagating part of the plant is rhizome, the under ground modified swollen stem. It's well established that the explant, its source, maturity, age, size and juvenility all have a determining role on the success of micro propagation.

In the recent time micro propagation in *Chlorophytum* has been attempted by several workers with reasonable success, using different explants such as shoot bud (Dave *et al.* 2004), shoot base (Basu and Jha, 2005; Pudake and Dhumole, 2003; Purohit *et al.*, 1994), stem disc and leaf explant (Gaikwad *et al.*, 2003). But there is no report of micro propagation in *Chlorophytum* using rhizome sections as explants, though rhizomes have been reported to be a very potential explant in a number of rhizome producing medicinal plant species (Vincent *et al.* 1992a; 1992b; Lakshmi and Mythili 2003; Swapna *et al.*, 2004; Shirin *et al.*, 2000; Jose *et al.*, 2002; Him-de-Freitez and Paez-de-Casares, 1997; Anand and Hariharan, 1997; Harikrishnan *et al.*, 1999; Hui *et al.*, 1996; Nguyen *et al.*, 2005; Begum *et*

al., 1995; Haque *et al.*, 1999; Malamung *et al.*, 1991; Salvi *et al.*, 2002; Borthakur *et al.*, 1998). Hence a program was undertaken to look into the various possibilities of *in vitro* regeneration using rhizomatous tissue as explant and also to compare the potentiality of rhizome explant with that of shoot base explant which has more frequently been used by earlier workers for developing micropropagation protocol in *Chlorophytum borivillianum*.

While initiating the present work such rhizome base sections formed the basic explant. As source of explant the mature rhizomes were kept under light sand layer during the initiation of monsoon season i.e. in the month of May – June in the University Medicinal Plant Garden. After 10-15 days when the buds sprouted the rhizomes along with the sprouting shoot buds were taken to the laboratory for initiating cultures.

As rhizomes grow under ground and often contain a high degree of microbial contaminations, it was compulsively needed to establish a suitable procedure of surface sterilization to obtain clean, healthy explants to initiate aseptic cultures. Hence, to determine the optimum level of sterilization with minimum degree of damage to the explants these were inoculated on gelled basal medium devoid of any growth regulators to marginalize wastage due to possible carryover culture infections.

Following finalization of the sterilization regime inevitably the next basic step was to identify an optimum nutritional requirement for sustainable growth response of the rhizome explants *in vitro*. While making the choice of basal medium for initiating the culture using the rhizome sections, beside MS (Murashige and Skoog, 1962) medium, which has frequently been used by previous workers for obtaining microshoots in *Chlorophytum* chiefly from shoot tip or shoot base explants, the choice was extended for B₅ (Gamborg *et al.*, 1968) medium too as the latter has been found to be effective for micro propagation in a number of rhizome/corm bearing species (Saha, 1997;

Sutter, 1986; Ziv, 1990, Phalisteen *et.al.*,2006; Liu *et.al.* 1988) through callogenesis and/or embryogenesis.

4.1.2 Methodology

Rhizome sections with one or two germinating buds were collected from the green house of Medicinal Plant Garden. The materials were thoroughly washed in running tap water for more than an hour and were washed in Triton-X detergent. As the explant was rhizome, an underground part of the plant, these were treated for 4-5 hours with fungicide Bavistin (1mg l^{-1} of water) prior to surface sterilization.

To find an effective method of sterilization, after thorough cleaning and washing and treating with fungicides, different sterilants were used individually or in combination and the explants were treated for different durations and the results were recorded after two weeks of inoculation on MS basal medium devoid of any growth substance.

For identifying a suitable media composition initially the rhizome sections were cultured in a separate sets of trial on agar gelled MS and B₅ medium containing varying concentrations of auxins (2,4-D, NAA 0.05 – 2.0 mg l^{-1} each and IBA 0.05 – 2.5 mg l^{-1}) with cytokinins (Kinetin and BA 1.0 -3.0 mg l^{-1} each).

Following inoculation cultures were maintained at $25\pm 1^{\circ}\text{C}$ and exposed to fluorescent light (500 lx) for 7 days. Then finally the cultures were transferred under light of higher luminescence (~2000 lx) maintaining a 16 h photoperiod. Following periodic chase final observations were recorded after 6 weeks.

4.1.3 Result and Discussion

Rhizome sections of *Chlorophytum borivillianum* *in vitro* frequently met with fungal infections destroying the cultures. Problem of contamination has frequently been experienced by many workers while initiating cultures using rhizome and corm sections as explant (Ziv, 1990; Saha, 1997; Liu *et al*, 1988; Phalirsteen *et al.*; 2006). An initial attempt to identify an effective regime of surface sterilization was extremely useful. Observations on the effect of sterilants on rhizome explants after 10 days of culture have been recorded in Table 4.1.1. It is evident that the effect of 5% calcium hypochlorite or 10% calcium hypochlorite responded moderately irrespective of time, while 0.5% silver nitrate was highly toxic when it was applied for 5 minutes or even for less duration. Similarly Mercuric chloride treatment of rhizome sections with 0.5% and 0.8% aqueous solutions for 3-5 minutes or at 0.1% for 6-7 minutes was also toxic. Best results were obtained when the explants were pretreated with Tween-20 for 10 minutes with 4-6 rinses /washes followed by a treatment with 0.1% mercuric chloride aqueous solution, for a period of 4-5 minutes.

Results of the initial trials with very low level of auxins (2, 4-D or NAA or IBA below 0.5mg l^{-1}) combined with low level of cytokinins (Kinetin or BA below 0.5mg l^{-1}) were not encouraging. Even the results of the trials with only auxins ($0.1, 0.5, 1.0 - 2.0\text{ mg l}^{-1}$) or only cytokinins ($< 0.5\text{ mg l}^{-1}$) were not satisfactory. Little swelling at the base of the rhizome sections were observed where auxin (2, 4-D, NAA) were used at $1-2\text{ mg l}^{-1}$ concentrations. But the growth failed to sustain and did not show any positive response. As there was no induction, the observations are not tabulated here. But the observations recorded after 6 weeks of culture on both MS and B₅ media using different auxins (2, 4-D $0.5 - 2.5\text{ mg l}^{-1}$, NAA $0.5 - 2.0\text{ mg l}^{-1}$ and IBA $0.5 - 2.0\text{ mg l}^{-1}$) with different combination of cytokinins (Kinetin $0.5-3.0\text{ mg l}^{-1}$ and BA $1.0 -3.0\text{ mg l}^{-1}$) have been summarized in the Table 4.1.2 and 4.1.3 respectively.

high NAA (2.0 mg l⁻¹) induced rhizogenesis (Fig 4.1 c). Most promising results were obtained with a combination of BA with IBA (BA 2.0 – 3.0 mg l⁻¹ and IBA 0.5 mg l⁻¹) yielded profuse shooting from the base of the rhizome sections, producing a cluster of microshoots (Fig 4.1 e,f) with reasonably high frequency (32.06 – 43.40 shoots / explant).

On the other hand supplementing B₅ medium with 2, 4-D and kinetin also failed to yield any positive result except inducing callus at higher concentrations (2, 4-D 2.0 – 2.5 mg l⁻¹ and kinetin 1.0 – 2.0 mg l⁻¹). But a combination of IBA 1.0 – 1.5 mg l⁻¹ and BA 1.0 – 2.0 mg l⁻¹ yielded proliferation of shiny globular embryoid like structures directly from certain locales of the rhizome sections (Fig 4.1 d).

The result of this initial study clearly revealed IBA as superior to other two auxins used in both MS and B₅ medium. It is also evident that the composition of MS medium is much more efficient as compared to B₅ medium for induction of callus, callus mediated morphogenetic changes and direct induction of shoot buds from the rhizome sections excepting for the formation of pro-embryoid like structures in B₅ medium supplemented with IBA and BA combinations. The MS medium combination has severally been reported to be highly effective for morphogenesis especially in monocotyledonous plants for its high NO₃⁻ and NH₄⁺ levels (George and Sherrington, 1984; Narayanaswamy, 1998; Amirato, 1984). This medium (Murashige and skoog, 1962) contained 40mM N as NO₃⁻ and 20 mM as NH₄⁺, that being of five fold increase in total nitrogen as compared to Miller (1956) medium, fifteen times as compared to Hildebrandt *et. al.* (1946) and nineteen times than that of Whites medium (White, 1943). Potassium and other macronutrients are also higher in MS but by smaller factors (George and Sherrington, 1984)

The present set of experiments indicated that a higher amount of NO₃⁻ and NH₄⁺ ions (40mM N as NO₃⁻ and 20 mM as NH₄⁺) of MS medium convincingly induced direct regeneration as well as recognizable changes in

callus morphology prone to indirect regeneration, while higher concentration of NO_3^- (25 mM) associated with lower concentration of NH_4^+ (1.0 mM) in B_5 medium favored the induction of embryoid like structures. These findings also support the earlier view that the principal supply of higher level of reduced nitrogen may be important for direct shoot formation (George and Sherrington, 1984; Yeoman, 1986). This initial study indicated a possibility that induction of organogenesis was brought about by high level of NH_4NO_3 in the medium along with high levels of auxin and cytokinins (IBA and BA), but these combinations were not favorable for the initiation of embryogenesis. It is believed that there must be a group of genes most likely to exert influence over differentiation and morphogenesis and capable of shifting plant development in different directions (Bayliss and Dunn, 1979; Saha, 1997). Such genes have been thought to be themselves regulated by the effective levels of endogenous and exogenous growth substances. However, to cast any light on these aspects has neither been the objective nor within the bounds of the present studies.

Precisely, the present results revealed three distinctly different courses of development following *in vitro* culture of rhizome sections of Safed Musli (*Chlorophytum borivillianum*):

- i) Direct organogenesis i.e., the induction of microshoots directly on the rhizome section of Safed Musli in MS medium containing only BA or different combination of IBA and BA. (Table 4.1.2)
- ii) Development of shiny globular embryoid like structures directly from certain locales of the rhizome sections cultured in B_5 medium containing different combination of IBA and BA. (Table 4.1.3)
- iii) Development of granular structures on the surface of the fast proliferating calluses in MS medium containing different concentration of IBA with kinetin. (Table 4.1.2)

Table 4.1.1: Relative response of various sterilization treatments followed to obtain clean rhizome cultures of *Chlorophytum borivillianum*

Treatments	Sterilants used	Conc. used (%)	Duration of treatment (min.)	No. of washes	Sterilizing efficiency
T ₁	Calcium hypochlorite	5.0	6	5-6	moderate
T ₂	Calcium hypochlorite	5.0	8	6-7	moderate
T ₃	Calcium hypochlorite	10.0	5-6	6-7	moderate
T ₄	Mercuric chloride	0.1	4-5	4-5	high
T ₅	Tween 20 pretreatment + Mercuric chloride	0.1	4-5	4-5	Excellent
T ₆	Mercuric chloride	0.1	6-7	4-5	high, toxic
T ₇	Mercuric chloride	0.5	5	6-7	high, toxic
T ₈	Mercuric chloride	0.8	3	6-7	toxic
T ₉	Silver nitrate	0.5	5	6-7	highly toxic

Table 4.1.2: Response of rhizome sections of *Chlorophytum borivillianum* to MS Medium supplemented with different combinations of growth regulators after 6 weeks of culture

Medium	Growth regulators(mgl ⁻¹)	Induction of callus	Nature of callus	Organogenic response of explants	Mean No. of shoots / explant	Transformed mean
MS	KIN(1.0)	-	-	-	0	0.71
	KIN(2.0)	-	-	-	0	0.71
	2,4-D(0.5) + KIN (1.0)	-	-	-	0	0.71
	2,4-D(0.5) +NAA (0.5) +KIN (2.0)	+	wht ,sft, sfr	-	0	0.71
	2,4-D(1.0) + KIN (1.0)	++	wht, sft, c	-	0	0.71
	2,4-D(1.0) + KIN (2.0)	++	wht ,sft, c	-	0	0.71
	2,4-D(2.0) + KIN (2.0)	+++	wht, sft, c	-	0	0.71
	NAA (0.5) + KIN (1.0)	-	-	-	0	0.71
	NAA (0.5) + KIN (2.0)	-	-	-	0	0.71
	NAA (1.0) + KIN (1.0)	++	Wht, sft, fr	-	0	0.71
	NAA (1.0) + KIN (2.0)	++	Wht, sft, fr	-	0	0.71
	NAA (2.0) + KIN (2.5)	+++	Wht, sft, fr	-	0	0.71
	IBA (1.0) + KIN (0.5)	-	-	-	0	0.71
	IBA (1.0) + KIN (1.0)	-	-	-	0	0.71
	IBA (1.0) + KIN (2.0)	+	wht, fr, gr	-	0	0.71
	IBA (2.0) + KIN (2.0)	+++	wht, fr, gr	Minute, granular structures over callus	0	0.71
	IBA (2.0) + KIN (3.0)	+++	wht, fr, gr	Minute, granular structures over callus	0	0.71
	BA (1.0)	-	-	-	0	0.71
	BA (2.0)	-	-	Shoot initials, no growth	8.86	3.05
	2,4-D (0.5) + NAA (0.5) +BA (1.0)	-	-	-	0	0.71
	2, 4-D (0.5) + BA (2.0)	-	-	-	0	0.71
	2, 4-D (0.5) + BA (3.0)	-	-	-	0	0.71
	2, 4-D (1.0) + BA (2.0)	+	wht,fr	-	0	0.71
	2, 4-D (1.0) + BA (3.0)	+	wht,fr	-	0	0.71
	NAA(0.5) +BA (1.0)	-	-	-	0	0.71
	NAA (0.5) +BA (2.0)	-	-	-	0	0.71
	NAA (1.0) + BA (2.0)	-	-	-	0	0.71
	NAA (2.0) + BA (3.0)	-	-	Direct rhizogenesis	0	0.71
	IBA (0.5) +BA (1.0)	-	-	-	0	0.71
	IBA (0.5) +BA (2.0)	-	-	Profuse shooting from explants	32.06	5.69
	IBA (0.5) +BA (3.0)	-	-	Profuse shooting from explants	43.4	6.52
	IBA (1.0) +BA (1.0)	-	-	-	0	0.71
	IBA (1.0) +BA (2.0)	-	-	-	0	0.71
	IBA (2.0) +BA (3.0)	+	wht, fr, gr	Granular structures on callus	0	0.71

-, no callus induced; +, poor callusing; ++, moderately proliferating callus; +++, fast proliferating callus
wht, whitish callus; c, compact in nature; fr, friable in appearance; gr, granular in appearance

Table 4.1.3: Response of rhizome sections of *Chlorophytum borivillianum* to B₅ medium supplemented with different auxin – cytokinin combinations after 6 weeks of culture

Medium	Growth regulators(mg l ⁻¹)	Induction of callus	Nature of callus	Organogenic response of explants	Mean No. of embryoids/ explant	Transformed mean
B5	KIN 0.5	-	-	-	0	0.71
	KIN 1.0	-	-	-	0	0.71
	2,4-D 1.0 +KIN 1.0	-	-	Swelling of the explant	0	0.71
	2,4-D 1.0 +KIN 2.0	-	-	-	0	0.71
	2,4-D 2.0 +KIN 1.0	++	wht, fr, sft	-	0	0.71
	2,4-D 2.5 +KIN 2.0	++	wht, fr, sft	-	0	0.71
	BA 1.0	-	-	Swelling of the explant	0	0.71
	IBA 1.0+ BA 1.0	-	-	Induction of globular shiny embryoids	22.53	4.77
	IBA 1.5 + BA 1.0	-	-	Induction of globular shiny embryoids	41.60	6.48
	IBA 1.5 + BA 2.0	-	-	Induction of globular shiny embryoids	31.73	5.68
	IBA 2.0 + BA 2.0	++	wht, fr	-	0	0.71
	IBA 2.0 + BA 3.0	++	wht, fr	-	0	0.71

-, no callus induced; +, poor callusing; ++, moderately proliferating callus; +++, fast proliferating callus
wht, whitish callus; fr, friable in appearance; sft., soft in appearance

Figure 4.1



Response of rhizome sections of *Chlorophytum borivillianum* to MS / B₅ medium supplemented with different combinations of growth regulators: **a**, white, soft, friable calluses induced in MS medium (NAA + kinetin); **b**, development of fast proliferating calluses with granular structures in MS (IBA 2.0 mg l⁻¹ + kinetin 2.0 - 3.0 mg l⁻¹); **c**, rhizogenesis induced in MS (BA 3.0 mg l⁻¹ + NAA 2.0 mg l⁻¹); **d**, proliferation of shiny globular embryoids like structures directly from rhizome sections in B₅ medium (0.8 - 1.0 mg l⁻¹ and BA 2.0 - 2.5 mg l⁻¹); **e-f**, cluster of microshoots produced from the base of the rhizome sections in MS (BA 2.0 - 3.0 mg l⁻¹ and IBA 0.5 mg l⁻¹).

Study on the morphogenetic response of preformed microshoots regenerated from rhizome explants of Safed Musli (*Chlorophytum borivillanum*)

4.2.1 Rationale

Of the three situations, as identified following 6 weeks culture of rhizome explants of Safed Musli in MS and B₅ medium (section 4.1), one was the production of adventive microshoots (Fig. 4.1 e,f) over the rhizome explants following culture in MS medium containing IBA-BA combinations. It became primarily essential to isolate the microshoots from the cluster and to introduce further hormonal manipulations for their sustainable growth and development into plantlets. Thus, while making such an attempt; it became a priority to keep the growth regulator combination (IBA - BA) used in the initial study unchanged to favor easy adaptation of microshoots following subculture. Instead the experiment was planned to find the effect of BA with and without IBA, but manipulating their concentrations to test whether any of these combinations is optimum to sustain the growth of the microshoots and their proliferation *in vitro*.

4.2.2 Methodology

Clusters of proliferating green micro shoots along with initial rhizome section at the base were aseptically withdrawn from the culture tubes. They were kept onto sterile petriplates. Microshoots were separated measuring about 5-9 mm and were inoculated in separate culture tubes / bottles containing MS medium with different IBA-BA combinations (Fig 4.2 a). Cultures were maintained in the culture room at 25 ±1° C temperature at higher light intensity (1500 – 2000 lx) provided by fluorescent tubes.

All the treatments were replicated three times. Each replication contained 10 individual cultures. Observations in respect of two morphological characters were recorded after six weeks of culture following the destructive method where 5 out of 10 tubes under each replication for individual treatment were chosen at random.

4.2.3 Results and Discussion

On the basis of the initial study, the isolated shoot initials were cultured in MS medium supplemented with varying concentrations of BA with / without any auxin and the morphogenetic response of the microshoot initials were recorded. For the purpose two morphological characters namely, height of microplants and number of leaves per plantlet were measured by destructive method after six weeks of culture and the observations are summarized in Table 4.2. Results (Table 4.2) indicated that BA below 1.0 mg l^{-1} was not responsive, while enhanced levels of BA ($1.0\text{-}2.5 \text{ mg l}^{-1}$) without auxin were responsive but failed to sustain normal growth and microshoots were lanky / elongated. With the reduction in BA concentrations the microshoots became increasingly lanky / elongated having increasing mean height of $4.30 - 5.35 \text{ cm}$, while the number of leaves per shoot was reduced. Normal shoot length with multiple shoot at base was observed when BA 2.5 mg/l and 3 mg/l were supplemented with IBA 1.0 mg/l and 0.5 mg/l respectively. However, in the former situation, mean number of leaves were significantly less as compared to the latter as they belong to different homogeneous subset (4.2.3). In contrast when the medium was supplemented by IBA 0.5 mg l^{-1} with a lower dose of BA (2.5 mg l^{-1}) or only with BA 2.0 mg l^{-1} , the mean height of the microshoots were statistically at par but they did not appear morphologically normal (Table 4.2.1, 4.2.2 and Fig 4.2b). Whereas, the mean number of leaves produced per microshoot found to differ significantly.

However, the results obtained with BA 3.0 mg l⁻¹ in presence of IBA 0.5 mg l⁻¹ seemed to be most favorable. It reduced the shoot length to 2.07 cm with an increase in the number of leaf (6.80 per plant) and the microshoots could undergo sustained normal morphogenetic development (Table 4.2.1).

These results clearly revealed a differential response of the isolated microshoots to varying auxin cytokinin concentrations and indicated that although BA at 2.0 – 3.0 mg l⁻¹ were conducive for the induction of morphogenesis and initiation of adventive shoots *in vitro* but presence of IBA at least at a low dose appeared to be essential for sustainable normal shoot growth in *Chlorophytum borivillianum* (Fig 4.2c).

A unique feature of the present finding is that only BA was not efficient for the healthy microshoot production instead a balance between auxin and cytokinins appeared necessary where although BA played the key role towards direct induction of adventive shoots but IBA had a critical role to sustain normal growth and development of microshoots.

As elsewhere mentioned micro propagation has been accomplished in *Chlorophytum borivillianum* with reasonable success by a number of workers in past (Basu and Jha , 2005; Pudake and Dhumale, 2003; Gaikwad *et.al.*; 2003; Purohit *et.al.*, 1994; Dave *et.al.*, 2004), but no report has yet been made on the use of rhizome as source of explant. Most probable reasons that might have prompted these workers to avoid rhizome as explant for micropropagation in *Chlorophytum* believed to be due to its chances of carrying contaminants and its being in a dormant state for long time. Although cultures are often best established from plant material that is in active growth but it is not always so that explants removed from plants in a naturally dormant state will not grow or proliferate *in vitro*. Sometimes dormancy is broken by excising meristems from an otherwise dormant source (Ochatt and Caso, 1982; Powell *et.al.*; 1980).

As commercial micropropagation depends not only on the rate of multiplication but on normal and rapid morphogenetic development as well, the two observations of the present findings are of considerable interest. Growth and proliferation of adventitious shoots from the primary explants (stage II of the micro propagation scheme) is often achieved in species having storage stems (tubers/rhizomes/corms/bulbs) with much ease. Sub-culturing made by transferring isolated shoots or shoot clumps to fresh medium, having same or altered medium composition, frequently induce further adventitious shoots from the base of the shoots formed initially as has also been documented (Saha, 1997; Shirin *et.al.*, 2000; Jose *et.al.*, 2002; Swapna *et.al.*, 2004) and reviewed (George and Sherington; 1984). In the present situation when single isolated shoots that initiated directly on rhizome section explants on transfer to the same medium fortified with high BAP (2.0-3.0 mg l⁻¹) at least a low level of auxin appeared to be essential to restore a normal morphogenetic development of the isolated *in vitro* shoots (Fig 4.2c). However, a continued growth on the same medium for prolonged period exceeding ten weeks again caused elongation and morphological deformities in the microshoots (Fig 4.2d). Evidently, a balance between auxin-cytokinin appeared to be most effective for direct shoot proliferation from rhizome sections, but possibly the regenerated microshoots acquired the potentiality to produce their own optimum endogenous hormonal balance within a short period. Hence, addition of exogenous phytohormones might have disturbed that balance, in turn, causing elongation and morphological deformities in the microshoots cultured on same medium for a prolonged period.

Table 4.2.1 Morphogenetic response of isolated microshoots of *Chlorophytum borivilianum* regenerated directly on the rhizome section after six weeks of their transfer to MS medium supplemented with different hormone combinations

Treatments	Growth regulators (mg l ⁻¹)		Morpho-genetic response of the shoot initials	Nature of shoot growth	Mean height of micro shoot (cm)		Mean no of leaves / plant	
	IBA	BA			Original mean	Transformed mean	Original mean	Transformed mean
T ₁	0.0	0.0	-	-	0.0	0.71	0.0	0.71
T ₂	0.0	0.5	-	-	0.0	0.71	0.0	0.71
T ₃	0.0	1.0	+	Thin, elongated shoot	5.35	2.41	2.27	1.63
T ₄	0.0	1.5	+	Thin, elongated shoot	4.41	2.38	1.93	1.48
T ₅	0.0	2.0	+	Elongated, deformed shoot	4.28	2.17	3.47	1.96
T ₆	0.5	2.5	+	Elongated, shoot	4.30	2.33	6.13	2.56
T ₇	1.0	2.5	++	Normal shoots, (more multiple shoots at base)	2.83	1.83	3.07	1.85
T ₈	0.5	3.0	++	Normal shoots, (few multiple shoots at base)	2.07	1.59	6.80	2.66

F value: 118.008
SE_m: ± 0.0126

F value: 37.371
SE_m: ± 0.0430

Table 4.2.2 Homogeneous subsets of Morphogenetic response of height of isolated microshoots of *Chlorophytum borivillianum* regenerated directly on the rhizome section

Treatments	Subset for alpha = 0.05				
	1	2	3	4	5
T ₁	0.71				
T ₂	0.71				
T ₈		1.5907			
T ₇			1.8280		
T ₅				2.1677	
T ₆				2.3287	2.3287
T ₄				2.3761	2.3310
T ₃					2.4140

Table 4.2.3 Homogeneous subset of Morphogenetic response of no of leaves *Chlorophytum borivillianum* regenerated directly on the rhizome section

Treatments	Subset for alpha = 0.05			
	1	2	3	4
T ₁	0.71			
T ₂	0.71			
T ₄		1.4890		
T ₃		1.6273	1.6273	
T ₇		1.8527	1.8527	
T ₅			1.9587	
T ₆				2.5597
T ₈				2.6563

Figure 4.2



Regeneration *in vitro* through multiple shooting in the rhizome sections of Safed Musli (*Chlorophytum borivillianum*): a, isolated microshoots showing differential growth and morphogenetic response to different nutritional compositions; b, c, adventive multiple microshoots undergoing sustained shoot growth; d, prolonged growth on growth hormone supplemented medium induced more elongated adventive shoots with morphological deformities

4.3

Morphogenetic response of embryoids of *Chlorophytum borivillianum* following their culture on B₅ medium supplemented with varying IBA – BA combination.

