IN VITRO STUDIES IN

Phyllanthus amarus Schum and Thonn

Thesis submitted in part fulfilment of the requirements for the degree of

Master of Science (Horticulture)

to the Tamil Nadu Agricultural University, Coimbatore-641 003.

By

R. CHITRA

I.D.No.99-620-004

DEPARTMENT OF FLORICULTURE AND LANDSCAPING

HORTICULTURAL COLLEGE AND RESEARCH INSTITUTE

TAMIL NADU AGRICULTURAL UNIVERSITY

COIMBATORE-641 003

2001

CERTIFICATE

This is to certify that the thesis entitled "***In vitro* STUDIES IN *Phyllanthus amarus* Schum. and Thonn."** submitted in part fulfilment of the requirements for the award of the degree of **Master of Science (Horticulture)** to the **Tamil Nadu Agricultural University, Coimbatore** is a **bonafide** record of research work carried out by **Ms.R.CHITRA** under my supervision and guidance and that no part of this thesis has been submitted for the award of any degree, diploma, fellowship or others similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

Date : 1.8.2001
Place : Coimbatore

(Dr.E.VADIVEL)
Chairman

Approved by