4.3.1 Rationale

Of the three situations identified in the initial trial (section 4.1) one that were of special interest was the induction of shiny globular structures developed following six weeks of culture of the rhizome explant onto B₅ medium containing IBA and BA (IBA 1.0-1.5 mg l⁻¹ and BA 1.0-2.0 mg l⁻¹) combinations. However, these auxin cytokinin combinations although were conducive for the induction of globular embryoids but were inadequate to support the growth of these embryoid like structures to undergo morphogenetic changes for the development of whole plants. Therefore, the present study was undertaken to find the effect of amended growth regulator combinations in the same medium (B₅) that may help these early adventive embryoids (or proembryoids) for undergoing sustained growth and pass through the advanced stages of embryogenesis leading to whole plant regeneration.

4.3.2 Methodology

The globular structures produced over the corm explants were separated in clusters along with corm portion at base by trimming the explants into pieces. These were then subcultured on the same medium containing different combination of IBA – BA. Initially the cultures were kept in dark for 4-5 days. Then they were kept under continuous diffused light (500 lx) for another 10 days and then transferred to the 16h light regime at 2000 lx illuminance.

Treatments were replicated three times; each replication contained 10 cultures. Observations were recorded following 4 weeks of culture following destructive method where 5 out of 10 tubes of each replication were chosen at random.

4.3.3 Results and Discussion

Observations on the morphogenetic response of embryoids of *Chlorophytum borivillianum* following their culture on B₅ medium supplemented with varying IBA – BA combination after 4 weeks of culture have been summarized in the Table 4.3.1. Observations were of great interest as they revealed certain distinct role of auxin-cytokinin balance on cytodifferentiation and morphogenesis leading to whole plant regeneration through somatic embryogenesis.

It is interesting to note B₅ medium supplemented with N⁶-benzyladenine (BA 1.0 mg l⁻¹) alone was not conducive for the induction of direct somatic embryogenesis (Table 4.3.1) but it proved to be adequate to sustain initial growth and morphogenesis in the embryoids that are once initiated (Fig 4.3a). At least 15.66 embryoids (Table 4.3.1) grew to advanced stages of embryonic development that later ceased (Fig 4.3b, d), while BA at a lower level (BA 0.5 mg l⁻¹) failed to support this growth. Whereas an increase in the BA concentration to 2.0 mg l⁻¹ has been found to be adequate not only in sustaining the growth and morphogenesis of induced adventive embryoids to advanced stages of development, but also in regenerating large number of microplanlets in absence of any auxin. Interestingly, addition of IBA at very low concentration (0.2 mg l⁻¹) was encouraging. When IBA 0.2 mg l⁻¹ was added in combination with BA 1.0 mg l⁻¹, it sustained the growth and production of embryoids *in vitro* but could not significantly enhance the number of embryoids produced per culture. In contrast, supplementing the

medium with IBA 0.2 mg l^{-1} + BA 2.0 mg l^{-1} not only enhanced the number of developed adventive embryoids per explant significantly, but also ensured normal morphogenesis in the globular embryoids for their regeneration into whole plants. Best results were obtained when the medium was fortified with IBA 0.2 mg l^{-1} + BA 2.0 mg l^{-1} producing 53 - 69 embryoids per explant (Fig 4.3c). A further increase in the auxin level (IBA $0.5 - 1.0 \text{ mg l}^{-1}$ and above) in the medium had a detrimental effect on growth and morphogenesis. IBA at 0.5 mg l^{-1} with BA $1.0 - 3.0 \text{ mg l}^{-1}$ showed initial growth of the embryoids but could not support their development into plantlets; at the same time the mean number of embryoids was found to be reduced from 64.80 to 7.46 (Table 4.3.1) . These results clearly revealed that supplementing the medium with cytokinin N⁶-benzyl adenine (BA) alone although supported adequately the production of adventive embryoids and their transformation into microplants, but the presence of an auxin IBA at very low dose (IBA 0.2 mg l^{-1}) seemed to be beneficial as is evident from the increased number of adventive embryoids reaching advanced stages of embryonic development and plantlet regeneration (Fig 4.3c-d).

It is well established that both direct and indirect organogenesis , as cells come to comprise the new meristems, they adopt a different inherent programming (or determination) which decides the subsequent pattern of development (Amirato, 1985) and this process of redetermination is apparently induced by the effect of growth regulators (in combination with the presence of correct nutritional factors). Although growth regulators may also help to induce direct organogenesis (shoot / embryos) *in vitro*, the cells of explant in many instances appear to be partially predetermined to a particular morphogenetic pathway, so that it takes only a slight change in environment to induce them to form morphogenetic meristems instead of progressing to become a differentiated cell within the intact plant (Thomas, 1978). However, the extent of pre determination varies between different tissues in the whole plant and between different plant species (Cassells,

1979). Pathway of regeneration via direct embryogenesis has been grouped into a number of rhizomatous species (Haque *et.al.*, 2000; Kacker, 1993; Saha, 1997).

In the present case germinating large rhizome sections formed the initial explant. Therefore, the rhizomatous tissue /cells, atleast at certain locales in the peripheral region were expected to be predetermined to produce meristems destined to undergo morphogenesis to form shoots. And the process got enhanced where the rhizome sections were put to nutrient medium (MS) rich in NO_3^- , supplemented with high cytokinin as has been observed in initial study (Section 4.1 and 4.2). On the contrary, following culture of the rhizome sections as explants on B_5 medium, (high NO_3^- with low reduced nitrogen source $(\text{NH}_4)_2\text{SO}_4$ content) supplemented with high auxin - cytokinin ($\text{IBA } 1.0\text{-}1.5 \text{ mg l}^{-1} + \text{BA } 1.0\text{-}2.0 \text{ mg l}^{-1}$) produced globular embryoids (Section 4.1) but the process ceased to continue until these early embryoids were transferred to a medium supported with high cytokinin but low or no auxin (Table 4.3.1).

As it appears from the present findings that the mature germinating rhizomes were predetermined for producing shoots, but possibly underwent a slightly changed pathway to produce adventitious embryos directly on the explant tissue under changed nutritional conditions as has been suggested earlier (Bhattacharya and Sen, 1980; Sharp *et. al.*, 1980).

These results further revealed that although redetermination of the rhizomatous cells to undergo embryogenesis was induced by a higher level of IBA ($1.0\text{-}1.5 \text{ mg l}^{-1}$) but induced cells with high auxin in the medium failed to support the embryogenic cells to grow beyond globular stage and the embryo development ceased (Fig 4.3a). It seemed necessary either to omit or to reduce auxin concentration for the development of the globular adventive embryos to heart and torpedo stages and finally to plants (Fig 4.3c-d). The process was

significantly enhanced (Table 4.3.2) where low dose of IBA (0.2 mg l^{-1}) was coupled with increased level of BA (2.0 mg l^{-1}), however, further increase in either IBA or BA both again inhibited the process (Tables 4.3.1 & 4.3.2).

These findings are in agreement with the fundamental postulations of Sharp *et. al.*, (1980) for the process of somatic embryogenesis, that it is often initiated in media containing high level of auxin, but embryos are not formed until the auxin concentration is reduced. In *Chlorophytum* too auxin induced embryogenic determination in a proportion of rhizomatous cells but at the same time caused these induced cells to cease division and the division in the 'embryogenic mother cells' or 'pro embryos' is only resumed in lower auxin or in no auxin conditions.

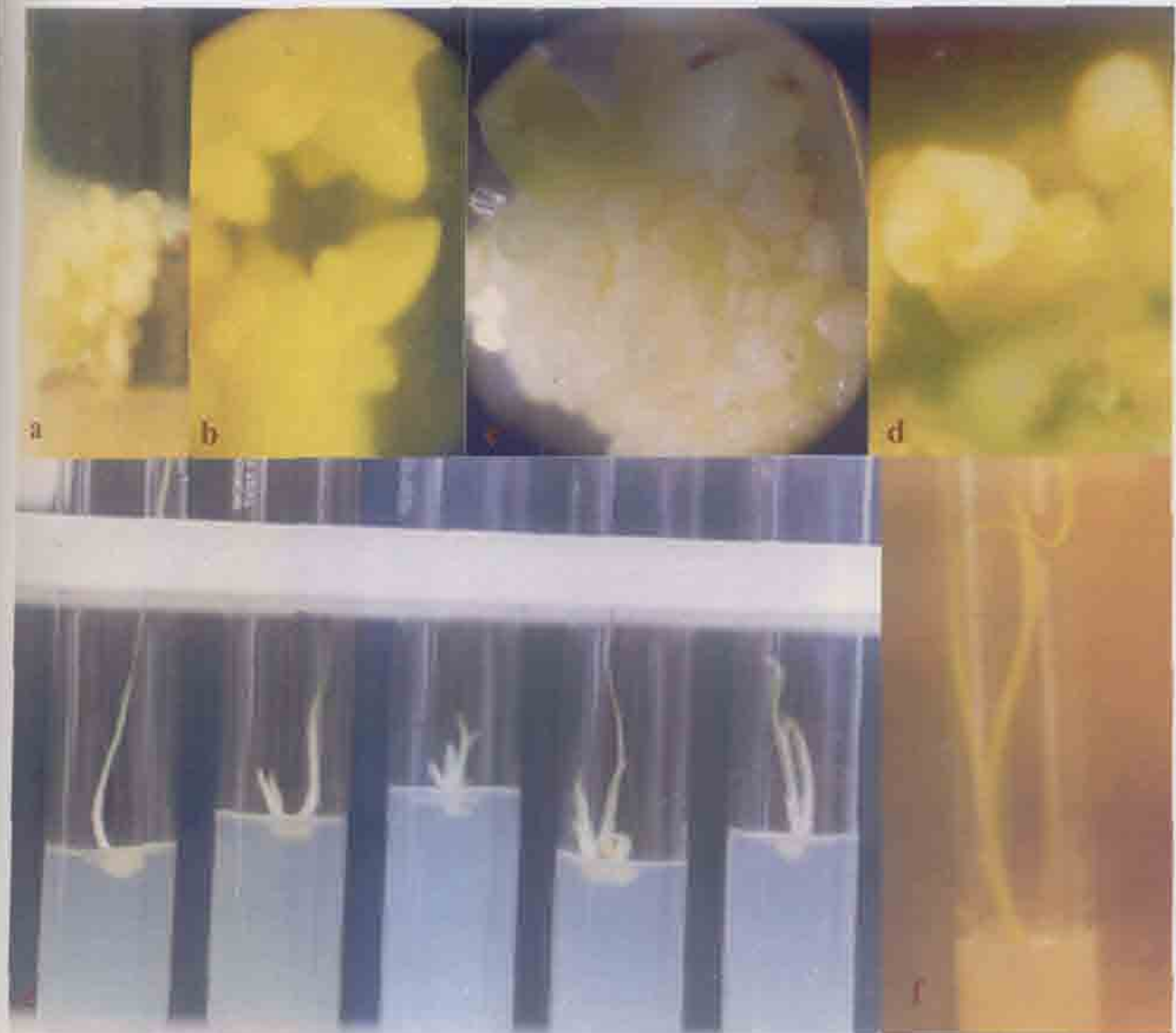
Plantlets regenerated through direct embryogenesis were isolated singly and transferred to B₅ basal medium depleted of growth regulators was beneficial for whole plant development and developed single plants could be raised within 10 – 15 days (Fig 4.3e, f).

The present protocol of cloning in *Chlorophytum* through direct embryogenesis appeared to be highly efficient particularly in terms of role of multiplication. Following transfer to modified B₅ medium for growth that is in the stage II ~64 adventive embryoids may be developed to advanced stages in every 4 week cycle having potentiality to regenerate into whole plants and the cycle may continue.

Table 4.3.2 Homogeneous subsets of morphogenetic response of embryoids of *Chlorophytum borivillianum*

Treatments	Subset for alpha = 0.05						
	1	2	3	4	5	6	7
T ₁	0.71						
T ₁₀	0.71						
T ₁₁	0.71						
T ₁₂	0.71						
T ₈		2.7900					
T ₆			3.3733				
T ₉				3.8267			
T ₇				3.9233	3.9233		
T ₂				4.0233	4.0233		
T ₄					4.2400		
T ₃						5.8833	
T ₅							8.0733

Figure 4.3



Regeneration *in vitro* through direct embryogenesis in the rhizome sections of Safed Musli (*Chlorophytum borivillianum*) in B₅ medium: a, bunch of globular embryoids originating from the upper end of the corm section; b - d, stereomicroscopic view of profusely growing embryoids at different stages (globular, heart, torpedo and early cotyledonary) of development; e, plantlets regenerated from isolated culture of mature embryoids obtained *in vitro*; f, plants growing in hormone depleted medium.

4.4

Indirect regeneration of microplants from intervening granular calluses of *Chlorophytum borivillianum*

4.4.1 Rationale

The last situation identified in the initial study (section 4.1) was also considered to be of immense interest as it produced profuse granular calluses from the rhizomatous tissue of the rhizome explants of *Chlorophytum borivillianum*, which continued to grow expanding the callus mass but did not show any marked morphogenetic change even after ten to twelve weeks of culture. The very presence of minute granular structures over the calluses drew attention and necessitated undertaking an experiment to try if this callus has any promise to undergo callogenesis to produce regenerated plants *in vitro*. Accordingly, an attempt was made to subculture the calluses in modified media combinations to find if any of these combinations is conducive towards induction of cytodifferentiation leading to the regeneration and growth of microshoots. Regeneration through rhizomatous callus has been reported in a number of rhizome bearing species viz., *Kaempferia galanga* (Vincent *et al.* 1992), *gladiolus* (Saha, 1997), *Alpina longa* (Anand *et.al.*, 1997) and in many others.

4.4.2 Methodology

Granular calluses induced over the rhizome sections in the initial trial were cut into pieces and separated along with the rhizomatous tissue aseptically and then subcultured on MS medium as was done earlier but in different treatment combinations of IBA (0.3 – 2.0 mg l⁻¹) with BA (1.0 – 3.0 mg l⁻¹) or Kinetin (1.0 – 3.0 mg l⁻¹) as treatments. The treatments were

replicated three times, each replication containing seven cultures. The cultures were maintained in the culture room having $25 \pm 1^\circ \text{C}$ temperature and 70% RH under 16h light phase of ~2000 lx illuminance. Observations were recorded following 4 weeks of culture following destructive method where five out of seven cultures were chosen at random.

4.4.3 Results and Discussion

Response of friable granular rhizome callus of *Chlorophytum borivillianum* subcultured on MS medium supplemented with varying concentrations of IBA - BA and IBA - KIN combinations after 4 weeks of culture were recorded and the observations are summarized in Table 4.4.1. It is clearly evident from the present observations that both kinetin and N⁶-benzyl adenine (BA) are potential cytokinins to induce cytodifferentiation leading to callogenesis but only in presence of a low level of auxin, IBA in this case (Fig 4.4a). Importantly, either a slight increase in the auxin concentration or its withdrawal from the medium totally inhibited the process of callogenesis revealing further a very critical role of auxin – cytokinin balance. However, kinetin appeared to be a more potent cytokinin as compared to BA at least for microshoot regeneration through an intervening callus phase, as is evidenced from the mean number of microshoots per explant. When the medium was fortified with IBA 0.5 mg l^{-1} and kinetin $1.0 - 3.0 \text{ mg l}^{-1}$ large number of microshoots were induced (Fig 4.4b) and the mean number varied from 11.27 to 47.67 (Table 4.4.1), highest being recorded for IBA 0.5 mg l^{-1} and Kinetin 2.0 mg l^{-1} . However, these shoots were stunted and relatively thick in nature. On the other hand, BA $1.0 - 3.0 \text{ mg l}^{-1}$ in presence of IBA $0.3 - 0.8 \text{ mg l}^{-1}$ although induced microshoots from the intervening callus but at significantly lower frequency ($4.13 - 17.53$ microshoots per explant) as is also evident from Table 4.4.2 and the microshoots were mostly lanky (Fig 4.4f). Unambiguously, it may be inferred that for developing a

protocol of micropropagation of Safed Musli via intervening callus through callogenesis IBA 0.5 mg l^{-1} in combination with Kin 2.0 mg l^{-1} seemed to be ideal, as its effect has been significantly superior to all other treatments (Table 4.4.2). However, what the present experiment failed to reveal if the initial culture of fresh rhizome explants on a high auxin cytokinin combination (IBA 2.0 mg l^{-1} and kinetin ($2.0\text{--}3.0 \text{ mg l}^{-1}$)) was essential for induction of the process of callogenesis in the rhizomatous tissue of *Chlorophytum borivillianum* as observed in the initial experiment (Section 4.1).

Direct adventitious shoot induction from shoot bud bearing rhizome explants of a number of rhizomatous species have been widely reported in the recent past (Ferwerda, 1994; Dogra *et al.*, 1994; Meenakshi *et al.*, 2001; Sunitibala *et al.*, 2001; Swapna *et al.* 2004). Relatively there are very few reports on indirect microshoot regeneration through callus induction on rhizome explants. Anand *et al.* (1997) reported callogenesis from rhizome callus. They found that for callus induction a high auxin (2,4-D 1.5 mg l^{-1} or NAA 1.0 mg l^{-1}) and low cytokinin (BA 0.5 mg l^{-1}) was necessary in *Curcuma aromatica* rhizomes while for complete organogenesis (shoot formation) low auxin–high cytokinin (IAA 0.5 mg l^{-1} –BA 1.0 mg l^{-1}) was essential. In case of indirect organogenesis in *Kaempferia galanga* too, as elsewhere mentioned (Section 5.1), callus from rhizome section was induced in MS medium supplemented with relatively higher concentration of auxins (2,4-D) but regenerated plantlets through embryogenesis (Vincent *et al.* 1992) or callogenesis Lakshmi and Mythili (2003) occurred only where MS medium was supplemented with relatively low level of auxin or no auxin at all.

These observations further confirmed the earlier contention (Section 4.3) that auxin concentration played a critical role in indirect organogenesis, where higher dose of auxin initiates cell division to produce callus and at the same time induce redetermination towards organogenic pathway (callogenesis)

or organogenesis) in otherwise predetermined rhizomatous cells of the germinating rhizome explants, but morphogenetic transformation occurred only following the transfer of the cultures to low auxin or no auxin medium.

Microshoots regenerated on IBA- Kinetin supplemented MS medium were aseptically withdrawn (Fig 4.4c) and isolated single shoots were transferred to hormone depleted medium for rooting (Fig 4.4d). Like the shoots the roots were also unusually thick and stunted (Fig 4.4e). However, on transfer to sand - soil (1:3) mixture these *in vitro* regenerated plantlets survived and rooted normally (Fig 4.4g).

Table 4.4.1: Response of friable granular callus of *Chlorophytum borivillianum* subcultured on MS medium supplemented with varying concentrations of IBA-BA and IBA-KIN combinations after four weeks of culture.

Treatments	Growth regulators (mg l ⁻¹)			Induction of callus	Mean no of microshoots / explant	Transformed mean	Mean height (cm) of the microshoots	Nature of shoot growth
	IBA	Kin	BA					
T ₁	0.0	1.0		+	0	0.71	-	-
T ₂	0.5	1.0		+	16.00	4.00	2.75	Thick, stunted
T ₃	0.5	2.0		+	47.67	6.53	4.70	Thick, stunted
T ₄	0.5	3.0		+	11.27	3.35	3.03	Thick, stunted
T ₅	0.8	1.0		++	0	0.71	-	-
T ₆	0.8	2.0		++	0	0.71	-	-
T ₇	0.8	3.0		+	0	0.71	-	-
T ₈	1.0	2.0		+++	0	0.71	-	-
T ₉	1.0	2.5		++	0	0.71	-	-
T ₁₀	2.0	3.0		+++	0	0.71	-	-
T ₁₁	0.0		1.0	+	0	0.71	-	-
T ₁₂	0.3		1.0	+	9.2	3.01	2.62	Thin lanky
T ₁₃	0.5		1.0	+	17.53	4.19	5.21	Thin lanky
T ₁₄	0.5		2.0	+	4.13	1.97	3.90	Thin lanky
T ₁₅	0.5		3.0	+	4.47	2.05	2.68	Thin, deformed
T ₁₆	0.8		3.0	++	4.6	2.11	1.31	Thin, deformed
T ₁₇	1.0		1.0	+++	0	0.71	-	-
T ₁₈	1.0		2.0	++	0	0.71	-	-
T ₁₉	2.0		3.0	+++	0	0.71	-	-

F-value: 81.140

SE_m ± : 0.262

Table 4.4.2 Homogeneous sub sets of the response of friable granular callus of *Chlorophytum borivilianum* after 6 weeks of culture

Treatments	Subset for alpha = 0.05				
	1	2	3	4	5
T ₁	0.71				
T ₅	0.71				
T ₆	0.71				
T ₇	0.71				
T ₈	0.71				
T ₉	0.71				
T ₁₀	0.71				
T ₁₁	0.71				
T ₁₇	0.71				
T ₁₈	0.71				
T ₁₉	0.71				
T ₁₄		1.9693			
T ₁₅		2.0493			
T ₁₆		2.1047			
T ₁₂			3.0093		
T ₄			3.3470		
T ₂				3.9993	
T ₁₃				4.2040	
T ₃					6.5250

Figure 4.4



Indirect regeneration of microplants from intervening rhizome calluses of *Chlorophytum borivillianum* in MS medium: **a**, callus proliferation from rhizome explant and initiation of callogenesis; **b**, indirect shoot development from subcultured granular calluses; **c**, aseptically withdrawn microshoots (relatively stunted in nature); **d**, single plant transferred for rooting; **e**, a rooted single plant; **f**, a lanky plant regenerated on IBA-BA medium; **g**, regenerated plants transferred to sand-soil mixture (1:3)

4.5

Effect of Gibberellic acid (GA₃) on the production and growth of *Chlorophytum* microshoots cultured *in vitro*

Rationale:

From the findings of the preceding section it can conclusively be inferred that for developing a protocol of micropropagation of Safed Musli via intervening callus through callogenesis IBA 0.5 mg l⁻¹ in combination with Kinetin 2.0 mg l⁻¹ seemed to be beneficial based on the number of microshoots per culture. However, the mean height of the *in vitro* shoots varied from 1.31 – 5.21 cm even after 4 weeks of culture. Evidently, there was a need to further manipulate the growth regulators, if possible, so that there is an enhancement in the frequency of adventive microshoot production which successively grows into large shoots as well as sustains normal morphogenesis. In a search for such growth regulator combination best responding combinations of IBA – Kinetin and IBA – BA were tried with / without varying concentrations of gibberellic acid, which is known to cause stem elongation.

Methodology:

Present trial was conducted to study the effect of GA₃ on the production and growth of preformed *Chlorophytum* microshoots *in vitro*. For the purpose two sets of trials were conducted. In the first phase the MS medium was supplemented with IBA 0.5 mg l⁻¹ + BA 2.0 mg l⁻¹ with different concentrations of GA₃ (0.0 – 2.5 mg l⁻¹). And in the second set the medium was supplemented with IBA 0.5 mg l⁻¹ + Kinetin 1.0 mg l⁻¹ along with same

concentrations of GA₃. Response on growth and morphogenesis were recorded for both sets of experiments after eight weeks of culture.

Results and Discussion

Observations in respect of growth and morphogenesis in terms of number of microshoots, mean height of the shoots and nature of growth were recorded separately for each set of trial and Table 4.5.1 represents the data of both sets of experiments. These observations clearly revealed that GA₃ has a synergistic effect on both the combinations of auxin – cytokinin (IBA – BA and IBA – Kinetin). The effect was more pronounced in case of IBA – Kinetin combinations (IBA 0.5 mg l⁻¹ + Kin 2.0 mg l⁻¹) at least for microshoot proliferation, as is evidenced through significantly higher increase (17.96 - 38.71 %) in microshoot production as compared to only 27.06 % increase for IBA – BA combination (IBA 0.5 mg l⁻¹ – BA 1.0 mg l⁻¹). On contrary, the number of large shoots per culture was enhanced by GA₃ (1.0-1.5 mg l⁻¹) more effectively in combination with IBA – BA as compared to IBA – Kinetin registering an increase of 256.15 - 262.16 % as against 168.37 - 221.40 % respectively, but there has been no difference in the final value being around 10.06 - 12.06 large shoots per culture in either of the situations. While increasing the mean height of the microshoots GA₃ at 1.0 mg l⁻¹ seemed to be more effective with IBA – Kin combination as compared to when GA₃ 1.5 mg l⁻¹ was added to IBA – BA combination registering an increase of 42.11 % and 34.21 % increase over controls (i.e., absence of GA₃) respectively.

Most interestingly, however, present observations further revealed an inversed correlation of number of microshoots to number of large shoots and mean height of the shoots. Treatment combination of IBA 0.5 mg l⁻¹ + Kin 2.0 mg l⁻¹ + GA₃ 1.5 mg l⁻¹ produced highest microshoots (66.26) per culture having 5.03 cm mean height and 8.40 large shoots per culture,

while IBA 0.5 mg l^{-1} + Kin 2.0 mg l^{-1} with lower GA₃ concentration of 1.0 mg l^{-1} produced 56.86 microshoots per culture having higher mean height (6.58 cm) and more large shoots (10.06) per culture. But under either of the treatments shoot growth was found to be normal. Treatments of IBA 0.5 mg l^{-1} + BA 1.0 mg l^{-1} with GA₃ 1.0 mg l^{-1} produced higher number of microshoots having lower mean shoot height as well as number of large shoots, while with GA₃ 1.5 mg l^{-1} it produced lesser number of microshoots but higher mean shoot height as well as number of large shoots (Table 4.5.1).

These results clearly confirm a synergistic role of GA₃ on the effect of both the auxin - cytokinin combinations (IBA - Kin and IBA - BA) that helped in enhancing the rate of microshoot production, increasing the number of large shoots per culture and as well as the mean height of the microshoots. It may well be concluded that supplementing the medium with a combination of IBA 0.5 mg l^{-1} + Kin $1.0\text{-}1.5 \text{ mg l}^{-1}$ + GA₃ 1.0 mg l^{-1} proved to be highly significant (4.5.2) and considered ideal to obtain highest number of microshoots with normal morphogenesis. Although mean number of large shoots and mean height of microshoots were significantly higher in other treatments.(Table 4.5.3 and 4.5.4).

GA₃ has been known to have a significant effect on stem elongation of genetically dwarf and rosette plants and promoting flowering etc. growing *ex vitro*. In such cases condensed internodes elongates with the application of GA₃. Several of these effects are known to be caused by stimulation in the synthesis and activity of specific enzymes or changed availability of endogenous auxins. It has also been thought that the growth promotive activity of GA₃ is due to enhanced transport of potassium ions (De La Guardia and Benlloch, 1980). Although GA₃ has been reported for its inhibitory action on rhizogenesis and callogenesis from callus (Murashige, 1961), there are reports of meristems and shoot tip cultures where GA₃ at

low concentrations significantly promoted the number of microshoots *in vitro* (Morel *et al*, 1968). Similar reports are available where addition of low concentrations of GA₃ at 0.03 mg l⁻¹ to the medium containing auxin and cytokinin or a cytokinin alone has improved the growth of the meristem tips, inhibited callus proliferation and increased the proportion of meristems that produced shoots in Cassava (Karthi *et al*, 1974). Reports where GA₃ accelerating the proliferation of axillary buds have been severally made in different species such as in Sisal, a rhizome bearing fibre crop (Hazra *et.al*, 2002), in tea (Nakamura, 1991; Saha, 1997) and in other ornamentals (Torres and Carlisi, 1986). The frequency of shoot proliferation has been reported to be markedly influenced by the synergistic action of gibberellic acid (GA₃) when used at an optimal concentration (0.4 mg l⁻¹) along with BA (2.0 mg l⁻¹) without any proximal callusing in *Vitex negundo*, a medicinal shrub, registering about 92% enhancement in axenic multiple shoot production (Sahoo and Chand, 1998).

Although rooting was successfully induced in hormone depleted medium, it was best induced (~85%) in shoots excised from proliferated shoot cultures on half-strength MS medium augmented with an optimal combination of indole-3-acetic acid and indole-3-butyric acid each at 0.5 mg l⁻¹.

It was interesting to note that adding GA₃ not only enhanced shoot length but also the number of regenerated shoots per culture, which consequently proved the regeneration via callus phase the most efficient of the three situations described. In fact the highest number of microshoots (~66 per culture) could be produced every 8 weeks cycle, if so continued under present conditions.

4-1

Table 4.5.1 Effect of Giberllic acid (GA) on the production and growth of *Chlorophytum* microshoots cultured *in vitro* after 8 weeks of culture

Treatments	Growth Regulators (mg l ⁻¹)				No. of microshoots / tube	No. of large shoots / tube	Mean height of the shoots (cm)	Nature of shoot growth
	IBA	Kin	BA	GA				
T ₁	0.5	2.0		0.0	48.20	3.40	4.63	Stunted
T ₂	0.5	2.0		1.0	56.86	10.06	6.58	Normal
T ₃	0.5	2.0		1.5	66.26	8.40	5.03	Normal
T ₄	0.5	2.0		2.0	28.0	2.53	4.94	Thin, lanky
T ₅	0.5	2.0		2.5	19.86	3.20	4.46	Thin, lanky
T ₆	0.5		1.0	0.0	16.26	3.23	4.94	Stunted
T ₇	0.5		1.0	1.0	20.66	11.86	5.97	Normal
T ₈	0.5		1.0	1.5	15.73	12.06	6.36	Thin
T ₉	0.5		1.0	2.0	11.40	7.06	5.03	Thin, deformed
T ₁₀	0.5		1.0	2.5	7.13	3.93	4.06	Thin, deformed

F-value: 205.35 **F-value:** 24.986 **F-value:** 3.672
SE_m ±: 6.377 **SE_m ±:** 1.733 **SE_m ±:** 0.409

Table 4.5.2 Homogeneous sub sets of the effect of gibberellic acid on number of microshoot production of *Chlorophytum borivillianum* after 8 weeks of culture

Treatments	Subset for alpha = 0.05						
	1	2	3	4	5	6	7
T ₁₀	7.1333						
T ₉	11.4000						
T ₈		15.7333					
T ₆		16.2667	16.2667				
T ₅		19.8667	19.8667				
T ₇			20.6667				
T ₄				28.0000			
T ₁					48.2000		
T ₂						56.8667	
T ₃							66.2667

Table 4.5.3 Homogeneous sub sets of the effect of gibberellic acid on number of large shoot production in *Chlorophytum borivillianum* after 8 weeks of culture

Treatments	Subset for alpha = 0.05			
	1	2	3	4
T ₄	2.5333			
T ₅	3.2000			
T ₆	3.2300			
T ₁	3.4000			
T ₁₀	3.9333			
T ₉		7.0667		
T ₃		8.4000	8.4000	
T ₂			10.0667	10.0667
T ₇				11.8667
T ₈				12.0667

Table 4.5.4 Homogeneous sub sets of the effect of gibberellic acid on mean height of shoots in *Chlorophytum borivillianum* after 8 weeks of culture

Treatments	Subset for alpha = 0.05		
	1	2	3
T ₅	4.5667		
T ₁	4.6300		
T ₁₀	4.6600		
T ₄	4.9400	4.9400	
T ₃	5.0300	5.0300	
T ₉	5.1100	5.1100	
T ₆	5.4133	5.4133	5.4133
T ₈	5.8733	5.8733	5.8733
T ₇		6.1267	6.1267
T ₂			6.5867

4.6

Multiple shooting in germinating shoot base in *Chlorophytum borivillianum*

4.6.1 Rationale:

Following the report of Purohit *et.al.* (1994) on micropropagation in *Chlorophytum borivillianum* using shoot base as explant a number of report on *in vitro* multiplication of this medicinal plant appeared in the recent past all using shoot base and/ or basal shoot disc as common explant (Gaikwad *et. al.*, 2003; Pudake and Dhumale, 2003; Basu and Jha, 2005) of course with variations in the growth regulator combinations and concentrations. These reports prompted the author to undertake a trial for comparison. Therefore, in a separate study an attempt was made to develop an efficient protocol of micropropagation in Safed Musli (*Chlorophytum borivillianum*) utilizing the shoot bases of germinating rhizomes as has been severally reported. The principal objective of the study had been to adopt the cultural as well as the nutritional conditions recommended by the earlier workers for micropropagation in Safed Musli and to introduce further amendments, if any, both in the culture technique and/or nutritional support required developing a more efficient protocol to scale-up *in vitro* cloning utilizing sprouting shoots as explant.

4.6.2 Methodology

Safed Musli rhizomes preserved in dormant condition under dry sand were planted in moist sand for germination i.e., sprouting of the shoot buds. The growing shoot buds were explanted with thin rhizome section at base. The germinating buds along with rhizome base were cultured aseptically following the normal sterilization procedure described earlier (Section 4.1) in

large bottles containing MS basal medium. Frequently the buds *in vitro* grew into solitary large shoots (Fig 4.6a). In some cases a few adventive microshoots developed from the base (Fig 4.6b) or callus was induced at basal region (Fig 4.6c), yet the apical dominance prevailed and the germinating shoot bud continued to grow into a large shoot. Expecting enhancement in the adventive multiple shoots, growing shoot buds were decapitated after 4 weeks and the shoot bases were removed from the rhizome section with the help of scalpel having a size of about 1-1.5 cm and were cultured in isolation in culture tubes containing MS medium supplemented with different combinations of IBA – BA or NAA – BA. A total of 29 treatment combinations, each with three replications were tried. Under each replication there were seven tubes. All tubes were incubated at an elevated temperature of $25 \pm 1^{\circ}\text{C}$ under 16 h light regime at 2000 lx illuminance. Observations were recorded after 4 weeks of culture following inoculation of the decapitated shoot bases. As all tubes under each replication failed to survive the data were recorded from the randomly chosen 5 tubes only under each replication of each treatment. The experiment was laid out following the complete randomized design (CRD).

4.6.3 Results and Discussion

Observations recorded on differential responses of shoot base explants after 4 weeks of culture of decapitated shoot bases have been summarized in the Table 4.6.1. The data were also analyzed using appropriate statistical methods.

These observations clearly revealed that although BA alone or in combination with either of the auxins IBA or NAA was conducive for induction of multiple shoots from the shoot bases, but the medium supplemented with BA 2.5 and 3.0 mg l⁻¹ alone yielded highest frequencies of multiple shoots (31.67 and 22.80 multiple shoots per explant respectively). Presence of either

of the auxins i.e., IBA or NAA either reduced the mean number of adventive shoots per shoot base or inhibited the process with the increase in concentration and frequently resulted in callus induction. Of the two auxins used IBA was found to be more compatible with BA in inducing adventive shoots from the shoot base explants *in vitro*, as is evident from ~20 adventive shoots in the combinations of IBA 0.5 mg l⁻¹ and BA 2.5-3.0 mg l⁻¹ (Fig 4.6d,e). However, the number of multiple shoots was reduced almost by 33% as compared to that of medium supplemented only with BA 2.5 mg l⁻¹. These results are in line with the earlier reports (Purohit *et al.*, 1994); Pudake and Dhumale, 2003; Basu and Jha, 2005).

Over 80% shoots rooted when transferred to MS medium with $\frac{3}{4}$ - strength inorganic and organic constituents with 3.0 mg l⁻¹ IBA (Fig 4.6g,h) and ~65% of the micropropagated plants were successfully established in pots. Such plants produced normal fasciculate storage roots following their transfer in pots as naturally occur.

Present findings clearly indicated a critical role of high cytokinin level in MS medium to favor both induction and development of adventitious multiple shoots from the *Chlorophytum* shoot base explants as has severally been reported by earlier workers (Purohit *et.al.* 1994) Gaikwad *et. al.*, 2003; Pudake and Dhumale, 2003; Basu and Jha, 2005). In the recent times direct multiple shoots from shoot base or young shoot bud explants have been reported in a number of rhizomatous species like *Scopolia perviflora* (Kang *et. al.*, 2004), *Curcuma zedoaria* (Nguyen *et.al.* 2005), *Glehnia littoralis* (Hui *et.al.*,1996), *Acorus calamus* (Harikrishnan *et.al.*,1997). Although varying nutritional and growth regulator combinations have been used by these authors, but presence of higher level of cytokinin was common. In tissue cultures, cytokinins appear to be necessary for plant cell division, while in their absence, metaphase but not prophase of mitosis is considerably

protracted and it has been suggested that cytokinins might be required to regenerate the synthesis of proteins involved in the formation as well as function of the mitotic spindle apparatus (Jouanneau, 1975; George and Sherrington, 1984). Therefore, cytokinins are very effective in promoting both direct and indirect shoot initiation often in combination with a low dose of auxin. Frequently a balance between auxin-cytokinin has been found most effective. In the present situation for direct shoot proliferation from shoot bases obtained from germinating rhizome bud cultures, best results were obtained when medium was supplemented with only BA (Table-4.6.1). Probably *Chlorophytum* bud sprouts are able to produce their own optimum endogenous auxin level required to balance the cytokinin level and an addition of exogenous auxin might have disturbed that balance and in turn reduced the frequency of microshoots.

In the present scheme of direct shoot formation from germinating shoot bases, stage I of micropropagation comprised of *in vitro* cultivation of germinating shoot buds on MS basal medium devoid of any growth regulators; while aseptic culture of decapitated shoot bases and production of adventive shoots in the same medium supplemented by relatively high cytokinin (BA) with low or no auxin (IBA or NAA) and subculture of shoot clumps in fresh medium comprised stage II. Isolating individual shoots to medium containing $\frac{3}{4}$ MS inorganics and organics supplemented with high auxin comprised stage III that prompted rooting.

TABLE 4.6.1: Multiple shooting in young shoot base culture of Safed Musli (*Chlorophytum borivillianum*) after 4 weeks of inoculation of decapitated shoot bases in MS medium

Treatments	Growth Regulators (mg l ⁻¹)	Callus Induction	Mean no of multiple shoots/plant	Transformed mean
T ₁	BA 1.0	-	0	0.71
T ₂	BA 1.5	-	6.07	2.96
T ₃	BA 2.0	-	16.67	4.18
T ₄	BA 2.5	-	31.67	5.65
T ₅	BA 3.0	-	22.80	4.60
T ₆	IBA 0.5 + BA 1.0	+	0	0.71
T ₇	IBA 0.5 + BA 2.0	-	4.47	1.57
T ₈	IBA 0.5 + BA 2.5	-	20.07	4.47
T ₉	IBA 0.5 + BA 3.0	-	20.13	4.49
T ₁₀	IBA 1.0 + BA 1.0	++	0	0.71
T ₁₁	IBA 1.0 + BA 2.0	++	0	0.71
T ₁₂	IBA 1.0 + BA 2.5	+	0	0.71
T ₁₃	IBA 1.0 + BA 3.0	-	0	0.71
T ₁₄	IBA 2.0 + BA 2.0	+++	0	0.71
T ₁₅	IBA 2.0 + BA 2.5	+++	0	0.71
T ₁₆	IBA 2.0 + BA 3.0	++	0	0.71
T ₁₇	IBA 2.0 + BA 3.5	+	0	0.71
T ₁₈	NAA 0.5 + BA 1.0	-	0	0.71
T ₁₉	NAA 0.5 + BA 2.5	-	0	0.71
T ₂₀	NAA 0.5 + BA 3.0	-	9.00	2.97
T ₂₁	NAA 0.5 + BA 4.0	-	12.27	3.48
T ₂₂	NAA 1.0 + BA 1.0	+	0	0.71
T ₂₃	NAA 1.0 + BA 2.5	++	0	0.71
T ₂₄	NAA 1.0 + BA 3.0	+	6.53	2.53
T ₂₅	NAA 1.0 + BA 4.0	+	9.40	3.05
T ₂₆	NAA 1.5 + BA 1.0	++	0	0.71
T ₂₇	NAA 1.5 + BA 2.5	+	0	0.71
T ₂₈	NAA 1.5 + BA 3.0	+	0	0.71
T ₂₉	NAA 1.5 + BA 4.0	-	0	0.71

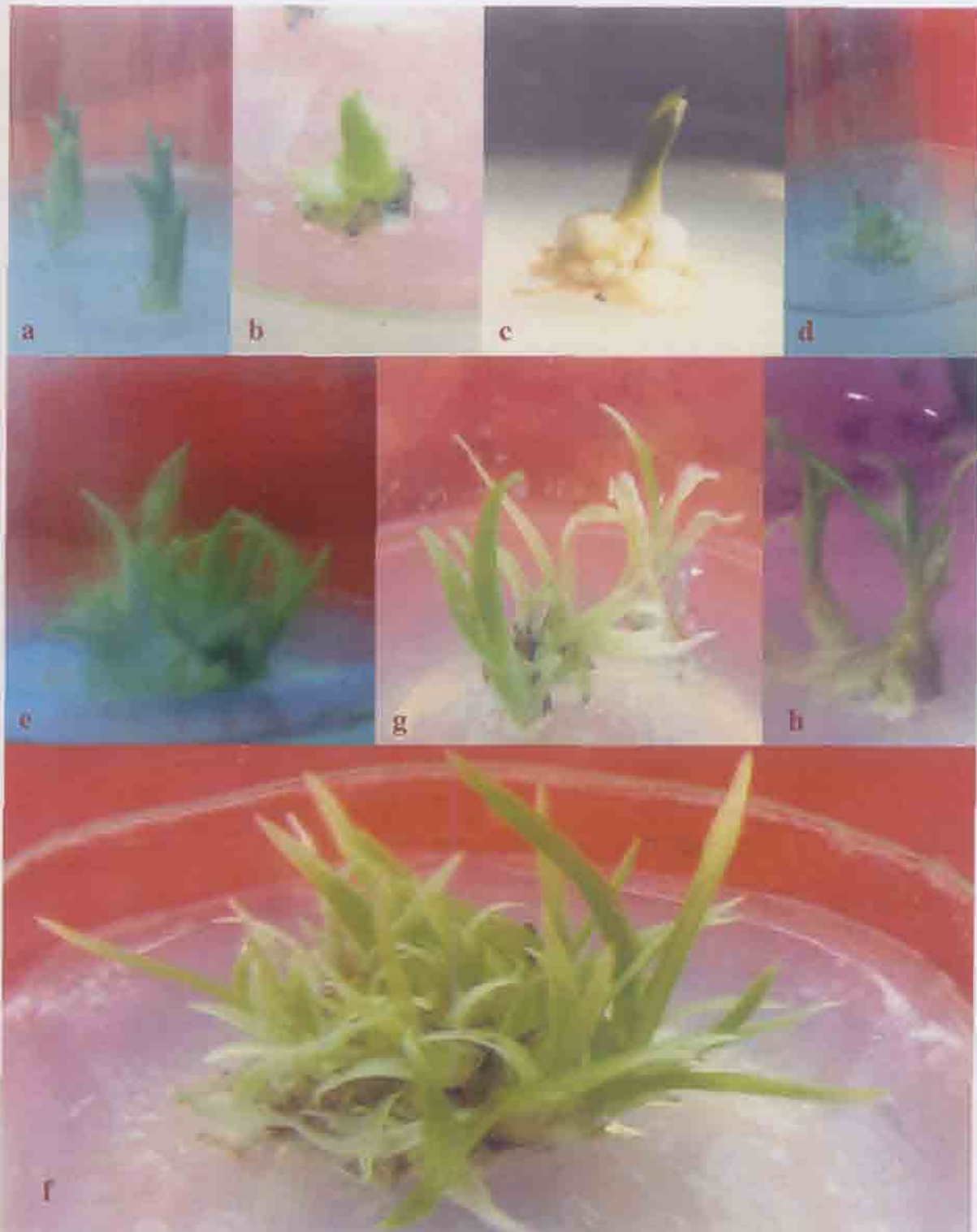
- no callusing, + poor callusing; ++ moderate callusing; +++ profuse callusing

F-value: 33.226; SE_m ± 0.113

Table 4.6.2 Homogeneous subsets of treatments for mean number of microshoots per explant in *Chlorophytum borivillianum* after 4 weeks of inoculation of decapitated shoot base

Treatments	Subset for alpha = 0.05							
	1	2	3	4	5	6	7	8
T ₁	0.71							
T ₆	0.71							
T ₁₀	0.71							
T ₁₁	0.71							
T ₁₂	0.71							
T ₁₃	0.71							
T ₁₄	0.71							
T ₁₅	0.71							
T ₁₆	0.71							
T ₁₇	0.71							
T ₁₈	0.71							
T ₁₉	0.71							
T ₂₂	0.71							
T ₂₃	0.71							
T ₂₆	0.71							
T ₂₇	0.71							
T ₂₈	0.71							
T ₂₉	0.71							
T ₇		1.5733						
T ₂₄			2.5300					
T ₂				2.9600				
T ₂₀				2.9700				
T ₂₅				3.0467				
T ₂₁					3.4767			
T ₃						4.1800		
T ₈						4.4667	4.4667	
T ₉						4.4900	4.4900	
T ₅							4.6033	
T ₄								5.6500

Figure 4.6



Multiple shooting in shoot base culture of Safed Musli (*Chlorophytum borivillianum*): a, solitary shoot growth showing apical dominance; b, rare initiation of a few adventive shoots from the shoot base; c, apical shoot bud culture and solitary growth of the apical shoot with compact callus at base without any adventive shoot development; d, initiation of multiple shoots from decapitated shoot bases; e-f, profuse multiple shooting from decapitated shoot bases that gradually turned brown if not subcultured periodically; g, isolated single shoot culture; h, isolated single shoots cultures giving out roots and more multiple shoots infrequently associated with callus.

4.7

Modifications in media constituents to scale-up *in vitro* production of multiple shoots in Safed Musli (*Chlorophytum borivilianum*)

4.7.1 Rationale

To meet the growing demand for planting material, a highly reproducible field-tested and cost-effective commercially viable micropropagation scheme is essential. Therefore, the preceeding study (section 4.6) was further extended to introduce possible modifications in the medium used to maximise the microshoot production. While doing so consideration was made for modifications in some inorganic and organic constituents of the MS medium which have been reported to be beneficial towards enhancement of microshoot production in rhizomatous herbs, such as Ammonium sulphate, Calcium nitrate (Basu and Jha ,2005) glycine and L-glutamine (Saha, 1997).

4.7.2 Methodology

The shoot initials produced in the previous combinations using the shoot base as explant in MS medium were isolated aseptically. The isolated single microshoots were cultured in culture tubes containing only BA with varying concentrations of different inorganic and organic constituents of the MS medium. Ammonium sulphate 100 mg l⁻¹, calcium nitrate 100 mg l⁻¹, glycine 10 mg l⁻¹ and L-glutamine 150 mg l⁻¹ were applied with BA 2.5 mg l⁻¹, either individually or in combinations. Each treatment contained three replications. Under each replication there were ten tubes. The subcultures were incubated at 25 ± 1°C under 16h light regime at 2000 lx illuminance. Observations were recorded from the randomly chosen 5 tubes under each

replication of the treatments after 5 weeks of culture. As all the external environments of the experiments were homogenized and experiment was done in laboratory condition, the experiment followed complete randomized design (CRD).

4.7.3 Results and Discussion

Table 4.7.1 shows the relative performance of microshoots production from the base of the isolated microshoots under various treatment conditions and the treatments were found to be highly significant (Table 4.7.2). Out of eight treatments one, which is taken as control, contained only BA 2.5 mg l^{-1} , as this was the best treatment for the induction of microshoots from shoot base explants (Section 4.6). Results (Table 4.7.1) showed clearly that supplementing the medium with organic constituents, glycine 10 mg l^{-1} and L-glutamine 150 mg l^{-1} did not show any beneficial effect towards enhancement in the mean number of microshoots. Average mean number of microshoots varied between 30.73 – 34.00 microshoots per shoot base. In contrast, supplementation of the medium with inorganic constituents ammonium sulphate (100 mg l^{-1}) singly or in combination with calcium nitrate (100 mg l^{-1}) both in presence of BA (2.5 mg l^{-1}) substantially stimulated the activity of adventive microshoot production as is evident from the increased mean number of microshoots (42.86 - 44.06 microshoots/shoot base). However, there has been no significant difference between effects of these two treatments as they belonged to the same homogenous subset (Table 4.7.2) revealing that ammonium sulphate singly played a major role in the process, while calcium nitrate had little or no effect. Interestingly, in other two treatments where MS medium was supplemented with BA + ammonium sulphate + glycine with or without calcium nitrate, recorded almost 93% and 83% increase in the microshoot production respectively over the control. Effects of these two treatments again belonged to the same homogenous group (Table 4.7.2) indicating clearly that there has been no significant difference between the treatments, which consequently revealed that calcium

nitrate, which has been reported earlier to promote microshoot production in *Chlorophytum* (Basu and Jha ,2005), did not contribute in a positive manner in the present situation. However, supplementing the medium with ammonium sulphate alone had a significant contribution in enhancing the microshoot production (42.86 microshoot per explant) and the same was further stimulated when it was added in combination with glycine 10 mg l⁻¹ (50.02 microshoots /explant).

It is well established that the growth and morphogenesis of tissue culture is markedly influenced by the availability of nitrogen and the form in which it is supplied. Divergent nitrogen sources (as nitrates or ammonium) are used in culture media, supplied in the form of well defined inorganic salts, amino acids, peptides, enzymatic digests of animal proteins, soya flour, malt or yeast. Among these, defined inorganic salts and amino acids have most frequently been used. As inorganic source most media contain more nitrate than ammonium ions and the form in which nitrogen is made available determine the nature of growth and morphogenesis. For instance it is reported that soybean callus grew better when NH₄NO₃ in MS was substituted by (NH₄)₂SO₄ of Gamborg's B₅ medium as a source of reduced nitrogen (Evans *et.al*, 1976). To support *in vitro* cell growth, cytodifferentiation and morphogenesis glycine is incorporated as the sole source of reduced nitrogen in many media formulations like SH (Schenk and Hildebrandt, 1972) media.

A supply of reduced nitrogen appears to be beneficial for at least two processes involved with cell division: the formation of cell wall (Meyer and Abe 1975) where glutamine does not substitute NH₄⁺; the synthesis of endogenous cell division factors where cytokinin requirement is reduced by the presence of glutamine or NH₄⁺.

48

Independent experimentation by several laboratories has clearly shown that NH_4 ions are necessary for somatic embryogenesis in cell and callus cultures (Reinert *et al.*, 1967; Walker and Sato, 1981). A supply of reduced nitrogen may be important for direct shoot formation in some species has also been reported by several authors in past (David, 1972; Gamborg *et al.* 1974). Direct adventitious shoot formation on cauliflower peduncle explants (Margara, 1969a, 1969b) and on Chrysanthemum pedicel explants (Roeset and Bokelmann, 1975) have been reported to affect the number of adventitious shoots by the increased nitrogen supply in the medium in a combined form of nitrate and ammonium.

Stimulation of direct shoot formation in species forming corms / rhizomes increasing the number of adventitious shoots by incorporation of reduced nitrogen as $(\text{NH}_4)_2\text{SO}_4$, glycine or glutamine have been severally reported in the recent times (Saha, 1997; Basu and Jha, 2005). Although the present results do fall in line with these reports but failed to record any beneficial effect of $\text{Ca}(\text{NO}_3)_2$ in enhancing shoot proliferation as reported by (Basu and Jha, 2005) in *Chlorophytum*.

The only organic source of reduced nitrogen which has responded positively is glycine in the present situation. It is interesting to note that glycine was ineffective when it was incorporated in the medium alone or with Glutamine, but its stimulating action was noted only in presence of $(\text{NH}_4)_2\text{SO}_4$ at low level and glutamine did not substitute for NH_4 as has earlier been reported (Meyer and Abel 1975; George and Sherington 1984). The stimulating effect of these reduced nitrogen sources may have alternative explanations (Mohanty and Fletcher, 1976; Dougall, 1977): i) nitrate reductase activity is induced by the presence of reduced nitrogen compounds, thus, facilitating the metabolism of nitrate nitrogen and increasing the availability of nitrogen – containing compounds within the cultured cells; ii) ammonium

ions effectively buffer plant nutrient media in the presence of nitrate and so enhance nitrate uptake that in turn stimulates microshoot production.

However, it was beyond the scope of present experiment to choose between the above possibilities, however, it may well be concluded that for an effective protocol of micropropagation in *Clorophytum* shoot base explants incorporating a combination of inorganic and organic sources of nitrogen along with BA is highly beneficial.

A comparison between different modes of *in vitro* regeneration in *Chlorophytum borivillianum* i.e., i) direct shoot proliferation from rhizome sections; ii) direct regeneration through adventive embryoid production from rhizome explants; iii) direct shoot proliferation from shoot base explants; and iv) indirect shoot production from intervening calluses convincingly revealed that the rate of multiplication has been highest by indirect regeneration through callogenesis (~66.26 microplants per explant every 20 weeks of culture cycle, Table 4.5.1), followed closely by microplants production through adventitious embryogenesis (~64.80 microplants per explant every 10 weeks of culture cycle, Table 4.3.1) and then by adventitious shoot production from shoot base explants (after 11 weeks cycle ~50.02 microshoots per explant, Table 4.7.1), while lowest rate of multiplication was recorded for direct microshoot production when rhizome sections were used as explant (43.40 microplants per explant after 13 weeks of culture, Table 4.1.2).

Therefore the most efficient mode of micropropagation in *Clorophytum* appeared to be through direct adventive embryoid production from rhizome explant, where regeneration of about 65 embryo plants in every 10 week cycle is achievable and the cycle continues.

Table 4.7.1 Effect of inorganic and organic constituents of MS medium in the production of multiple shoots from isolated microshoots of shoot base origin in *Clorophytum*

Treatment No.	Nutritional modifications (mg l ⁻¹)	Number of multiple shoots / explant	Mean number of multiple shoots / explant
T ₁	BA 2.5	29 – 34	31.00
T ₂	BA 2.5 + (NH ₄) ₂ SO ₄ 100.0	39 – 47	42.86
T ₃	BA 2.5 + (NH ₄) ₂ SO ₄ 100.0 + Ca (NO ₃) ₂ 100.0	38 - 49	44.06
T ₄	BA 2.5 + Glycine 10.0	32 – 38	34.00
T ₅	BA 2.5 + Glutamine 150.0	26 – 34	30.73
T ₆	BA 2.5 + Glycine 10.0 + Glutamine 150.0	28 – 36	33.46
T ₇	BA 2.5 + (NH ₄) ₂ SO ₄ 100.0 + Glycine 10.0	47 – 53	49.62
T ₈	BA 2.5 + (NH ₄) ₂ SO ₄ 100.0 + Ca (NO ₃) ₂ + Glycine 10.0	43 - 53	47.86

F value: 101.698; **SE_m ± :** 1.081

Table-4.7.2 Homogeneous subsets of treatments showing the effect of inorganic and organic constituents of MS medium

Treatments	Subset for alpha = 0.05				
	1	2	3	4	5
T ₅	30.7333				
T ₁	31.0000	31.0000			
T ₆		33.4667	33.4667		
T ₄			34.0000		
T ₂				42.8667	
T ₃				44.0667	
T ₈					47.8600
T ₇					49.6200

Chapter V

Micropropagation in Ekangi (*Kaempferia galanga*)

5.1

Initiation of culture and *in vitro* response of rhizome sections of Ekangi (*Kaempferia galanga* L.) to different nutritional combinations

5.1.1 Rationale

As mentioned earlier (Chapter III) Chandramul or Ekangi (*Kaempferia galanga*) is an aromatic rhizome bearing perennial herb belonging to the family Zingiberaceae. *Kaempferia galanga* is a valuable medicinal plant and is native to India, but currently it is cultivated mainly in South East Asia and China. The maximum used parts of this plant for medicinal purpose is rhizome, the modified swollen underground stem and roots. Conventionally, Ekangi is propagated vegetatively by the stored rhizomes but disease susceptibility and higher cost of production have restricted its cultivation (Rahman *et al.*, 2004). Micropropagation techniques can profitably be used to overcome such constraints and at same time may provide a ready to use protocol for geneticists interested in introducing variability in this species. Therefore, an attempt was made for rapid cloning.

Rhizome being the natural propagule, its *in vitro* regenerative capacity is considered to be highest as compared to any other plant part and hence, has been chosen as explant for present micropropagation study. As MS(Murashige and Skoog,1962) is the most frequently used medium for micropropagational studies in *Kaempferia galanga* (Swapna *et al.*, 2004; Jose *et al.*, 2002; Vincent *et al.* 1992a,1992b; Lakshmi and Mythili 2003, Shirin *et al.*, 2000) and in many other rhizomatous species e.g., *Curcuma longa*, *C. caesia*, *C. zedoaria*, *C. aromatica*, *Zingiber officinale*, *Alpinia galanga*, *K.*

rotunda (Him-de-Freitez and Paez-de-Casares, 1998; Anand and Hariharan, 1997; Anand *et al.*, 1999; Harikrishnan *et al.*, 1999; Hui *et al.*, 1996; Nguyen *et al.*, 2005; Borthakur *et.al.* 1998), the present program of work was also initiated with MS basal medium.

5.1.2 Methodology

The propagating time of Ekangi is mid May to June i.e., during pre-monsoon to early monsoon season. The axillary shoot buds of rhizome sprout under warm wet climate. The matured sprouting rhizomes which were ready for the normal propagation were utilized as explant.

Matured rhizomes with 3-5 sprouted axillary shoot buds were collected from the university medicinal plant garden. The materials were taken to the laboratory and after initial cleaning with forceps the rhizomes were kept under running water for 30 – 40 minutes, followed by a thorough washing with Triton – X. To disinfect the rhizomes were treated with Bavistin 0.1 mg l⁻¹ of water for 2 hours and then by rinsed with distilled water. The rhizomes were cut into large pieces and then were surface sterilized as done in *Chlorophytum* (Section 4.1). It was identified that 5 – 6 minutes incubation in 0.1% mercuric chloride solution was optimum for Ekangi rhizomes. Following proper rinsing with sterile water rhizome pieces were cut into 5-6 mm sections.

To screen out carryover contaminations with explants, initially the rhizome sections were aseptically cultured in agar gelled MS basal medium devoid of any growth hormone. Following 7 days incubation the sterile explants were transferred to same medium containing auxins (2, 4-D, NAA, IBA each 0.5 – 1.5 mg l⁻¹) and cytokinin (BA 0.1 - 2.5 mg l⁻¹).

Following inoculation, cultures were maintained in the culture room at $25 \pm 1^\circ \text{C}$ and exposed to diffused light of 500 lx illumination for 10 days. Later the cultures were maintained under of 2500 lx illuminance maintaining 16h photoperiod. Each treatment had three replications and under each replication five tubes were maintained. Data was recorded after 4 weeks of culture. Due to fungal contamination some tubes had to be discarded and observations were finally recorded from 3 tubes under each replication of each treatment. The experimental condition was homogenous, so all the trials were conducted following the Complete Randomized Design.

5.1.3 Results and Discussion

Observations on initial response were recorded after 5 weeks of culture and are presented in Table 5.1.1. At this initial phase of the study as the objective was to identify a nutritional composition favourable towards successful culture initiation for micropropagation using the rhizome sections, only visual observations of the response effects were recorded.

Observations recorded in the table clearly indicated that a very low level of BA i.e., $0.1 - 0.5 \text{ mg l}^{-1}$ in combination with either of the auxins used (NAA, IBA or 2,4-D each at $0.5 - 1.5 \text{ mg l}^{-1}$) failed to give any beneficial result towards adventive *in vitro* shoot formation or shoot bud development in the rhizome sections except varying degree of explant swelling, callus induction and rhizogenesis (Fig. 5.1 a-d). However, a clear variation was observed in respect of the effectiveness of the three auxins. So far as callus induction is in question NAA appeared to be the weakest while 2, 4-D being the most potential auxin to induce callus growth from rhizome explants. Both IBA and 2,4-D at 1.5 mg l^{-1} induced prolific callus almost covering the entire explant (Fig. 5.1 a), while NAA at 1.5 mg l^{-1} with BA 0.5 mg l^{-1} induced vigorous root development all along the rhizome explant (Fig. 5.1 b), with little or no callus induction. However, with increase in BA

concentration above 0.5 mg l^{-1} (BA $1.5 - 2.5 \text{ mg l}^{-1}$) callus induction virtually ceased, sprouting and growth of axillary shoot buds along with the induction of adventive shoot buds even over the cut explant surface was observed irrespective to the kind of auxin used (Fig. 5.1 c). A combination of BA 2.5 mg l^{-1} with NAA 1.5 mg l^{-1} was found to be conducive for distinct organogenesis producing 4-6 developed adventive shoot buds, infrequently associated with adventive rhizogenesis from some locales of the explant (Fig. 5.1 d).

These observations were corroborative with the understanding about the process of *in vitro* organogenesis from rhizomatous tissue as witnessed in case of *Chlorophytum* (Chapter IV) and also with the earlier findings in *Kaempferia* rhizome explant culture experiments (Swapna *et al.*, 2004; Jose *et al.*, 2002; Shirin *et al.*, 2000), as well as in other rhizome bearing herbs (Him-de-Freitez and Paez-de-Casares, 1998; Anand and Hariharan, 1997; Harikrishnan *et al.*, 1999; Hui *et al.*, 1996; Nguyen *et al.*, 2005; Begum *et al.*, 1995; Haque *et al.*, 1999; Malamung *et al.*, 1991) of medicinal importance.

In Ekangi (*Kaempferia galanga*) indirect plantlet formation has been reported both through calogenesis (Vincent *et al.*, 1991) and embryogenesis (Vincent *et al.* 1992a; 1992b; Lakshmi and Mythili 2003), however, in all these reports the micropropagation protocol required varying growth regulator combination for Stage I and Stage II cultures. On the contrary, direct adventive shoots from rhizome explants have been reported both in one step (Stage I and Stage II combined) protocol (Swapna *et al.*, 2004; Shirin *et al.*, 2000) and in two step protocol (Jose *et al.*, 2002). In the latter report nutritional requirement for culture establishment with subsequent induction of adventitious shoot buds (Stage I) and for adventive shoot proliferation (Stage II) have been different. Similarly, in the present study

too, although adventive shoot buds were induced and grew in size in MS medium augmented with BA 2.5 mg l⁻¹ and NAA 1.0–1.5, but these buds ceased to grow beyond a stage without developing into plantlets even after 7-8 weeks, instead infrequently further adventive buds proliferated if culture continued on the same medium. This necessitated further study to identify suitable nutritional requirements for Stage II to support morphogenesis in these adventive shoot buds developing into complete regenerated plantlets.

Table 5.1.1 Initial response of rhizome section of *Kaempheria galanga* in vitro in MS medium supplemented with different growth regulator combinations after four weeks

Treatments	Growth regulators (mg l ⁻¹)				Response
	BA	NAA	IBA	2,4-D	
T ₁	0.1	0.5			Swelling of explant, no growth
T ₂	0.1	1.0			Initiation of callus
T ₃	0.1	1.5			Initiation of callus
T ₄	0.1			0.5	Initiation of callus
T ₅	0.1			1.0	Callus proliferation
T ₆	0.1			1.5	Vigorous callus
T ₇	0.5	0.5			Initiation of callus and roots
T ₈	0.5	1.0			Initiation of callus and roots
T ₉	0.5	1.5			Vigorous root growth all along the explant
T ₁₀	0.5		0.5		Only swelling of explant
T ₁₁	0.5		1.0		Swelling of explant and callus initiation
T ₁₂	0.5		1.5		Callus proliferation
T ₁₃	0.5			0.5	Swelling of explant, no growth
T ₁₄	0.5			1.0	Swelling of explant, no growth
T ₁₅	0.5			1.5	Vigorous callus growth
T ₁₆	1.5	0.5			Swelling of explants
T ₁₇	1.5	1.0			Swelling of explants and initiation of roots
T ₁₈	1.5	1.5			Vigorous root growth all along the explant
T ₁₉	2.5		0.5		Swelling of explants, growth of rhizome buds
T ₂₀	2.5		1.0		Swelling of explants, growth of rhizome buds
T ₂₁	2.5		1.5		Swelling and rhizome bud development
T ₂₂	2.5	0.5			Swelling of explant
T ₂₃	2.5	1.0			Development of 2-3 adventive shoot buds
T ₂₄	2.5	1.5			4-6 adventive shoot buds with infrequent roots
T ₂₅	2.5			0.5	Swelling and rhizome bud growth
T ₂₆	2.5			1.0	Swelling and rhizome bud growth
T ₂₇	2.5			1.5	Shoot bud growth

Figure 5.1



***In vitro* response of rhizome sections of Ekangi (*Kaempferia galanga*): a, profuse callus induction from rhizome sections; b, rhizogenesis in rhizome sections; c, shoot meristemoids developing directly on the rhizome sections; d, meristemoid enlargement and healthy shoot bud growth.**

5.2

Effect of different BA-NAA combinations on *in vitro* morphogenetic response of the isolated microshoots and adventitious shoot bud production in *Kaempferia galanga* L.

Rationale

As evidenced from the preceding trial the adventive shoot buds induced through culture of rhizome section ceased to grow beyond certain stage and failed either to develop into whole plants or to produce large number of multiple shoots. Hence, it became primarily essential to culture the aseptically isolated shoot buds in a modified medium for their possible transformation into multiple shoots and/or for further production of shoot buds.

To make such an attempt it became a priority to manipulate hormone combinations keeping initial media composition unchanged. Hence, the effect of NAA – BA combinations with varying concentrations needed to be studied. Thus, while planning the experiment essentially a change in the cytokinin – auxin was avoided to favor easy adaptation of the adventive shoot buds following their isolation and subsequent transfer to fresh medium.

Methodology

The adventive buds along with rhizome section were aseptically withdrawn from the culture tubes on the sterile petriplates. Then the buds

were isolated aseptically with sterilized forceps and were inoculated into the tubes containing MS medium with different combination of NAA – BA. Further manipulation of the concentration of NAA and BA was done. The concentration of NAA ranged from 1.0 to 1.5 mg l⁻¹ while concentration of BA ranged from 2.0 to 3.5 mg l⁻¹.

Cultures were maintained under high light intensities (2000–2500 lx) for 16 h photoperiod. The temperature was maintained at 25±1° C, Observations in respect of mean number of adventive shoot bud development and the nature of the shoot buds were recorded after 5 weeks of culture where each treatment was replicated 3 times and each replication contained 7 tubes. Out of 7 tubes in each replication only 5 tubes were chosen randomly and observations were recorded following destructive method. The trials were designed following the Complete Randomized Design.

Results and Discussion

Observations recorded after 5 weeks of culture have been summarized in Table 5.2. Considering the potentiality of the responsive concentrations of NAA and BA as recorded in the preceding section (Table 5.1) favoring induction and growth of adventive shoot buds , in the present trial minor manipulations were introduced in the auxin and cytokinin levels (NAA 1.0 – 1.5 and BA 2.0 – 3.5 mg l⁻¹) of the same medium (MS). Growth response was studied after regular interval, while final data on adventive shoot production and morphogenetic response were recorded after five weeks following transfer to new medium. Data were recorded and statistically analyzed.

It is clearly evident from the table that with a gradual increase in the concentration of BA from 2.0 to 2.5 mg l⁻¹ there has been a gradual increase in the mean number of microshoots produced (i.e., 1.4 to 7.26 per culture),

associated with distinct morphogenetic changes. A remarkable change was noticed following further increase in the cytokinin level (BA 3.0 mg l⁻¹). Within two weeks of culture base of the enlarged shoot buds swelled and burst out to produce large number of adventitious axillary shoot buds (Fig 5.2 a) which subsequently developed into large shoot apices (Fig 5.2 b). However, a further increase in the cytokinin concentration (BA 3.5 mg l⁻¹) resulted in a drastic diminution in the morphogenetic activity irrespective to auxin concentrations used (Table 5.2). Interestingly, adventive shoot proliferation has been at its peak when MS medium was supplemented with BA 3.0 mg l⁻¹ and NAA 1.0 mg l⁻¹ giving out 15.6 microshoots per culture, but an increase in the auxin level (NAA 1.5 mg l⁻¹) caused an effective reduction in the mean number of microshoots per culture. Nevertheless, the present study proved to be helpful to identify the BA – NAA balance in MS medium to enhance the microshoot production (~ 3 fold increase) and their development into grown up shoot apices, yet complete morphogenesis remained an impasse.

However, to accomplish whole plant regeneration *in vitro* microshoots were further isolated singly and aseptically put onto MS medium ($\frac{3}{4}$ macroelement) devoid of any growth regulator. The medium devoid of any growth regulator was found to be adequate for normal transformation of the microshoots into rooted plants in more than 80% microshoots. Finally, the rooted plants (Fig 5.2.c) were successfully transferred to pots filled with 3 parts soil : 1 part sand.

Based on these results it may precisely be inferred that for bud breaking and induction of adventive microshoot directly from the rhizome explant a BA-NAA ratio of ~1.33 (BA 2.0 mg l⁻¹ – NAA 1.5 mg l⁻¹) seemed to be adequate while an enhanced BA-NAA ratio of 3.0 (BA 3.0 mg l⁻¹ – NAA 1.0 mg l⁻¹) appeared to be essential for adventive axillary shoot bud proliferation

and sustenance of their growth into healthy shoot apices. Probably a continued growth on high exogenous cytokinin – auxin containing medium enhanced the endogenous hormone level of the growing shoot apices and in turn affected the morphogenetic development adversely. Following the transfer of the isolated microshoots to hormone depleted medium ($\frac{3}{4}$ MS macroelement) morphogenesis was resumed within a week leading to the normal plantlet development and no auxin was needed for rooting as has been reported earlier (Jose *et al.*, 2002; Swapna *et al.*, 2004).

It is well established that growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium, and the growth substances produced endogenously by cultured cells. Besides exerting a direct effect on cellular mechanism, many synthetic regulants may in fact modify the level of endogenous growth substances, sometimes in a fashion which is heritable over many cell generations (Amirato, 1977, 1985). In the present situation for direct adventive shoot production an auxin is invariably required to promote the initial growth of meristemoids and adventitious shoot buds, however, a low auxin concentration in conjunction with high levels of cytokinin reduced the apical dominance and led to the proliferation of adventive axillary shoot buds as frequently reported in isolated microshoot culture (Bhattacharya *et al.*, 2003; Saha, 1997). Probably the exogenous synthetic growth substances supplied in the Stage II medium raised the endogenous hormone status to a level optimum for ensuring the morphogenetic changes following a passage in the growth substance depleted medium ($\frac{3}{4}$ MS macroelement). This passage in a diluted medium promoted satisfactory rooting in the *in vitro* shoots of *Kaempferia galanga*. It is, in fact, a common practice to transfer shoots to be rooted, from high strength media (such as MS) to less concentrated solutions, or a dilution of the same medium used at Stage II. There is then less likelihood of plantlets becoming vitrescent. According to a

random sampling by George and Sherrington (1984) of 54 papers describing *in vitro* rooting, it was found that full strength MS macronutrients were used at Stage III in only 44% of the cases, whereas they had been used in 91% of the preceding Stage II cultures. Dilute media formulations commonly used for root initiation are $\frac{1}{4}$ - $\frac{1}{2}$ MS (macronutrient 23.3 – 46.7 mM). It is believed that the favourable effect of macroelement concentration on rooting is probably less due to total ionic concentration than to the need for only a small amount of total nitrogen (Hyndman *et al.*, 1982).

The present *in vitro* plant regeneration protocol directly from rhizome sections essentially contained three steps: I. Culture establishment and adventive shoot bud induction in MS medium containing BA 2.5 mg l⁻¹ + NAA 1.0-1.5 mg l⁻¹; II. Multiple shoot development through adventive axillary bud proliferation; III. Whole plant regeneration with rooting in hormone depleted $\frac{3}{4}$ (macroelement) MS medium.

Most noteworthy is the potentiality of the *in vitro* microshoots to produce more microshoots when isolated and cultured in the next cycle and thus the cycle continued producing ~15 microshoots per culture every 4 week cycle. Hence, using present protocol the rate of microshoot production is convincingly many folds faster as compared to the protocol of microshoot multiplication in *Kaempferia* reported in the recent past (Jose *et al.*, 2002; Swapna *et al.*, 2004).

Table 5.2.1 Effect of different BA-NAA combination in MS medium for the production of multiple shoots on rhizome sections of *Kaempferia galanga* in vitro after five weeks

Treatments	Growth regulators (mg l ⁻¹)		Mean no of adventive shoot buds developed / culture	Response
	BA	NAA		
T ₁	2.0	1.0	1.4	Minute shoot bud initiation
T ₂	2.0	1.5	2.33	Minute shoot bud initiation
T ₃	2.5	1.0	4.6	Growth of green shoot apices
T ₄	2.5	1.5	7.26	Growth of green shoot apices
T ₅	3.0	1.0	15.6	Bud break, adventive axillary bud proliferation and healthy green multiple shoots developed
T ₆	3.0	1.5	6.67	Bud break, adventive axillary bud proliferation and healthy green multiple shoots developed
T ₇	3.5	1.0	3.87	Shoot bud initiation and growth ceased in later phase
T ₈	3.5	1.5	2.73	Shoot bud initiation and growth ceased in later phase

F- value: 48.576;

SE_m ± : 1.246

Table 5.2.2. Homogeneous subsets of the effect of different BA-NAA combination in the MS medium in the production of adventitious buds on rhizome sections *in vitro*

Treatments	Subset for alpha = 0.05			
	1	2	3	4
T₁	1.4000			
T₈	2.7333	2.7333		
T₂	2.8667	2.8667		
T₇		3.8667		
T₃		4.6000		
T₆			6.8667	
T₄			7.2667	
T₅				15.6000

Figure 5.2



Regeneration of whole plant of Ekangi (*Kaempferia galanga*) *in vitro* from rhizome sections: **a**, large number of adventitious shoots developing from the base of the *in vitro* shoot buds; **b**, numerous proliferating shoots; **c**, a regenerated whole plant *in vitro*.

Chapter VI

Micro-cloning in Kaclus (*Atalantia malaberica*)

6.1

Initiation of culture and *in vitro* response of seed embryo with cotyledon explants of *Atalantia malaberica* to different nutritional combinations

6.1.1 Rationale

Atalantia malaberica, commonly known as Kaclus is a woody shrub / tree normally propagated by seed but infrequently also propagated by vegetative means. Seed raised plant population is genetically heterogeneous and at the same time seed to seed generation is too long, while vegetative propagation using conventional asexual methods although can produce clonal population having perpetual homogeneity but are slow and inadequate to produce large population in a short time. Therefore a program of work was undertaken to develop an *in vitro* protocol for rapid cloning of this important medicinal plant.

It is known that the explant, its source, size and juvenility – all play very important role on the micropropagational achievement. Therefore, to initiate the program on multiple shoots production in Kaclus, the zygotic embryo with or without cotyledon (EWC / EWOC) was taken as primary explant because its high regenerative capacity, as has been reported by many early workers in woody shrubs like *Camellia* sp. (Borthakur and Mitra, 1990; Saha, 1997), *Citrus* relatives (Teo *et al.*, 1990; Huang and Xiong, 1992; Parthasarathy and Nagaraju, 1994; Das *et al.*, 2000), including *Atalantia ceylanica* (Ling and Iwamasa, 1997) and *Atalantia bilocularis* (Jumin, 1995) and many other species.

As stated earlier this woody plant belongs to the family *Rutaceae* i.e., the citrus family and production of embryoids and regenerated shoots from mature and immature embryos and cotyledons in *Rutaceae* members have been reported by many workers.

6.1.2 Methodology

Young fruits of *Atalantia malaberica* were collected from a commercial field during the month of May – June of 2004 and 2005. Fruits were peeled out and the mature seeds were put in moist sand for germination. Germinated seeds were washed and treated in an aqueous solution of mercuric chloride (0.1% w/v) for 7 minutes and rinsed 5-6 times in sterile double distilled water. After removal of the seed coat, two cotyledons were aseptically separated with sterile scalpel and the embryo with / without cotyledon section (EWC / EWOC) were then placed onto the tubes containing nutrient medium supplemented with different hormone combinations.

The explants were cultured on MS medium supplemented with different kind of auxins and cytokinins in varying concentrations. Auxins were added at relatively lower dose (0.5 and 1.0 mg l⁻¹ while cytokinins were added relatively at higher dose (1.0 mg l⁻¹ to 4.0 mg l⁻¹). The inoculated tubes were kept in dark for 48 h and then transferred to 2000 lx illuminance maintaining a 16 h light phase at 25 ± 1°C under 70 – 75% RH. The experiment was conducted following Complete Randomized Design (CRD) and statistical analyses were done accordingly. Each treatment was replicated three times. Initially 6 tubes were kept under each replication, but observations were recorded based on three tubes only, since all the six tubes in each replication did not survive mostly due to infection.

On the basis of the observations of the trial further attempt was made to achieve an optimum auxin–cytokinin combination for increasing the production of multiple shoots. For this purpose it became primarily essential to culture the microshoots in isolation and to introduce further manipulation in the favorable hormonal combinations in MS medium and eleven different combinations of IBA (0.0 – 3.0 mg l⁻¹) with BA (0.0 – 3.0 mg l⁻¹) were tried.

6.1.3 Results and Discussions

Under this study, initially two types of explants were used, one consisting embryo with cotyledon part (EWC) and the other consisting embryo without cotyledon part (EWOC). Expectedly, the responses of two explants were different. EWC explants followed direct regeneration by production of multiple shoots whereas EWOC explants infrequently produced a small amount of callus and ceased to grow in the later phase. So, the work progressed with EWC explants only and the observations are recorded in Table 6.1.1.

The embryo of EWC explants showed varying degree of response in different treatments. Amongst different hormone combinations used, kinetin in combination with 2,4-D were found to be totally non-responsive (Fig 6.1a) except occasional swelling of the cotyledon and seed embryo, rarely giving out a few protrusions (Fig 6.1b). While combinations of kinetin with both NAA and IBA responded poorly and in some cases the plumular end underwent a solitary growth giving out tufted shoot with a stunted growth of the radical axis, infrequently developing into plantlet with inadequate root growth (Fig 6.1c-d). Relatively, the response of 2,4-D and BA combinations has been moderate. 2,4-D 1.0 mg l⁻¹ when applied with higher concentrations of BA (2.0 and 3.0 mg l⁻¹) the mean number of the adventive shoots from the plumular axis varied between 2.29 – 2.33 microshoots per plumular axis. However, there was no significant

difference between these two treatments and these belonged to the same homogenous group (Table 6.1.2). The EWC explants did not show any improved response even when medium (MS) was supplemented with NAA (1.0 mg l^{-1}) and BA (3.0 mg l^{-1}) and produced only 2.68 microshoots per plumular axis. In contrast, the mean number of microshoots per explant increased 2-3 fold ($3.20 - 6.99$ microshoots) when the medium was supplied with IBA 1.0 mg l^{-1} and BA $2.0 - 4.0 \text{ mg l}^{-1}$ and the response has been significantly higher with IBA 1.0 mg l^{-1} and BA 3.0 mg l^{-1} giving out ~ 7 adventive microshoots per explant (Fig 6.1e).

Observations on varying growth response following 5 weeks of culture of isolated microshoots of EWC origin were recorded and the same has been summarized in Table 6.1.3. These observations clearly indicated that there has been a complete cessation of growth and no microshoots production when the medium was devoid of any growth regulator, while supplementing the medium with only IBA (1.0 mg l^{-1}) failed to support any sustainable growth.

Interestingly, following the transfer of the microshoots to same nutritional combinations IBA (1.0 mg l^{-1}) and BA ($2.0 - 3.0 \text{ mg l}^{-1}$) was found to be the only condition that not only supported the growth of the microshoots but was conducive for further production of adventive microshoots from the base of the inoculated one (Fig 6.1f).

Whereas, an increase in the concentrations of either IBA or BA had adverse effects on the growth and morphogenesis of the microshoots causing frequent abnormalities. Increasing the IBA level to 2.0 mg l^{-1} , irrespective of the cytokinin doses, induced callusing at the base of the microshoots and also extreme narrowing of the leaves (Fig 6.1g). A further increase in IBA ($2.5 - 3.0 \text{ mg l}^{-1}$) caused profuse callusing, some time covering the entire shoot and at times induced a range of morphological deformities (Table 6.1.3; Fig 6.1h).

As elsewhere mentioned *in vitro* plant regeneration from juvenile nucellar and zygotic embryo culture has been reported in a number of *Rutaceae* members like sweet orange cv Musambi (Parthasarathy and Nagaraju, 1994; Das *et al.*, 2000), *Citrus microcarpa* (Teo *et al.*, 1988) *Citrus reticulata* (Huang and Xiong, 1992). In former two species shoot buds were induced in MS medium supplemented with NAA and malt extract or GA₃, while in the latter regeneration was achieved in MT medium supplemented with IBA and BA. In the present study regeneration has been achieved in MS medium, but the most effective growth regulator combination for the induction of adventive microshoots from the plumular meristems of the EWC explants as well as for their proliferation in isolated cultures has been IBA - BA.

Regeneration through zygotic embryo culture in *Atalantia malabérica* although has little significance in micropropagation since the plant is cross pollinated and the seeded plants differ from their parents. Yet for the studies involving interspecific hybridization where hybrid seeds are produced in limited numbers, zygotic embryo culture may play an important role in multiplying them in a short time. Nadamitsu *et al.*, (1986) produced interspecific hybrids between *C. vietnamensis* and *C. crisantha* where only three seeds were viable but large number of hybrid plants could be obtained using this method.

It is worthwhile to mention that leaving the reports of some success in plant regeneration via somatic embryogenesis in *Atalantia ceylanica* (Ling and Iwamasa, 1997) and *Atalantia bilocularis* (Jumin, 1995), this may be the first report on *in vitro* microshoot production in *Kaclus* (*Atalantia malabérica*). However rooting in the microshoot remained unachieved.

Table 6.1.1 *In vitro* response of seed embryo with cotyledon explants (EWC) of *Atalantia malaberica* to various growth regulator combinations cultured on MS medium

Treatments	Growth regulators (mg l ⁻¹)					Growth response of plumular axis	Mean no of adventive shoots / plumular axis	Transformed mean
	2,4-D	NAA	IBA	Kin	BA			
T ₁	0.5			1.0		-	0	0.71
T ₂	0.5			2.0		-	0	0.71
T ₃	1.0			1.0		-	0	0.71
T ₄	1.0			2.0		-	0	0.71
T ₅		1.0		1.0		-	0.60	1.03
T ₆		1.0		2.0		+	0.22	0.84
T ₇			1.0	1.0		+	0.77	1.13
T ₈			1.0	2.0		+	0.44	0.97
T ₉	0.5				2.0	+	0.66	1.07
T ₁₀	0.5				3.0	+	1.44	1.39
T ₁₁	1.0				2.0	+	2.33	1.68
T ₁₂	1.0				3.0	+	2.29	1.66
T ₁₃		1.0			2.0	+	1.76	1.49
T ₁₄		1.0			3.0	++	2.68	1.78
T ₁₅			1.0		2.0	++	4.78	2.30
T ₁₆			1.0		3.0	++	6.99	2.74
T ₁₇			1.0		4.0	++	3.20	1.92

-, no response; +, poor callusing; ++, moderate callusing

F-value: 70.480
SE_m : ± 1.005

Table 6.1.2 Homogeneous subsets of *in vitro* response of EWC explants of *Atalantia malaberica* to various growth regulator combinations cultured in MS medium

Treatments	Subset for alpha = 0.05								
	1	2	3	4	5	6	7	8	9
T ₁	0.71								
T ₂	0.71								
T ₃	0.71								
T ₄	0.71								
T ₆	0.8431	0.8431							
T ₈		0.9664	0.9664						
T ₅		1.0297	1.0297						
T ₉			1.0709						
T ₇			1.1263						
T ₁₀				1.3862					
T ₁₃				1.4929	1.4929				
T ₁₂					1.6653	1.6653			
T ₁₁					1.6803	1.6803			
T ₁₄						1.7825	1.7825		
T ₁₇							1.9223		
T ₁₅								2.2970	
T ₁₆									2.7363

Table 6.1.3 Isolated microshoots cultured on MS medium supplemented with various growth regulators combinations

Treatments	Growth regulators (mg l ⁻¹)	Response recorded after 5 weeks
T ₁	None	Cessation of growth
T ₂	IBA 1.0 + BA 0.0	No sustainable growth
T ₃	IBA 1.0 + BA 2.0	Initiation of more adventive shoots
T ₄	IBA 1.0 + BA 3.0	Initiation of more adventive shoots
T ₅	IBA 2.0 + BA 1.0	Callusing of base and needle like narrowing of leaves.
T ₆	IBA 2.0 + BA 2.0	Callusing of base and needle like narrowing of leaves.
T ₇	IBA 2.5 + BA 1.0	Profuse callusing, deformed leaves
T ₈	IBA 2.5 + BA 2.0	Profuse callusing, deformed leaves
T ₉	IBA 3.0 + BA 1.0	Profuse callusing, deformed leaves
T ₁₀	IBA 3.0 + BA 2.0	Profuse callusing associated with morphological deformities
T ₁₁	IBA 3.0 + BA 3.0	Profuse callusing associated with morphological deformities

Figure 6.1



Seed embryo culture for micropropagation in Kaclus (*Atalantia malabérica*): **a**, embryo with cotyledon section (EWC) cultured without appreciable growth of the embryo; **b**, occasional swelling of the cotyledon and seed embryo, rarely giving out a few protrusions; **c**, culture of isolated embryo attached to a section of cotyledon showing normal germination but thickening of the root-shoot axis; **d**, culture of germinated seed embryo detached from cotyledon section neither sustained growth nor induced adventitious shoots; **e**, culture of isolated embryo attached to a section of cotyledon (EWC) produced multiple adventive shoots; **f**, isolated single shoots detached from cotyledon section underwent further multiple shooting with callus growth at base but failed to sustain normal development; **g**, increasing the IBA level to 2.0 mg l^{-1} induced callusing at the base of the microshoots and also extreme narrowing of the leaves; **h**, IBA $2.5 - 3.0 \text{ mg l}^{-1}$ caused profuse callusing with a range of morphological

Multiple shoot culture from nodal segments of young *Atalantia malaberica* plants

6.2.1 Rationale

In view of the limitations of microshoot production from zygotic embryos while initiating the second phase of work on micropropagation of *Atalantia malaberica*, the most important and critical consideration had been to choose the plant part other than the seed embryo which may be taken as explant and used for rapid cloning. Therefore, while initiating the present work, nodal segment of young plants of *Atalantia malaberica* were preferred as the primary explant since it is considered to be potential for giving out multiple shoots from the nodal tissue destined for axillary shoot bud production in many woody species (Bhattacharya *et al.*, 2003).

6.2.2 Methodology

Young nodal segments of young plants were collected with 2 – 3 young leaves and axillary buds. The field grown explants were thoroughly washed in running tap water and then treated with Triton-X for 20 minutes. Then the materials were thoroughly rinsed with double distilled water. For surface sterilization then the plant materials, after trimming the leaves, were treated with 0.1 % mercuric chloride solution for 6 minutes under aseptic environment. Just prior to inoculation, the nodal segments were treated with ready for use sterilized antioxidant mixture (Murashige and Skoog, 1962) for 15 minutes and initial inoculation was done in MS medium with half strength of inorganic salts in large vials (Fig 6.2a). Later, after a week nodal explants were transferred singly to tubes containing full strength MS basal medium with various growth regulator combinations (Fig 6.2b).

Growth regulators (IBA, NAA and BA) were added to the medium in various combinations and concentrations. There were two batches of culture of nodal explants. In the first batch different combination of NAA and BA were added. But in the second batch medium was supplemented with various combinations of IBA and BA. These comprised of twelve treatments. Each treatment had three replications and for each replication 7 tubes were initially cultured, but due to infection problem the observations were recorded from 6 tubes only. Following inoculation, cultures were initially maintained in diffused light (500 lx) for a week and then transferred to 2000 lx illuminance under 16 h photoperiod at $25 \pm 1^\circ\text{C}$. The experiments followed the CRD. Observations were recorded after 10 weeks of culture the data were statistically analyzed.

For sustained growth of microshoots of nodal origin with restoration of normal morphogenesis culture of the isolated microshoots was necessary. While making such attempt both manipulating the IBA – BA concentrations and reduction in the inorganic salts in the medium. For isolated microshoot culture initially adventive microshoots were aseptically taken out in the petriplates and then the individual microshoots were separated and inoculated in the tubes containing the varying treatment combinations of IBA – BA in MS medium containing either full or half inorganic salts. Each treatment was replicated three times. The observations were recorded from 5 tubes of each replication after 6-8 weeks of culture. Only visual observations were taken and the same has been summarized. The cultures were kept at $25 \pm 1^\circ\text{C}$ under higher light intensity of 2000 lx with 16 h photoperiod.

6.2.3 Results and Discussion

Initial hazard of phenolic oxidation could be successfully overcome with a pre-treatment of the explant prior to inoculation with an antioxidant mixture (Murashige and Skoog, 1962) solution for 15 minutes and by the

reduction of the inorganic salts to half strength in the MS medium followed by maintaining the cultures initially for a week in diffused light of 500 lx. To overcome phenolic oxidation in woody species pretreatment with antioxidant mixture prior to inoculation (Sarin and Bagga, 1983; Saha, 1997; Bhattacharya *et al.*, 2003) with a reduction in inorganic salts in the medium (Sarwar, 1984; Bhattacharya *et al.*, 2003) as has severally been recommended in past. Further, initial exposure of the cultures to dark or diffused light also proved to be highly beneficial in the present situation to reduce phenolic oxidation hazards, strikingly similar to the earlier observations in teak (Devi *et al.*, 1994) and tea (Bhattacharya *et al.*, 2003).

Observations recorded on the development of adventive microshoots development after 10 weeks of culture are summarized in the Table 6.2.1. The pooled data obtained from two batches of experiments, conducted under same cultural conditions, were statistically analyzed and the distribution of data in different homogeneous subsets is presented in Table 6.2.2.

In the 1st set of experiment, NAA at 0.5 mg l⁻¹ and 1.0 mg l⁻¹ were added to the medium with higher concentrations of BA (2.0 – 4.0 mg l⁻¹). Visual observation indicated that NAA both at 0.5 mg l⁻¹ and 1.0 mg l⁻¹ with gradual increase in BA concentrations up to 3.0 mg l⁻¹ was responsive towards gradual increase of adventive microshoot development from the base of nodal segments (1.33 – 1.61 and 2.16 – 2.88 microshoots per nodal explant respectively). However, there have been no significant difference between these effects as they all belong to the same homogenous subset (Table 6.2.2), except the effects observed in T₅ (NAA 1.0 mg l⁻¹ with BA 3.0 mg l⁻¹), where mean number of microshoots were found to increase significantly (2.88 per explant) and belonged to a different homogenous subset with profuse callus from base (Fig 6.2c). A further increase in BA concentration to 4.0 mg l⁻¹, however, reduced the number of adventive microshoots irrespective of NAA levels in the medium. In all above

treatments adventive microshoots were induced along with callus at base of the explant in different propensities.

In the second phase of trial varying concentrations of BA were applied with varying concentration of IBA and observations are summarized in Table 6.2.1. The combinations of IBA 0.5 mg l^{-1} with varying concentrations of BA ($2.0 - 4.0 \text{ mg l}^{-1}$) was conducive for callus induction at explant base. But with IBA – BA combinations callusing was relatively low as compared to NAA – BA combinations while in respect of mean number of adventive shoot production IBA – BA combinations induced better result than NAA – BA combinations. At the same time IBA 1.0 mg l^{-1} with varying concentrations of BA produced much desired result than that of IBA 0.5 mg l^{-1} in combination with the same variation of BA. Gradual increase in BA up to 3.0 mg l^{-1} with IBA 0.5 mg l^{-1} was responsible for gradual increase in mean number of microshoots. BA at 4.0 mg l^{-1} with IBA 0.5 mg l^{-1} , however caused a reduction in the mean number of microshoots. Statistical analysis revealed that IBA 1.0 mg l^{-1} and BA 3.0 mg l^{-1} was found to be the best combination for microshoot induction (Fig 6.2d), producing highest mean number of microshoots (6.66 adventive shoots per nodal segment) which is significantly higher than all other treatment combination (Table 6.2.2).

Although microshoots were produced with low or high frequency in both the sets of experiments, but most important observation which drew attention was that the growth of the microshoots were more or less abnormal under either of the treatment conditions (Fig. 6.2c,d). The leaves of the microshoots produced abnormal thickening and narrowing, very similar to what has been observed also in case of adventive shoot production from seed embryo explant. These observations necessarily demanded for further manipulation in the medium for culture of these morphologically abnormal microshoots following their isolation.

Observations on morphological development of isolated microshoots after 6-8 weeks of culture were recorded and summarized in Table 6.2.3. The microshoots initially cultured in hormone depleted MS medium failed to sustain any growth. On the basis of the previous response of induction of microshoots from the nodal segment of *Atalantia*, IBA 1.0 mg l⁻¹ and BA 2.0 – 3.0 mg l⁻¹ was added to full MS medium. Observations clearly indicated that these combinations failed to sustain growth of the microshoots instead prolonged culture induced prolific callus at base frequently covering the entire shoot (Fig 6.2e). Supplementing the medium with GA₃ (2.0 – 3.0 mg l⁻¹) along with IBA and BA (1.0 and 2.0 mg l⁻¹ respectively) did not show any normal morphogenetic growth but caused callusing at base and narrowing of leaves. Therefore, no combination was found to be suitable for the normal development of microshoots in these growth regulator combinations in MS medium.

In the other set of experiment instead of MS medium, half strength of inorganic salts in MS medium were used along with same treatment combinations of IBA, BA and GA₃. The varying IBA – BA concentrations did not improve the status of morphogenetic development anyway. But interestingly, the GA₃ with IBA 1.0 mg l⁻¹ + BA 2.0 mg l⁻¹ was responsive for the induction of compact greenish callus from the base that turned out to be regenerative at a later stage following subculture on the same medium and large number of minute shoot buds developed through callogenesis (Fig 6.2f). However, such *in vitro* regenerated shoot buds when cultured on same medium in isolation although produced more microshoots as well as very little callus at base but could not restore normal morphogenesis (Fig 6.2g,h).

Most noteworthy is the potentiality of the *in vitro* microshoots of nodal origin to produce more adventive microshoots when isolated and cultured singly in the next cycle and the cycle continued.

It is again worth mentioning that as there is no report yet of any *in vitro* study in this mediant species this becomes the first report of adventive multiple shoot culture from nodal segments in *Atalantia malaberrica*. However, restoration of normal morphogenesis with their rooting remained unachieved and needed to be achieved in future for developing a protocol.

Table 6.2.1 *In vitro* response of nodal explants of *Atalantia malaberica* in MS medium supplemented with different combinations of growth regulators as recorded after 10 weeks of culture

Treatments	Growth regulators (mg l ⁻¹)			Mean no of microshoots/explants	Nature of growth response
	NAA	IBA	BA		
T ₁	0.5		2.0	1.33	Initiation of callus at base
T ₂	0.5		3.0	1.72	Initiation callus at base
T ₃	0.5		4.0	1.61	Initiation callus at base
T ₄	1.0		2.0	2.16	Profuse callus at base
T ₅	1.0		3.0	2.88	Profuse callus at base
T ₆	1.0		4.0	2.33	Callus at base
T ₇		0.5	2.0	2.66	Initiation callusing at base
T ₈		0.5	3.0	3.22	Initiation callusing at base
T ₉		0.5	4.0	3.55	Callusing at base
T ₁₀		1.0	2.0	4.72	Low callusing at base
T ₁₁		1.0	3.0	6.66	No callus
T ₁₂		1.0	4.0	3.77	No callus

F value: 18.314

SE_m: ±0.371

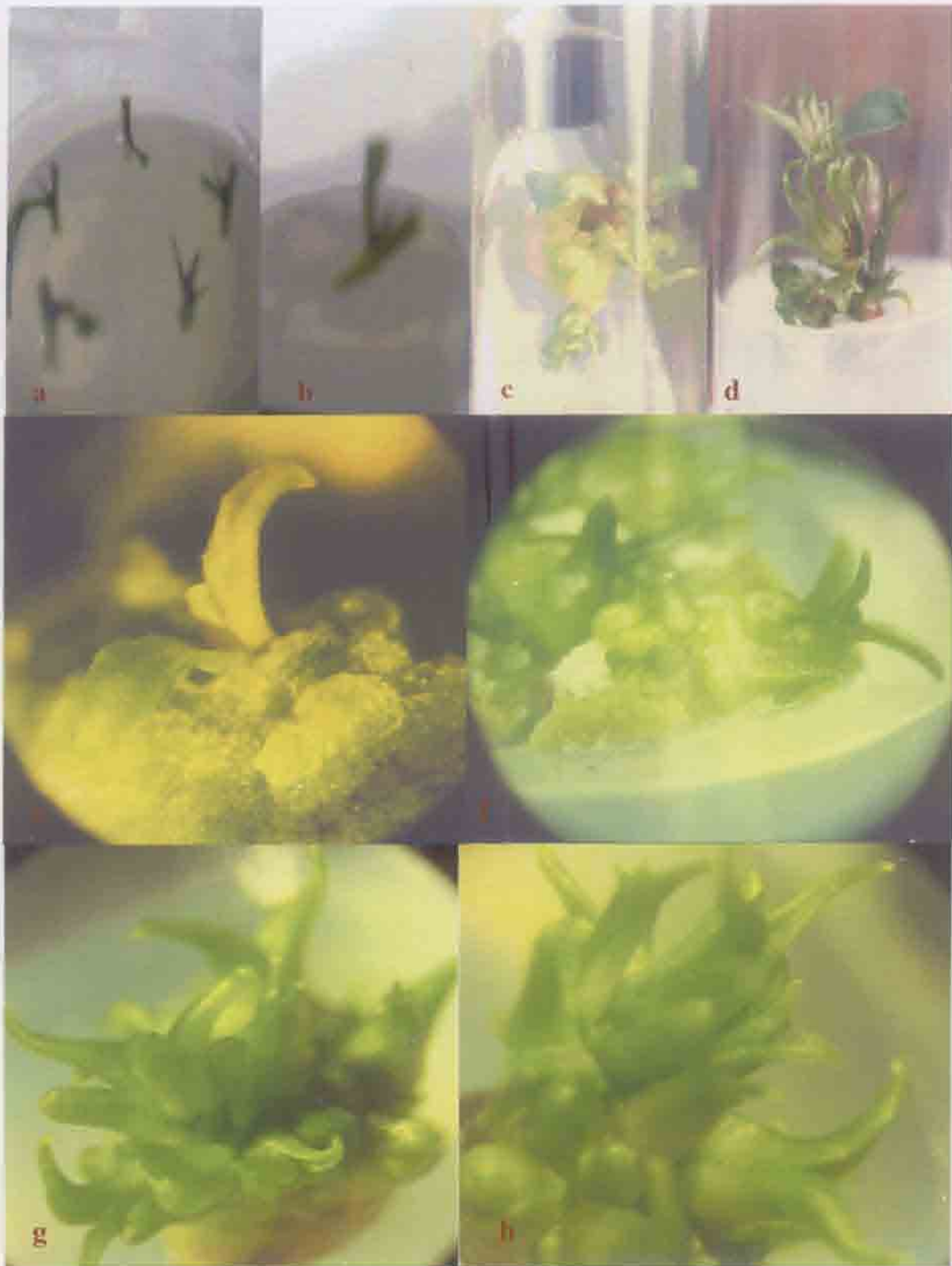
Table 6.2.2 Homogeneous subsets for *in vitro* response of nodal explants of *Atalantia* in MS medium supplemented with different combinations of growth regulators

Treatments	Subset for alpha = 0.05					
	1	2	3	4	5	6
T ₁	1.3300					
T ₃	1.6100	1.6100				
T ₂	1.7200	1.7200				
T ₄	2.1600	2.1600	2.1600			
T ₆	2.3300	2.3300	2.3300			
T ₇		2.6633	2.6633	2.6633		
T ₅			2.8867	2.8867		
T ₉			3.2200	3.2200		
T ₈				3.5500		
T ₁₂				3.7733	3.7733	
T ₁₀					4.7200	
T ₁₁						6.6633

Table 6.2.3 *In vitro* culture of isolated micro shoots of nodal origin on various nutritional combinations after 6-8 weeks of culture

Treatments	Medium	Growth regulators (mg l ⁻¹)	Nature of growth
T ₁	MS	None	No sustainable growth
T ₂		IBA 1.0 + BA 2.0	Prolific callusing covering of entire shoot
T ₃		IBA 1.0 + BA 2.5	Prolific callusing covering of entire shoot
T ₄		IBA 1.0 + BA 3.0	Prolific callus from base
T ₅		IBA 1.0 + BA 2.0 + GA 2.0	Callus at base and narrowing of leaves
T ₆		IBA 1.0 + BA 2.0 + GA 3.0	Callus at base and narrowing of leaves
T ₇	½ MS	None	No sustainable growth
T ₈		IBA 1.0 + BA 2.0	Cessation of growth , gradual callusing
T ₉		IBA 1.0 + BA 2.5	Cessation of growth , gradual callusing
T ₁₀		IBA 1.0 + BA 3.0	Callusing at base, cessation of growth
T ₁₁		IBA 1.0 + BA 2.0 +GA 2.0	Compact greenish callus from base turned out to be regenerative
T ₁₂		IBA1.0+BA2.0 +GA 3.0	Compact greenish callus from base turned out to be regenerative

Figure 6.2



Multiple shoot culture of *Atalantia malabérica* from nodal sections *in vitro*: a, initial culture of nodal segments in $\frac{1}{2}$ MS basal medium; b, the nodal explants transferred to tubes containing full MS medium c, multiple shoots originating from the nodal explants with profuse callus at base; d, adventive shoots produced from nodal segments; e, isolated single microshoots underwent profuse callusing from base covering entire shoot; f, microshoots derived calluses subcultured on same medium turned out to be highly regenerative; g-h, isolated microshoots cultured singly on the same medium sustained growth and further adventive shooting but failed to show normal morphogenesis.

6.3

Anther culture studies in *Atalantia malaberica*

6.3.1 Rationale

Being a perennial woody species *Kaclus* requires a long developmental phase before reaching reproductive stage, hence obtaining transgressive segregants with beneficial genetic combinations takes too many years. In consequence, any useful recombinants if at all produced frequently remain unidentified and unevaluated. Moreover, owing to highly out breeding nature of most perennial woody species producing inbreds is a difficult proposition, if not impossible, due to high inbreeding depression (Bezbaruah and Saikia, 1977; Saha, 1997). Production of haploids, which has tremendous breeding potential, is almost impossible through conventional methods as has been emphasized by several workers in past (Singh, 1980; Raina and Iyer, 1983). Therefore, attempts to produce haploids / homodiploids through anther / microspore culture remained as the only potential option, so some preliminary studies have been made in that direction

6.3.2 Methodology

The flower bud of *Kaclus* was collected from farmer's field at Kaltala near Mohanpur, Nadia. The plants flowered profusely for a short period during February to April 2006. The flower buds 3 – 4 days prior to bloom were collected and taken to the laboratory. Following an initial cleaning with a mild detergent the flower buds were surface sterilized with aqueous mercuric chloride solution (0.1 % w/v) for 2 minutes and washed thoroughly with sterile double distilled water. 6 –

8 excised anthers from each flower bud were aseptically inoculated in the slant culture each containing 10-15 ml of N₆ (Nitsch and Nitsch, 1969) medium.

To study the *in vitro* response, the excised anthers were cultured on agar gelled N₆ medium containing different auxin – cytokinin combinations and the tubes were incubated in the dark for initial two weeks at 25 ± 1°C temperature. Responsive anthers with tiny callus growth were then transferred under continuous diffused soft light (500 lx) for a week and then to higher illuminance of 2000 lx for 16 h light regime and 70 - 75% RH.

Each treatment was replicated three times and 7 tubes were cultured under each replication, however, visual observations were recorded periodically from 5 tubes under each replication for each treatment and the same has been summarized.

To induce morphogenesis the anther calluses were transferred to new medium with different growth regulators. Therefore, the calluses were transferred to both agar-gelled and liquid half MS medium supplemented with different NAA – BA combinations. Liquid suspension cultures in conical flasks kept on a gyratory shaker maintained ~70 rpm under continuous soft illuminance (~500 lx). The observations were taken visually and observations were recorded.

6.3.3 Results and Discussion

Initial swelling of the excised anthers followed by rupture of pollen sacs was noticed within 10 – 12 days of dark incubation in the N₆ medium. Induction and growth of whitish anther callus was observed in about ~21 - 87% of the inoculated anthers. Maintaining the induced anthers on the same medium with same growth regulator under continuous light for 5 weeks did not show any positive response

towards morphogenesis. Instead, the whitish initial callus grown in dark gradually developed creamish to pinkish pigmentation when exposed to 2000 lx illuminance for 16 h light regime (Fig 6.3a).

A differential response was recorded in terms of nature and growth of callus induced in various auxin – cytokinin combinations. It is evident from the observations summarized in Table 6.3.1 that varying combinations of IBA - BA was not at all responsive. No callus induction was noticed on these combinations. In contrast, the NAA - BA combinations were highly responsive in respect of callus induction and growth. About 20 - 40 % of the inoculated anthers produced callus on a medium supplemented with NAA 0.1 mg l⁻¹ and BA 0.5 - 2.0 mg l⁻¹, although the rate of callus growth remained relatively slow (Table 6.3.1). Above 70 - 80 % of the inoculated anthers produced callus following their culture in N₆ medium supplemented with higher concentrations of NAA (1.0 mg l⁻¹) and varying concentrations of BA (1.0 - 2.0 mg l⁻¹). Evidently, there has been an increase in frequency of induced anthers with a decrease in the auxin – cytokinin ratio from 1.0 to 0.5, strikingly similar to the observations by Pedroso and Pais (1993) in *C. japonica* and by Saha (1997) in *C. sinensis*. On these treatment combinations callus growth was found to be prolific but the nature of the callus appeared to be characteristically different as these calluses underwent androgenesis with the development of a few shiny globular structures over the growing anther calluses, but did not show any further morphogenetic changes. For induction of morphogenesis these calluses (Fig 6.3b) were subcultured with further modifications in the medium.

Those modifications were introduced in two sets of experiments. Both sets of experiments had same growth regulator combinations in varying concentrations. The only difference being the type of culture; in one set the medium was gelled with agar and in the other no agar was added the cultures were maintained in suspension.

The visual observations recorded in Table 6.3.2 revealed that all combinations of NAA – BA in the agar gelled $\frac{1}{2}$ MS medium were responsive for moderate to prolific growth of callus. Both the combinations of NAA 0.5 mg l⁻¹ with BA (0.5 and 1.0 mg l⁻¹) were found to be adequate to support prolific anther callus growth, but NAA 0.5 mg l⁻¹ with BA 1.0 mg l⁻¹ responded towards morphogenetic changes and conducive for rhizogenesis, producing healthy root growth along with the development of clusters of shiny globular embryo like structures (Fig 6.3c) that continuing on same medium failed to undergo any further morphogenetic changes.

On the other hand, anther calluses cultured in liquid $\frac{1}{2}$ MS medium with same hormonal treatment combinations could not show any remarkable change in the single cells / cell aggregates. However, two notable observations were the production of whitish minute globular structures in a combination of NAA 0.1 mg l⁻¹ with BA 0.5 mg l⁻¹ and fast increase in cell density in medium containing NAA 0.5 mg l⁻¹ with BA 0.5 – 1.0 mg l⁻¹ (Table 6.3.2). The minute whitish globular structures on transfer to the same medium gelled with agar produced enlarged round unidentifiable structures with varied pigment development (Fig 6.3d). Alternative culture in liquid and solid culture has been reported to be beneficial towards increasing the regenerative ability in some Chinese medicinal herbs (Chang *et al.*, 1992). However, in the present study no recognizable morphogenetic changes occurred in these structures following their transfer to solid medium and no defined organ developed even after 90 days of culture on same medium.

Anticipating a possible stimulating effect of elevated temperature on the production of meristemoid and morphogenesis in anther callus as reported by Saha and Bhattacharya, 1992, some cultures were

exposed to $30 \pm 1^{\circ} \text{C}$ for 4-5 days but that did not yield any beneficial effect, so not reported in detail.

As this is the first attempt towards androgenic plant development in *Atalantia* the present results may be considered reasonably positive and encouraging. The study needed to be carried forward with fresh attempts to accomplish androgenic plant development. As that required fresh anthers to be collected next season, the study remained unconcluded and time became the limiting factor.

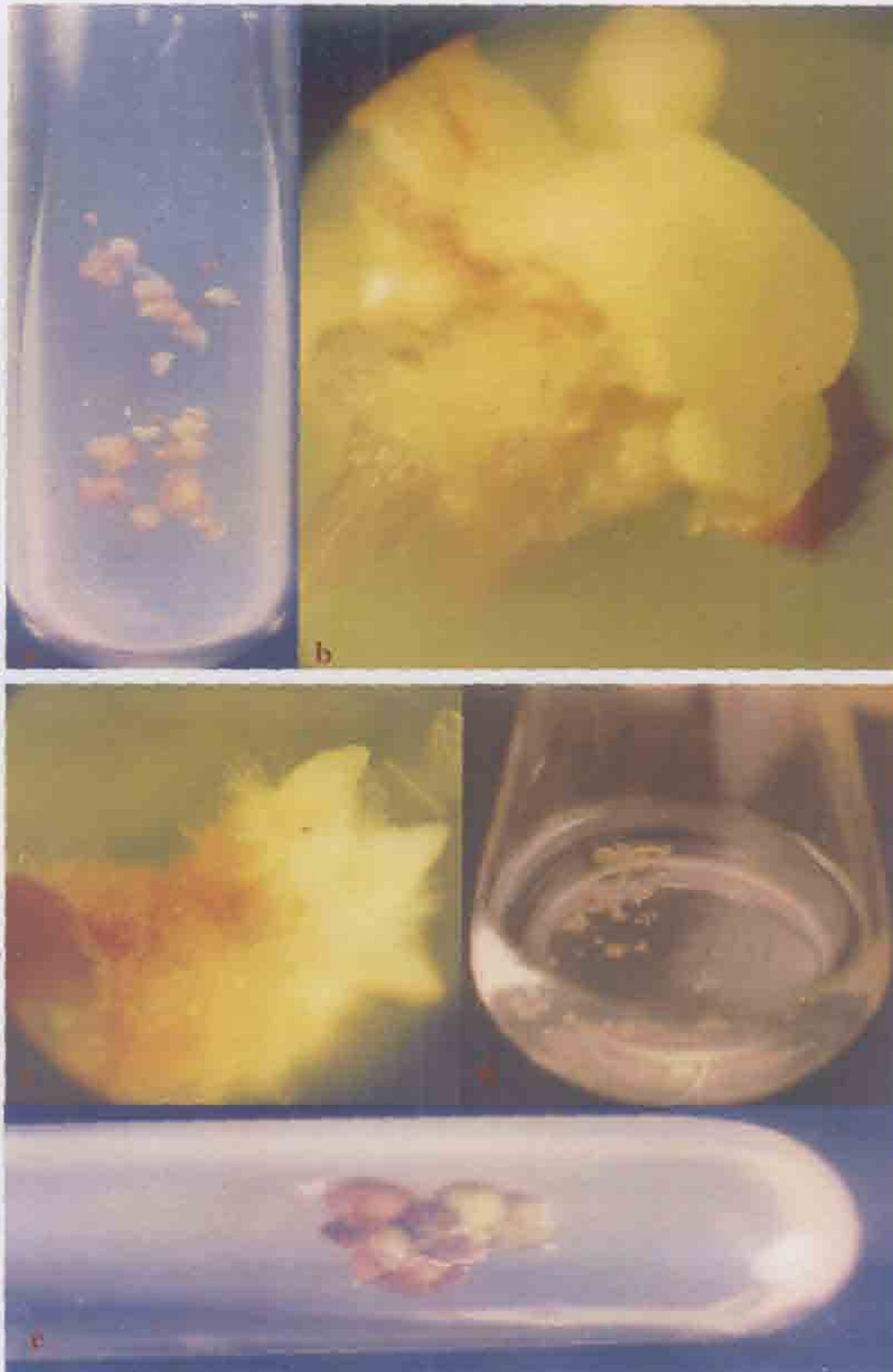
Table 6.3.1 Response of *Atalantia malaberica* anthers cultured in N₆ medium supplemented with various growth regulator combinations

Treatments	Growth regulator (mg/l)			Anthers produced callus (%)	Response effect after 5 weeks	
	NAA	IBA	BA		Induction of callus	Remarks
T ₁	0.1		0.5	23.33	+	Slow callus growth
T ₂	0.1		1.0	21.42	+	Slow callus growth
T ₃	0.1		2.0	24.24	+	Slow callus growth
T ₄	0.5		1.0	40.00	++	Slow callus growth
T ₅	0.5		2.0	31.42	+	Slow callus growth
T ₆	1.0		1.0	73.68	+++	Prolific callus growth
T ₇	1.0		2.0	86.84	+++	Prolific callus growth
T ₈		0.1	0.5	-	-	No response
T ₉		0.1	1.0	-	-	No response
T ₁₀		0.1	2.0	-	-	No response
T ₁₁		0.5	1.0	-	-	No response
T ₁₂		0.5	2.0	-	-	No response
T ₁₃		1.0	1.0	-	-	No response
T ₁₄		1.0	2.0	-	-	No response

Table-6.3.2 Subculture of anther derived callus with low level of auxin-cytokinin combinations both in agar gelled and without agar (liquid) $\frac{1}{2}$ MS medium

Treat ments	Medium	Growth regulators (mg l ⁻¹)		Relative callus growth	Morphogenic response
		NAA	BA		
T ₁	$\frac{1}{2}$ MS medium (agar gelled)	0.1	0.1	++	No morphogenetic response
T ₂		0.1	0.5	++	No morphogenetic response
T ₃		0.1	1.0	++	No morphogenetic response
T ₄		0.5	0.5	+++	Prolific callus growth, friable in nature
T ₅		0.5	1.0	+++	Prolific callus, rhizogenesis with shiny globular growth
T ₆	$\frac{1}{2}$ MS medium (liquid)	0.1	0.1	+	No morphogenetic response
T ₇		0.1	0.5	+	Globular unidentifiable whitish minute structure
T ₈		0.1	1.0	+	No morphogenetic response
T ₉		0.5	0.5	++	Fast increase in cell density
T ₁₀		0.5	1.0	++	Fast increase in cell density

Figure 6.3



Anther culture in Kaclus (*Atalantia malabérica*): a, anther callus induction showing whitish to creamish and pinkish calluses; b, anther callus showing shiny globular meristemoid like structures; c, rhizogenesis from parts of shiny globular calluses; d, globular shiny calluses grew into round embryoid like structures without further morphogenetic changes when cultured in liquid medium; e, globular shiny calluses subcultured on agar gelled medium developed into large unidentifiable round structures that failed to undergo further morphogenetic changes.

Chapter VII

Summary & Conclusions

Application of cell and tissue cultures in medicinal plant cloning and improvement is relatively a newer area of research. It is worth mentioning that the know how of *in vitro* regeneration of plants in a wide range of plant species is always important, even if it has no immediate commercial value. *In vitro* plant regeneration through cell, tissue and organ culture has a pivotal role in exploiting the advantages of the modern researches in molecular biology and genetic engineering for possible production of genetically modified plants. At the same time the advanced biotechnological methods of culturing plant cells and tissues should provide new means of cryoconserving and rapidly propagating valuable, rare, and endangered medicinal plants. However, of these alternatives the clonal propagation of plants *in vitro* has become the first major practical application of plant tissue culture.

In the above back drop a program of work was undertaken to study comprehensively the cell, tissue and organ culture aspects in three important medicinal plants namely, *Chlorophytum borivillianum* (Safed Musli), *Kaempferia galanga* (Ekangi / Chandramool) and *Atalantia malaberica* (Kaculus). Though the studies widely involved cultural and nutritional management for successful initiation and maintenance of cultures in these medplant species for micropropagation but precisely progressed in the following two major directions:

- i) Studies aimed at developing *in vitro* plant production system for rapid cloning.

- ii) Studies aimed at developing technical protocol for microspore / anther culture *in vitro* wherever needed.

Micropropagation in Safed Musli (*Chlorophytum borivillianum*)

To identify a suitable media composition initially the rhizome sections were cultured on agar gelled MS and B5 medium containing varying concentrations of auxins (2,4-D, NAA 0.05 – 2.0 mg l⁻¹ each and IBA 0.05 – 2.5 mg l⁻¹) with cytokinins (Kinetin and BA 1.0 -3.0 mg l⁻¹ each).

Precisely, the present results summarized in Section 4.1 revealed three distinctly different courses of development following culture of rhizome sections of Safed Musli (*Chlorophytum borivillianum*) *in vitro*.

- i) Direct organogenesis i.e., the induction of microshoots directly on the rhizome section of Safed Musli in MS medium containing only BA or different combination of IBA and BA.
- ii) Development of granular structures on the surface of the fast proliferating calluses in MS medium containing different concentrations of IBA with kinetin.
- iii) Development of shiny globular structures directly from certain locales of the rhizome sections cultured in B5 medium containing different combination of IBA and BA.

All the three above situations have been dealt with three separate series of studies and the results have been summarized and discussed accordingly under different sections (Section 4.2 to 4.7).

Regeneration through direct microshoot production:

To study the first situation, the clusters of proliferating green microshoots along with initial rhizome part were separated and subcultured in MS medium with different IBA-BA combinations. These results, as documented in Section 4.2, clearly revealed a differential response of the isolated microshoots to varying auxin cytokinin concentrations and indicated that although BA at 2.0 – 3.0 mg l⁻¹ were conducive for the induction of morphogenesis and initiation of adventive shoots *in vitro* but presence of IBA at least at a low dose (0.5 mg l⁻¹) to be essential for sustainable normal shoot growth in *Chlorophytum borivillianum*.

A unique feature of the present finding is that only BA was not efficient for the healthy microshoot production instead a balance between auxin and cytokinins appeared necessary where although BA played the key role towards direct induction of adventive shoots but IBA had a critical role to sustain normal growth and development of the microshoots.

Regeneration through embryogenesis:

The second situation identified in the initial trial was the induction of shiny globular structures developed following six weeks of culture of the rhizome explants onto B₅ medium containing IBA and BA (IBA 1.0-1.5 mg l⁻¹ and BA 1.0-2.0 mg l⁻¹) combinations. Since these were conducive for the induction of globular embryoids but could not support the growth of these embryoid structures towards the development of whole plants, a study was undertaken to amend the growth regulator combinations in the same medium (B₅) to achieve sustained growth and embryogenesis. For the purpose these early adventive embryoids (or proembryoids) were subcultured on the same medium containing different combination of IBA – BA and maintained initially under diffused light but later transferred to higher illuminance. Section 4.3 documents the results.

The results revealed that although redetermination of the rhizomatous cells to undergo embryogenesis was induced by a higher level of IBA ($1.0\text{--}1.5\text{ mg l}^{-1}$) but the induced cells with high auxin in the medium failed to support the embryogenic cells to grow beyond globular stage and the embryo development ceased. It seemed necessary either to omit or to reduce auxin concentration for the development of the globular adventive embryos to heart and torpedo stages and finally to plants. Plantlets regenerated through direct embryogenesis were isolated singly and transferred to B₅ basal medium depleted of growth regulators was beneficial for whole plant development.

Regeneration through callogenesis:

In the last situation identified in the initial study was production of profuse granular calluses from the rhizomatous tissue of *Chlorophytum borivillianum*, which continued to grow expanding the callus mass but did not show any marked morphogenetic change. An attempt was made to subculture the calluses in modified media combinations (IBA $0.3\text{--}2.0\text{ mg l}^{-1}$ with BA $1.0\text{--}3.0\text{ mg l}^{-1}$ or Kinetin $1.0\text{--}3.0\text{ mg l}^{-1}$) to induce cytodifferentiation leading to callogenesis and the results are described under Section 4.4.

The observations confirmed that auxin concentration played a critical role in indirect organogenesis, where higher dose of auxin initiates cell division to produced callus and at the same time induced redetermination towards organogenic pathway in otherwise predetermined rhizomatous cells of the germinating rhizome explants, but morphogenetic transformation occurred only following the transfer of the cultures to low auxin or no auxin medium and microshoot developed from the intervening calluses.

Microshoots regenerated on IBA- Kinetin supplemented MS medium on transfer to hormone depleted medium for rooting developed plants with unusually thick and stunted shoots – roots axis. However, on transfer to sand - soil (1:3) mixture these *in vitro* regenerated plantlets survived and rooted normally.

In yet another trial (Section 4.5) the effect of GA₃ on the production and growth of preformed *in vitro Chlorophytum* microshoots was studied. For the purpose two sets of trials were conducted. In the first phase the MS medium was supplemented with IBA 0.5 mg l⁻¹ + BA 2.0 mg l⁻¹ and IBA 0.5 mg l⁻¹ + Kinetin 1.0 mg l⁻¹ with or without GA₃ (0.0 – 2.5 mg l⁻¹).

These results clearly confirmed a synergistic role of GA₃ on the effect of both the auxin - cytokinin combinations that helped in enhancing the rate of microshoot production, increasing the number of large shoots per culture as well as the mean height of the microshoots. Rooting was best induced (~85%) in shoots excised from proliferated shoot cultures on half-strength MS medium augmented with an optimal combination of indole-3-acetic acid and indole-3-butyric acid each at 1.0 mg l⁻¹.

Regeneration through shoot base culture:

Section 4.6 describes the findings of another attempt that was made to develop an efficient protocol of micropropagation in Safed Musli (*Chlorophytum borivillianum*) utilizing the shoot bases of germinating rhizomes as has been severally reported. The principal objective of the study had been to adopt the cultural as well as the nutritional conditions recommended by the earlier workers for micropropagation in Safed Musli and to introduce further amendments, if any, for *in vitro* cloning utilizing sprouting shoot bases as explants.

The findings clearly revealed that although BA alone or in combination with either of the auxins IBA or NAA was conducive for induction of multiple shoots from the shoot bases, but the media supplemented with BA 2.5 and 3.0 mg l⁻¹ alone yielded best results (31.63 and 22.80 multiple shoots per explant respectively). Presence of either of the auxins i.e., IBA or NAA either reduced the mean number of adventive shoots per shoot base or inhibited the process with the increase in concentration and frequently resulted in callus induction. Over 80% shoots rooted when transferred to MS medium with $\frac{3}{4}$ -strength inorganic and organic constituents with 3.0 mg l⁻¹ IBA and ~65% of the micropropagated plants were successfully established in pots. Such plants produced normal fasciculate storage roots following their transfer in pots as naturally occur.

Scaling-up *in vitro* multiple shoots production in shoot base cultures:

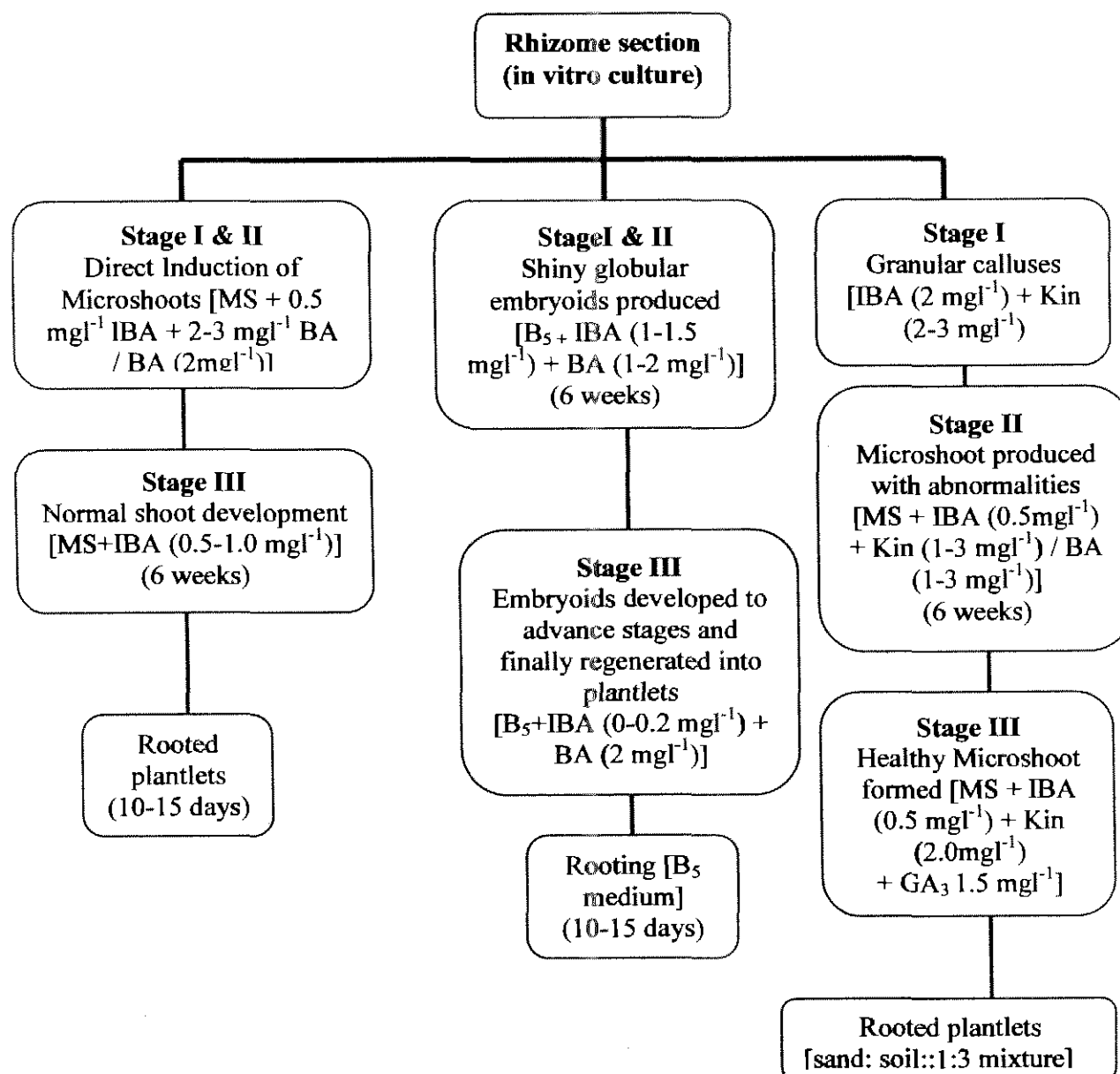
The preceeding study was further extended under Section 4.7 to scale-up the microshoot production. For the purpose the isolated single microshoots were cultured in MS medium containing only BA with varying concentrations of different inorganic and organic constituents. Ammonium sulphate 100 mg l⁻¹, Calcium nitrate 100 mg l⁻¹, glycine 10 mg l⁻¹ and L-glutamine 150 mg l⁻¹ were added with BA 2.5 mg l⁻¹, either individually or in combinations. The only organic source of reduced nitrogen which has responded positively in the present situation is glycine and its stimulating action was noted only in presence of (NH₄)₂SO₄ at a low level.

A comparison:

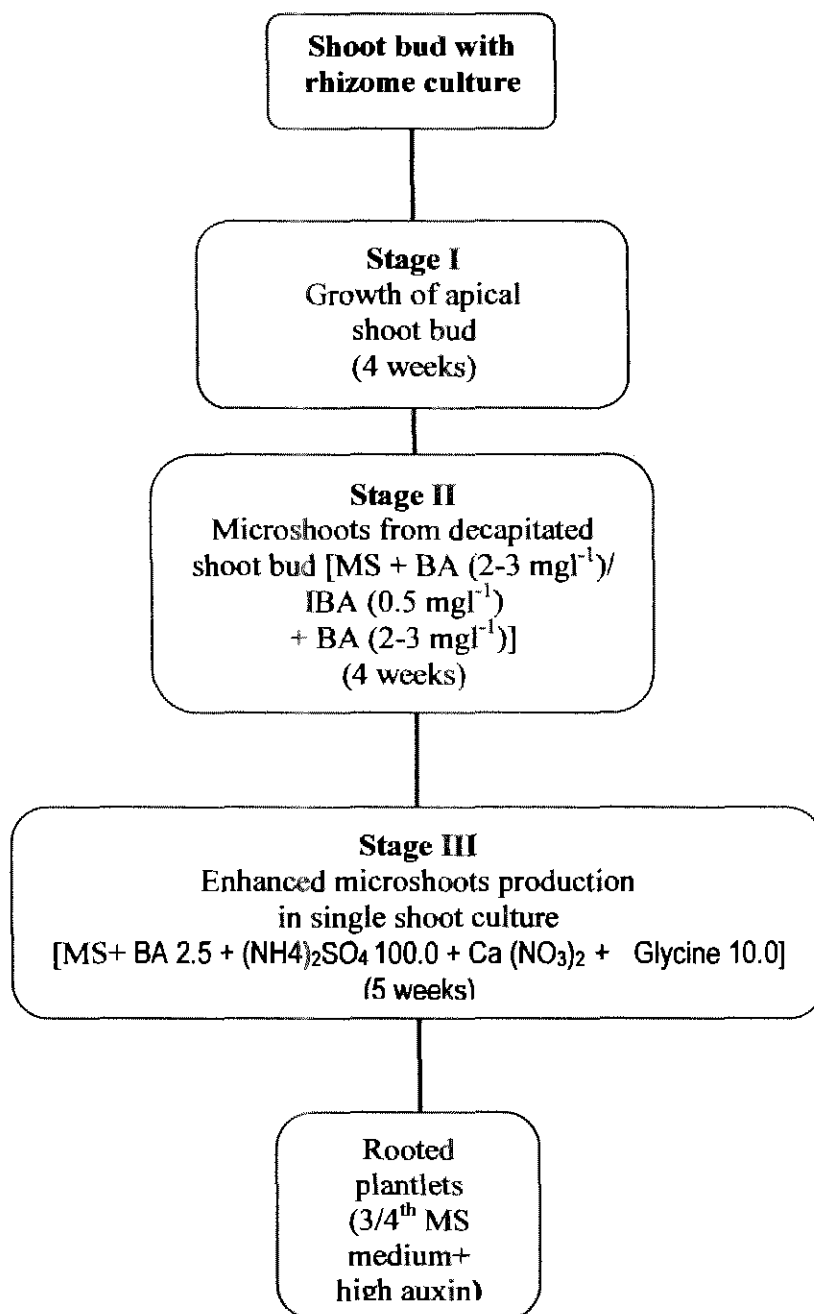
Different modes of *in vitro* regeneration in *Chlorophytum borivillianum* i.e., i) direct shoot proliferation from rhizome sections; ii) direct regeneration through adventive embryoid production from rhizome explants; iii) direct shoot proliferation from shoot base explants; and iv)

indirect shoot production from intervening calluses convincingly revealed that the rate of multiplication has been highest by indirect regeneration through callogenesis (~66.26 microplants per explant), followed closely by microplant production through adventitious embryogenesis (~64.80 microplants per explant) and then by adventitious shoot production from shoot base explants (~50.02 microshoots per explant), while lowest rate of multiplication was recorded for direct microshoot production when rhizome section were used as explants (43.40 microplants per explant).

Protocol for micropropagation of *Chlorophytum borivilianum* using rhizome as explant



**Protocol for micropropagation of *Chlorophytum borivilianum*
using decapitated shoot base as explant.**



Micropropagation in Ekangi (*Kaempheria galanga*)

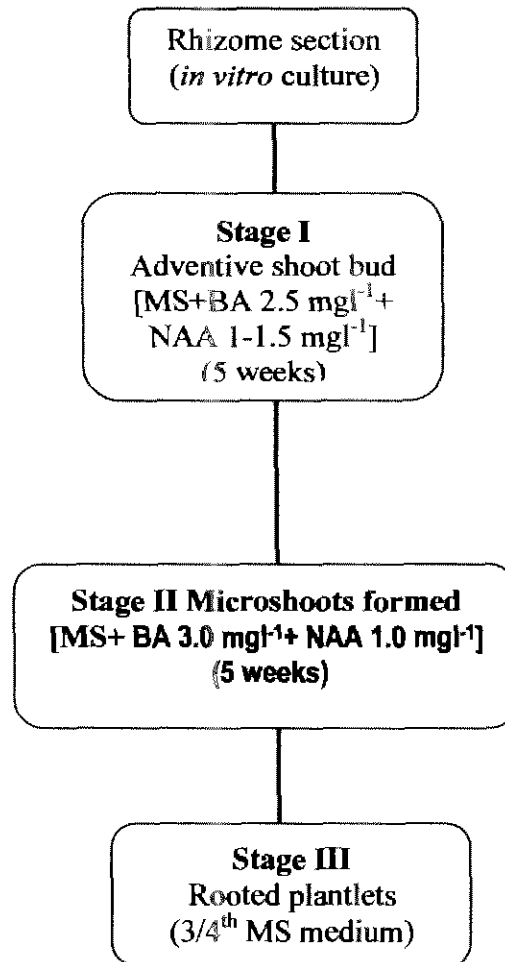
Regeneration by of adventive multiple shoots culture:

Rhizomes being the natural propagule, matured rhizomes with 3-5 sprouted axillary shoot buds were chosen as explant for present micropropagation study duly described in Section 5.1. Adventive shoot buds were induced and grew in size in MS medium augmented with BA 2.5 mg l⁻¹ and NAA 1.0–1.5, but these buds without developing into plantlets ceased to grow beyond a stage.

To identify the suitable nutritional requirements to support morphogenesis in these adventive shoot buds developing into complete regenerated plantlets the study was further extended and the same has been documented in Section 5.2. The aseptically isolated adventive buds were cultured in MS medium with different combination and concentrations of NAA – BA (1.0 to 1.5 mg l⁻¹ and 2.0 to 3.5 mg l⁻¹ respectively).

Based on these results it may precisely be inferred that for bud breaking and induction of adventive microshoots directly from the rhizome explant a BA-NAA ratio of ~1.33 (BA 2.0 mg l⁻¹ – NAA 1.5 mg l⁻¹) seemed to be adequate while an enhanced BA-NAA ratio of ~3.0 (BA 3.0 mg l⁻¹ – NAA 1.0 mg l⁻¹) appeared to be essential for adventive axillary shoot bud proliferation and sustenance of their growth into healthy shoot apices in *Kaempheria galanga*. Following the transfer of the isolated microshoots to hormone depleted medium (¾ MS macroelement) morphogenesis was resumed within a week leading to the normal plantlet development and no auxin was needed for rooting. Most noteworthy is the potentiality of the *in vitro* microshoots to produce more microshoots when isolated and cultured in the next cycle and thus the cycle continued producing ~15 microshoots per culture in every 4 week cycle.

**Protocol for micropropagation of *Kaempferia galanga*
using rhizome as explant**



Micro-cloning in Kaclus (*Atalantia malabérica*)

A program of work was undertaken to develop an *in vitro* protocol for rapid cloning in *Atalantia malabérica*, an outbreeding woody shrub / tree and described in Section 6.1. The zygotic embryo with / without cotyledon section (EWC / EWOC) were taken as explants and were cultured on MS medium supplemented with different kind of auxins and cytokinins in varying concentrations. Auxins were added at relatively lower dose (0.5 and 1.0 mg l⁻¹) while cytokinins were added relatively at higher dose (1.0 mg l⁻¹ to 4.0 mg l⁻¹). The response has been significantly higher with IBA 1.0 mg l⁻¹ + BA 3.0 mg l⁻¹ giving out ~7 adventive microshoots per explant. Interestingly, following the transfer of the microshoots to same nutritional combinations IBA (1.0 mg l⁻¹) and BA (2.0 – 3.0 mg l⁻¹) was found to be conducive for further production of adventive microshoots from the base of the inoculated one. It is worthwhile to mention that this is the first report on *in vitro* microshoot production in Kaclus (*Atalantia malabérica*). However rooting in the microshoot remained unachieved.

Multiple shoots from nodal segments of *Atalantia malabérica*:

In view of the limitations of microshoot production from zygotic embryos while initiating the second phase of work on micropropagation of *Atalantia malabérica*, the nodal segment of young plants of *Atalantia malabérica* were considered to be potential for giving out multiple shoots from the nodal tissue destined for axillary shoot bud production. Results are described in Section 6.2 revealed that IBA 1.0 mg l⁻¹ and BA 3.0 mg l⁻¹ was found to be the best combination for microshoot induction producing highest mean number of microshoots (6.66 adventive shoots per nodal segment).

For sustained growth of microshoots of nodal origin with restoration of normal morphogenesis isolated microshoots were cultured in MS medium containing either full or half inorganic salts with varying

hormonal combinations. Although an increase in the frequency of microshoots could be successfully achieved but the microshoots were more or less abnormal under either of the treatment conditions.

Anther culture studies in *Atalantia malabérica*:

Being a perennial woody species production of haploids in *Kaclus*, which has tremendous breeding potential, is almost impossible through conventional methods, therefore, attempts to produce haploids / homodiploids through anther / microspore culture remained as the only potential option, so some preliminary studies have been made in that direction. To study the *in vitro* response, the excised anthers were cultured on agar gelled N₆ medium containing different auxin – cytokinin combinations. A differential response was recorded in terms of nature and growth of callus.

To induce morphogenesis the anther calluses were transferred to new medium with different growth regulators in both agar-gelled and liquid half MS medium supplemented with different NAA – BA combinations and the observations are discussed in Section 6.3. NAA 0.5 mg l⁻¹ with BA 1.0 mg l⁻¹ responded towards morphogenetic changes and conducive for rhizogenesis, producing healthy root growth along with the development of clusters of shiny globular embryo like structures but regeneration of whole plants remained an impasse.

As this is the first attempt towards androgenic plant development in *Atalantia* the present results may be considered reasonably positive and encouraging. The study needed to be carried forward with fresh attempts to accomplish androgenic plant development. As that required fresh anthers to be collected next season, the study remained unconcluded as time became the limiting factor.

Bibliography

- Afza, R.; Duren, M. Van, Morpurago, R. and Van Duren, M. (1996), Regeneration of *Ensete ventricosum* through somatic embryogenesis and adventitious buds. *Plant cell Reports* 15(6) : 445-448.
- Ahn, M.S.; Lim, H.C.; Choi, D.C.; Choi, J.S.; Choi, Y.G. and Pak, Y.J. (2002), Effect of basal media, growth regulators and addition agents on callus formation and plant regeneration for anther culture of *Hemerocallis* spp.. *Korian Journal of Horticultural Science and Technology* 20(2) : 124-129.
- Alferman, A.W. and Reinhard, E. (1980), Production of natural compounds by Cell culture method. *Proc. Symp. Plant Cell Culture*, Tubingen, 1980.
- Amirato, P. (1985) patterns of development in culture In : Henke, R.; Hughes, K.; Constantin, M. and Hollendear, A. (eds.): *Tissue culture in forestry and agriculture*. Penum Press, N.Y. : 9-62.
- Amirato, P. V. (1977), Hormonal control of somatic embryo development from cultured cells of caraway. *Plant Physiol* 59:579-586
- Anand, P.H.M. and Hariharan, M. (1997), *In vitro* multiplication of greater galangal (*Alpinia galanga* (Linn.) Wild)- a medicinal plant. *Phytomorphology* 47(1): 427-429
- Anand, P.H.M.; Hariharan, M. and Kishor P.B.K. (1999), *In vitro* plant regeneration from rhizome bud derived callus in yellow zedoary (*Curcuma aromatica* Salisb.) –a medicinal plant. *Plant Tissue Culture and Biotechnology, emerging trends. Proceeding of a symposium held at Hyderabad, India. 29-31 January 1997.* :187-192.
- Anand, P.H.M.; Hari Krishnan, K. N., Martin, K.P. and Hariharan, M. (1997), *In vitro* propagation of *Kaempferia rotunda* Linn. "*Indian crocus*" – a medicinal plant. *Phytomorphology* 47(3): 281-286.
- Anjum, M.A. and Ali, H. (2004), Effect of culture medium on direct organogenesis from different explants of various potato genotypes. *Biotechnology* 3(2) 187-193
- Anjum, M.A. and Ali, H. (2004), Effect of culture medium on shoot initiation from calluses of different origin of potato (*Solanum tuberosum* L.). *Biotechnology* 3(2) : 194-199
- Appleton H.R.; Staden J. Van; Van Staden J.; Vainstein, A. (ed.) and Weiss, D. (1995), *In vitro* propagation of a miniature Hypoxis, *H. angustifolia* Ornamental Plant Improvement. Classical and Molecular Approaches XVIIIth EUCARPIA symposium, section ornamentals, Tel. Aviv, Israel, 5-9 March, 1995. *Acta Horticulturae*. 420.: 95-97.
- Babu, K. N., Regy Lukose, Ravindran, P. N. and Vidhyasekaran, P. (1993), Tissue Culture of tropical spices Genetic Engineering. *Molecular Biology and Tissue Culture for Crop Pest and Disease Management*. 257-267
- Basu, S and Jha, T.B. (2005), *In vitro* propagation of rare medicinal plant. *Proceedings National Symposium on biotechnology. CIMAP* :102
- Bayliss, M.W. and Dunn, S.D.M. (1979), *Plant Sci. Lett.* 14. 311-316. cited in: *Plant propagation by Tissue culture* George, E. F. and Sherington. Exegetics Ltd. Rverseley, England :1-708.
- Begum, S.; Hakim, L. and Azam, M.A. (1995), Efficient Plant regeneration via somatic embryogenesis in ginger. *Plant Cell Tissue and Organ Cult.* 32: 289- 292

- Beura, S.; Singh, R and Jagadev P.N.(2003), *In vitro* shoot proliferation and corm production and corm production in Gladiolus cultivar American Beauty. Journal of Ornamental Horticulture New Series. 6(3) :195-201.
- Bezbaruah, H. P. and Saikia, L. R. (1977), Variation in cell and cross compatibility in tea (*C. sinensis* L.) and *C. japonica* L. In: Proc. Indian Acad. Sci. 76:219-220.
- Bhattacharya, N. M., Hazra, S. K. and Saha, (2003), A. Multiple shoot culture from nodal segments of Tea (*Camelia Sinensis* L.O. Kuntze.). Journal of Plant Biology 30(3):307-311.
- Bhattacharya, N.M. and Sen, S.K. (1980), Production of plantlets through somatic embryogenesis in *B. campestris* Z. Pflanzephywol. 99:357-365.
- Borthakur, M.; Hazarika, J.; Singh R.S. (1998), A protocol for micropropagation of *Alpinia galanga*. Plant Cell, Tissue and Organ Culture, 55: 231-233,
- Borthakur, M.K. and Mitra, G. C. (1990) Nutrient requirements for growth and multiplication of tea plants *in vitro*. Bangladesh. J. Bot. 19(1). :65-71.
- Cassells, A.C. (1979). Physiol. Plant 46:159-164. cited in: Plant propagation by Tissue culture. George, E. F. and Sherington. Exegetics Ltd. Rverseley, England:1-708.
- Chandal, K. P. S.; Shukla G. and Sharma, N. (1996), Biodiversity in medicinal and aromatic plants. Conservation and Utilization. :1-239. N.B.P.G.R., New Delhi.
- Chandel K.P.S. and Sharma, N.(1993), *In vitro* strategy for the conservation of medicinal and aromatic plants in India. G-15 GEBMAP Newsletter 3 and 4.
- Chang R.C.O. and Goh, C.J.(1994), High frequency direct shoot regeneration from corm axillary buds and rapid clonal propagation of taro, *Colocasia Esculanta* var. *esculanta* (L.) Schott.(Araceae). Plant Science Limerick 104(1): 93-100.
- Chang W.D.; Gau, T.G. ; Chang, D.D.; Chern, Y.C.; Chen, C.C.; Yeh, F.T.; Chang, Y.S.; Hsieh, M.T.; Chu, N.Y. and Tsay, H.S. (1992), Rapid clonal propagation and production of useful compounds of Chinese medicinal herbs by tissue and cell suspension culture. Special Publication Taichung District Agricultural Improvement Station. 29:207-211; in the impact of biological research on agricultural productivity. Proceedings of the SABRAO International symposium 10-13 March.
- Charlwood, B.V.; Rhodes, M.J.C.(1990), Secondary products from plant tissue culture (eds.) Chalwood B.V.; Rhodes, M.J.C. Proc. Intl. Symp. Secondary Compounds in Plant Tissue Culture, Kings College, London, Dec. 1988:288.
- Constabel, F.(1990), Medicinal plant biotechnology. Planta Med. 56: 421-425.
- Cook, E.L.; Cunningham, A. and Staden, J.V. (1988), The tissue culture of an exploited Zulu medicinal plant, *Bowiea volubilis*. South African Journal of Botany. 54(5) :509-510
- Das, A. ; Paul, A.K. and Chaudhury, S. (2000), Micro propagation of sweet orange *Citrus csinensis* for the development of the nucellar seedlings. Indian Journal of Experimental Biology. 38(3): 269-272.
- Dave, A; Joshi, N; Purohit, S.D.(2004), *In vitro* propagation of *Chlorophytum bolivirianum* using encapsulated shoot buds. European Journal of Horticulture Science 69 (1):37-42.
- David, A.(1972), Compt. Rend. Acad. Sci. Paris 275 D:857-860 . cited in: Plant Propagation by Tissue Culture, George, E. F. and Sherington. Exegetics Ltd. Rverseley, England. :1-708.

- De La Guardia, M.D. and Benlloch, M. (1980), *Physiol. Plant.* 49: 443-448. cited in: *Plant Propagation by Tissue Culture* George, E. F. and Sherington. Exegetics Ltd. Rverseley, England:1-708.
- Devi, S. Y.; Mukherjee, B.B. and gupta, S. (1994), Rapid cloning of teak (*Tectona glandis* L.) by in vitro multiple shoot production. *Indian Journal Experimental Biology* 32: 668-671
- Dinkelman, K. and Staden, J.V(1988), Micropropagation of *Ixia maculata* L. *South African Journal of Science* 84(7) :589.
- Dogra, S.P.; Korla, B.N. and Sharma, P.P. (1994), In vitro clonal propagation of Ginger (*Zingiber officinal* Rosc.) *Horticultural Journal.* 7(1). :45-50.
- Dougall, D.K. (1977),:76-84. in Barz et al. (eds.) 1977 (q.v.). cited in: *Plant Propagation by Tissue Culture* George, E. F. and Sherington. Exegetics Ltd. Rverseley, England. :1-708.
- Drewes, F.E. Bayley, A.D. Staden, J.Van and Van Staden J (1993),Tissue Culture of *Schizobasis intricata*, a medicinal plant. *South African Journal of Botany.*59(1) :105-106.
- Esna-Asri,M. and Villiers, T.A.(1998), Plant regeneration from tuber discs of potato (*Solanum tuberosum* L.) using 6- benzylaminopurine (BAP).*Potato Rsearch.* 41 (4). :371-382.
- Evans,D.A.; Sharp,W.R. and Paddock,E.F.(1976), *Phytomorph.* 26: 379-383. cited in: *Plant propagation by Tissue culture* George, E. F. and Sherington. Exegetics Ltd. Rverseley, England. :1-708.
- Ferwerda, F.H. (1994) Clonal propagation of Galanga (*Languas galangal*(L.) Stuntz.) through tissue culture. *Journal of Agriculture of the University of Puerto-Rico.* 78 (3-4). :147-152.
- Fujiwara, A. (1982) *Plant Tissue culture* (ed.) A. Fujiwara, Proc. 5th Intl. Cong. on Plant Cell and Tissue Culture, Tokyo, Japan,1982.
- Gaikward, P.D.; John, S.C and Billiore , M.(2003) , callus induction and regeneration studies in Safed musli (*Chlorophytum bolivirianum* Sant and Fern) *Research on Crop.* 4 (2): 249-253
- Gamborg, O.L., Miller, R.A. and Ojhima (1968). Nutrient requirements of suspension culture of soyabean root cells. *Exptl.Cell Res.* 50:151-158.
- Gamborg, O.L.; Constabel,L. and Miller, R.A. (1970), *Physiol. Plant* 30: 125-128. cited in: *Plant Propagation by Tissue Culture* George, E. F. and Sherington. Exegetics Ltd. Rverseley, England. 1-708.
- George, E. F. and Sherington. .(1984), In: *Plant Propagation by Tissue Culture*, Exegetics Ltd. Rverseley, England:1-708.
- Geraci, G. and Starrantino, A. (1990), Attempts of regenerate haploid plants from *in vitro* culture of Citrus anthers. *Acta Horticulturae.* 280: 315-320.
- Germana, M.A.(2003), Somatic embryogenesis and plant regeneration from anther culture of *Citrus aurantium* and *C. reticulata* *Biologia Bratislava* 58(4):843-850.
- Germana, M.A.; Wang, Y.Y.;Barbagallo, M.G. Iannolino, G. and Crescimanno, F.G.(1994), Recovery of haploid and diploid plantlets from anther culture of *Citrus Clementina* Hort. Ex Tan. And *citrus reticulata* Blanco. *Journal of Horticultural Science.* 69(3):473-480.
- Grosser, J. W.; Gmitter, F. G.; Castele, W.S. and Chandler, J. L.(1998), Somatic Hybridization : a new approach to Citrus rootstalk improvement. *Fruits Paris* : 53(5) :331-334.

- Haensch K.T. (1994), Plant regeneration through somatic embryogenesis in different genotypes of liliun hybrids Gartenbauwissenschaft 61(5). :214-218.
- Haque, M.I., S. Perveen and R.H. Sarker, 1999, *In vitro* propagation of ginger (*Zingiber officinale* Rose.). Plant Tissue Cult., 9: 44-51.
- Harikrishnan, K.N.; Hariharan, M. and Kishore, P.B.K.(1999), *In vitro* clonal propagation of Sweet flag (*Acorus calamus* L.)- a medicinal plant Plant Tissue Culture and Biotechnology: emerging trends. Proceedings of a symposium held at Hyderabad, India.29-31 January 1997 :220-222
- Harikrishnan, K.N.; Martin K.P., Anand, P. H. M. and Hariharan, M.(1997) ,Micropropagation of Sweet flag (*Acorus calamus*) a medicinal plant . Journal of Medicinal and Aromatic Plant Sciences, 19(2) :427-429.
- Hazra, S.K., Das,S. and Das,A.K. (2002), Sisal plant regeneration via organogenesis. Plant Cell Tissue and Organ Cult. 70:235-240.
- Hidaka, T. and Omura, M. (1989), Origin and development of embryoids from microspores in anther culture of Citrus. Japanese Journal of Breeding. 39(2) :169-178.
- Hildebrandt, A. C., Rikor, A. J. and Duggar, B. M. (1946), The influence of the composition of the medium on growth *in vitro* of excised tobacco and sunflower tissue cultures. Amer. J. Bot.33. :591-597.
- Him-de-Freitez, Y. and Paez-de-Casares, J. (1997) Micropropagation of ginger (*Zingiber officinale* R.) by organogenesis and embryogenesis Proceedings of Interamerican Society for Tropical Horticulture 41:70-76.
- Hong W. and Debergh, P. C.(1995), Plant –Cell- Tissue –and –Organ- Culture. 43(1) :21-28.
- Huang, S.Z. and Xiong, D.H.(1992) Juvenile embryo culture and plant let clonal propagation of the jianshui seed less tangerine orange (*Cytrus reticulata*) *in vitro*. Acta Botanica Yunnanica 14 (1) :73-79.
- Hui,Hong; Jiang , Niag; Liu, Qi Xin(1996), On the culture of test tube plant let from rhizomes of threatened plant *Glehnia littoralis* Fr. Schmidt ex Miq. Journal of Plant Resources and Environment 5(4):57-58.
- Hussain C.T.S.;Geetha, C.K.; Rajeevan, P.K. and Valsalakumari P.K. (1997) ,Effect of triadimefon and sucrose on *in vitro* corm production in gladiolus cv. Friendship. Journal of Ornamental Horticulture 3(1-2) :36-40.
- Hyndman,S.E.; Hasegawa,P.M. and Bressan,R.A. (1982 b), Plant cell tissue organ culture 1:229-238.cited in: Plant Propagation by Tissue Culture George, E. F. and Sherington. Exegetics Ltd. Rverseley, England:1-708.
- Jose, ASR, Thomas R. and Nair, G.M. (2002), Micropropagation of Kaempferia galanga. Linn. Through high frequency *in vitro* shoot multiplication . Journal of Plant Biology. 29 (1):97-100.
- Jouanneau, J.P. (1975), *Esp. cell. Res.* 91. :184-190. cited in: Plant propagation by Tissue culture George, E. F. and Sherington. Exegetics Ltd. Rverseley, England. :1-708.
- Jumin, H.B.(1995), Plant regeneration via somatic embryogenesis in Citrus and its relatives. Phytomorphology. 45 :1-2.

Kang, Y.M.; Min, J.Y.; Moon, H. S.; Karigar, C.S.; Prasad, D.T. ; Lee, C.H. and Choi, M. S. (2004), Rapid *in vitro* adventitious shoot propagation of *Scopalia parriflora* through rhizome cultures for enhanced production of tropane alkaloids. *Plant Cell Reports* 23(3). :128-133.

Kartha, K.K.; Gamborg, O.L. and Constable, F. (1974), *Physiol Plant*.31: 217-220 cited in: *Plant propagation by Tissue culture* George, E. F. and Sherington. Exegetics Ltd. Rverseley, England :1-708.

Kurz, W.G.W. (1989), Primary and secondary metabolism of plant cell culture II (ed.) Kurz, W.G.W. *Proc Symposium on Regulation of Primary and Secondary Metabolism in Plant Cells*. Plant Biotech. Institute, NRC, Canada 1988.

Lakshmi, M and Mythili, S(2003), Somatic embryogenesis and plant regeneration from callus cultures of *Kaemferia galanga* a medicinal plant. *Journal of Medicinal and Aromatic Plant Sciences* 25(4). : 947-951

Li, W.; Gao, H.H.; Lu, R. Guo, G.Q. and Zheng, G.C. (2002) Direct plantlet regeneration from the tuber of *Stachys sieboldii*. *Plant Cell Tissue and Organ Culture*.71 (3) :259-262.

Lim, H. T.; Song, Y. N.; Kim, W. B. Lee, E.A. Rubatzky, V.E.(ed.); Chen, Hang(ed.) and Petron, J. Y.(1998), Plant regeneration via organogenesis and somatic embryogenesis and micro multiplication of *Allium victorialis* var. *Platyphyllum* Makino. Thired International Symposium on Diversification of Vegetable Crops, Beijing, China 24-27 Sept. 1996. *Acta Horticulturae*.467 :193-197

Ling, J.T. and Iwamasa, M. (1997), Plant regeration from embryogenic calli of six Citrus related genera. *Plant Cell Tissue and Organ Culture* 49(2): 145-148

Liu, L.J.Y, Rosa Marquez, E., Licha, M. and Biascoechea, M.L.(1988). Tanier (*Xanthosoma spp*) propagation *in vitro*. *Journal of Agriculture of the University of Puerto-Rico*. 72 (3). :413-426 .

Maiti, S. and Geetha, K.A. (2005), Characterization, genetic improvement and cultivation of *Chlorophytum borivillianum* –an medicinal plant of India. *Plant-Genetic-Resource:- characterization- and – utilization*.3(2), :264-272.

Malamung, J.J.F., H. Inden and T. Asahira, (1991), Plantlet regeneration and propagation from ginger callus. *Scientia Hort*.48: 89-97

Manimaran, S.; Loganathan, V.; Akilandeswari, S.; Jaswantb, A.; Sathya, S. and Ruckmani, K.(2002), Wound healing and antimicrobial activity of formulated cream of leaf volatile oil of *Atalantia monophylla* Correa. *Hamdard Medicus* 45(4), :59-62.

Margara, J. (1969 a), *Ann. Physiol. Veg.* 11 :95-112. cited in: *Plant Propagation by Tissue Culture* George, E. F. and Sherington. Exegetics Ltd. Rverseley, England. :1-708.

Margara, J. (1969 b), *Compt.Rend. Sead. Sci. Paris*. 268 D :686-688. cited in: *Plant Propagation by Tissue Culture*. George, E. F. and Sherington. Exegetics Ltd. Rverseley, England. :1-708.

Meenakshi, N; Sulikeri, G.S. ; Krishnaoorthy, V and Hegde, R. V.(2001), Standardization of chemical environment for multiple shoot induction of Turmeric (*Curcuma longa* L.) for *in vitro* clonal propagation. *Crop Research Hisar* (3). :449-453.

Mendelsohn, R. and Balick, M. (1994), The value of undiscovered pharmaceuticles in tropical forest. *Econ. Bot.* 94(2) :223-228.

Meyer, Y. and Abel, W.O. (1975), *PLanta* .123 :33-40. cited in: *Plant propagation by Tissue culture*. George, E. F. and Sherington. Exegetics Ltd. Rverseley, England. : 1-708.

- Millar, C. O.; Skoog, F.; Okumura, F. S.; Von Saltza, M. H. and Strong, F. M. (1956), Isolation, Structure and synthesis of kinetin, a substance promoting cell division. J. Am. Chem. Society. 78:1375-1380.
- Mogor A. F.; Mogor, G.; Ono, E. O., Rodrigues, J.D. ; Snoke, E.(ed.) Mathe, I.(ed.) Blunden, G. (ed.) Kery A. (2003), Effect of Benzylaminopurine (BAP) on clonal propagation rate of *Curcuma longa* L. Proceedings of the International Conference on *Medicinal and Aromatic Plants*, Budapest, Hungary, 8-11 July, 2001 Part II. Acta Horticultura, 597:321-323.
- Mohanty, B. and Fletcher, J.S. (1976), Plant Physiol 58 :152-155. cited in: Plant Propagation by Tissue Culture George, E. F. and Sherington. Exegetics Ltd. Rverseley, England :1-708.
- Mohanty, R. B. (2005), Indigenous use of *Atalantia malabérica* (Rutaceae) to treat lumbago. Ethnobotany 17(1/2), : 205.
- Morel, G.; Martin, C. and Muller, J.F. (1968), Ann. Physiol. Veg. 10 :113-139. cited in: Plant Propagation by Tissue Culture. George, E. F. and Sherington. Exegetics Ltd. Rverseley, England. :1-708.
- Mukhopadhyay, M. J. Mukhopadhyay, S. and Sen, S. (2002), *In vitro* propagation of *Iphigenia indica*, an alternative source of colchicine. Cell Tissue and Organ Culture. 69 (1). :101-104.
- Murashig, T. (1961), Science 134 :280. cited in: Plant Propagation by Tissue Culture George, E. F. and Sherington. Exegetics Ltd. Rverseley, England. :1-708.
- MURASHIGE, T. and SKOOG, F. (1962), A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum, 15:473-497
- Nadamitsu, S. Y. ; Anodh, K. ; Kondo, and Agawa. M.S. Interspecific hybrids between *C. vietnamensis* and *C. chrysanth* by cotyledon culture . Jap. J. Breed. 36 (30). :309-313.
- Naik, G. R. (1998), Micropropagation studies in medicinal and aromatic plants. In: I. K. Khan and A. Khanun (eds.) Role of biotechnology in Medicinal and Aromatic Plants, Hyderabad : 50-56.
- Nakamura, Y. and Shizuoka, I. S., (1991), *In vitro* propagation techniques of tea plants. Tokyo, Japan: 436-440.
- Nalawade, S.M.; Sagare, A. P.; Lee, C. y.; Kao, C.L. and Tsay, H. S. (2003), Studies on tissue culture studies in chinese medicinal plants. Resource in Taiwan and sustainable utilization. Bot. Bull. Sead. Sin. 44: :79-98.
- Narayanaswamy, S. (1998), Plant Cell and Tissue Culture. Pub. Tata McGraw-Hill, New Delhi: 1-225.
- Ndong, Y. A.; Wadouchi, A.; Sangwan, Norreel, B S; Sangwan R.S (2006), Efficient *in vitro* regeneration of fertile plants from corm ex plants of *Hypoxis hemerocallidea* landrace Gaza- the "African Potato". *Plant cell Reports*. 25(4):265-273.
- Negash, A.; Puite, K.; Schaart, J.; Visser, B. and Krens, F. (2000), *In vitro* regeneration and micro propagation of enset from Southwestern Ethiopia. Plant Cell Tissue and Organ Culture. 62(2) :153-158.
- Nguyen, H.L., Doan Trong, Duc:Kown, TaeHo and Yang, Moonsik (2005), Micropropagation of Zedoary (*Curcuma zedoaria* Roscoe) a valuable medicinal plant. Plant Cell Tissue and Organ Culture 81(1) :119-122.

- Nitsch, J. P. and Nitsch, C. (1969), Haploid plants from pollen grains. *Science*, 163:85-87.
- Ochatt, S.J. and Cso, O. H. (1982) *Plant physiol.* 69 (Suppl.) 34 (Abst. 188). cited in: *Plant Propagation by Tissue Culture* George, E. F. and Sherington. Exegetics Ltd. Rverseley, England.:1-708.
- Parthasarathy, V. A. and Nagaraju, V.(1994), Effect of NAA and malt extract on in vitro growth of excised embryos of citrus. *International Journal of Tropical Agriculture*.12(1-2) :107-111.
- Pedroso, M.C. and Pais, M. S. (1993), Direct embryo formation on leaves of *C. japonica* L. *Plant. Cell Reports*. 12(11) : 639-643.
- Phalisteen, Sultan; Shawl, A.S.; Ramtake, P.W; Arif , Jan; Nahida, Chisti ; Neelafar, Jabeen and Shabir, S. (2006), *In vitro* propagation for mass multiplication of *Podaphyllum hexandrum*: a high value medicinal herb. *Asian Journal of Plant Science* 5 (2) :179-184 .
- Powell, L.E.; Borkowska,B. and Singha, S. (1980) *Plant Physiol.* 65 (suppl.) 92. cited in: *Plant propagation by Tissue culture* George, E. F. and Sherington. Exegetics Ltd. Rverseley, England: 1-708.
- Pudake,R.N. and Dhumale, D.B. (2003), In vitro multiplication of *Chlorophytum bolivirianum* Sant. And Fern. *Journal of Maharashtra Agriculture Universities* 28(3) :265-267.
- Purohit, S.D.; Dave, and Kukda, g. (1994), Micro propagation Safed musli (*Chlorophytum bolivirianum*), a rare Indian medicinal herb . *Plant –Cell- Tissue –and – Organ- Culture* 39(1) : 93-96
- Rahman M.M., Amin, M.N. ; Ahamed, T. ; Ali M. R. and Habib, 'A. (2004), Efficient plant regeneration through somatic embryogenesis from leaf base-derived callus of *Kaempferia galanga*. *Asian Journal of Plant Sciences* 3 (6): 675-678
- Raina, S. K. and Iyer, R. D. (1983), Multicelled pollen embryoids and callus formation in tea anther cultures. Cited in: C. S. VenkataRam.Comm.Vol.J. *Plant Crops* (eds. M. K. Nair, V. Ranganathan, V. S. Sharma and T. Premkumar) 11: 63-67.
- Raju, B.;Das, A. and Kalita, M.C.(2005), *in vitro* clonal propagation of *Curcuma caesia* Roxb. And *Curcuma Zedoaria* Rosc. from rhizome bud explants. *Journal of Plant Biochemistry and Biotechnology* 14(1):61-63.
- Rani,G. and Grover, I.S.(1999), In vitro callus induction and regeneration studies in *Withenia somnifera* Plant –Cell- Tissue –and – Organ- Culture 57(1):23-27.
- Refrogiato,R.G.(1999), *Biotechnology in Citrus production*. *Agrumicoltura Italiana: Problemi e Prospettive*, Firenze, Italia, 13 Novembre1998. Georgofili.1998Publ.1999,45: 5: 123-133.
- Reinert, J. ; Tazawa, m. and Semenoff, S. (1967) , *Nature* 216 :1215-1216. cited in: *Plant Propagation by Tissue Culture* George, E. F. and Sherington. Exegetics Ltd. Rverseley, England: 1-708.
- Roest, S. and Bokelmann, G.S. (1975), *Ssientia Hort* 3:317-330. cited in: *Plant propagation by Tissue culture* George, E. F. and Sherington. Exegetics Ltd. Rverseley, England. : 1-708.
- Rumynin, V. A. and Slyusarenko, A. G. (1989), Mass clonal propagation of Lilies. *Byulletin' Glavnogo Botanicheskogo Sada* 153 : 62-69

- Saha, A. (1997), Ph.D. thesis: Tissue Culture Studies in Certain Economically Important Plants. B.C.K.V., W.B., Nadia
- Saha, D.K. and Bhattacharya, N.M. (1992), Stimulating effect of temperature treatments on the production of meristemoids from pollen callus of tea, *C. sinensis* Indian Journal of Exptl. Biol. 30:83-86.
- Sahoo, Y. and Chand, P. K. (1998), Micropropagation of *Vitex vegando* L. a woody aromatic medicinal shrub, through high frequency axillary shoot proliferation . Plant Cell Report. 18(3-4) :301-307.
- Salvi, N.D.; George, L.; Eapen, S. (2002), Micropropagation and field evaluation of micropropagated plants of tumeric. Plant Cell, Tissue and Organ Culture, 68:143-151,
- Sarin, N.B. and Bagga, S. (1983), In vitro culture of embryos and other parts of *Cocos nucifera*. Cited in: Proc. Natl. Sem. Plant Tissue Culture. 2-4 March: 1983 at CPCRI: Kasergod: India:132-139.
- Sarwar, M. (1984), Callus formation from explanted organs of tea. (*Camellia sinensis*). Sri Lanka J. Tea Sci. 54: 18-22.
- Shark, R. V. and Hildebrandt, A. C. (1972), Can J. Bot. cited in: Plant Propagation by Tissue Culture. George, E. F. and Sherington. Exegetics Ltd. Rverseley, England :1-708
- Sharma, P.; Sharma Y.D.; Dhiman, S.R. and Gupta, Y.C. (2003), Studies of clonal propagation of *Gloriosa*. Journal of Ornamental-Horticulture-New-Series 6(4):347-351.
- Sharma, P.; Sharma, Y. D.; Dhiman, S.R. and Gupta, Y. C. (2003), Studies on clonal propagation of *Gloriosa*. Journal of Ornamental Horticulture New Series. 6(4):347-351.
- Sharp, W.R. ; Sondahl, M.R. ; Caldas, L.S. and Maraffa, S.B. (1980), *Hort. Rev.* 2 :268-310. cited in: Plant propagation by Tissue culture George, E. F. and Sherington. Exegetics Ltd. Rverseley, England:1-708.
- Shirin, F.; Kumar, S. and Mishra, Y. (2000), *in vitro* plantlet production system for *Kaemferia galangal*, a rare Indian medicinal herb. Plant –Cell- Tissue –and – Organ- Culture 63(3) :193-197 .
- Shrunk, R. U. and Hildebrandt (1972), Medium and Techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50:199-204.
- Singh, I. D. (1980), Non conventional approaches to the breeding of tea in North East India. Two and a bud. 27:3-6
- Srivastava, L. J. and Raina, R. (1997), Domestication of medicinal plants. Kurukshetra Dec. 1997 : 26-28.
- Stefaniak, B. (1994), Somatic embryogenesis and plant regeneration of *Gladiolus*. Plant cell Reports. 13(7) :386-389
- Sunitibala, H ; Damayanti, M and Sharma G.J. (2001), *in vitro* propagation and rhizome formation of *Curcuma longa* Linn, Cytobios 105(409):71-82.
- Sutter, E. G. (1986), Micropropagation of *Isia viridifolia* x *Homoglossum* hybrid. Scientia Horticulturae. 29 (1/2) :181-189.

- Swapna, T. S., Binitha, M ; Manju, T.S. (2004). In vitro multiplication in *Kaempferia galanga* Linn. Applied Biochemistry and Biotechnology 118(1/3) :233-242.
- Teng, W.L. An alternative propagation method of Ananas through nodal culture.(1997), Plant cell Reports. 16(7) :454-457.
- Teo, L. ; Rao,A.N. ; Loh, C.S. and Bay,P.J. (1988), Cotyledon tissue culture and shoot buds on roots. Cell and tissue culture in field crop improvement. Proceedings 38 : 115-127.
- Thomas, E. ; Kochba, J. and Wernicke,W.(1978), Experientia. 34 :959. cited in: Plant Propagation by Tissue Culture George, E. F. and Sherington. Exegetics Ltd. Rverseley, England:1-708.
- Torres, K. C. and Carlisi, J. A. (1986), Micropropagation of *Cammlia*. cited in: American Camelia Year Book. :1-7
- Vincent , K.A., Hariharan, M., and Mathew K. M. (1992a), Embryogenesis and plantlet formation in tissue culture of *Kaempferia galanga* L. a medicinal plant. Phytomorphology 42(3-4). :253-256.
- Vincent K. A. , Mathew, K. M and Hariharan M (1992b), Micropropagation of *Kaempferia galanga* L. — a medicinal plant. Plant Cell, Tissue and Organ Culture. 28(2) :229-230
- Vincent, K.A., Bijoy, M., M. Hariharan and K.M. Mathew, (1991), Plantlet regeneration from callus culture of *Kaempferia galanga* L. Indian J. of Plant Physiol., 34: 396-400.
- Walker, K.A. and Sato, S. J. (1981) ,Plant cell tissue organ culture. 1 :109-121. cited in: Plant propagation by Tissue culture George, E. F. and Sherington. Exegetics Ltd. Rverseley, England. :1-708.
- Wawrosch, C.; Mala,P.R. and Copp,B.(2001),Clonal propagation of *Lilium Nepalense* D. Donna a threatened medicinal plant of Nepal. Plant Cell Reports.20(4): 285-288.
- Wu,L.F.;Qu,Y.H. and Qu, S.P.(1999), Tissue culture of coloured common Callalily (*Zantedeschia aethiopica*). Journal of Southwest Agricultural University 21(5) :423-426.
- Yan, R.L.; Zhang, C.J.; Zhang, L. and Liu, G.R. (1991), A study on the *in vitro* anther culture of *Asperagus* and haploid plant regeneration. Acta Agriculturae Boreali Sinica 7(1):75-82.
- Yeoman, M. M. (1986), In: Plant Cell Culture Technology. Pub. Blackwell Scientific, Oxford. : 1-375.
- Zakutskaya, E.S. aand Murin, A. V. (1990), Use of the tissue culture method in propagating *Gladiolus*. Izvestiya Akademii Nauk SSR Moldova Biologicheskie i Khimicheskie Nauki. 6(63).
- Ziv, M. (1990), The effect of growth retardants on shoot proliferation and morphogenesis in liquid culture *Gladiolus* plants. Acta Horticulturae 28 :207-214.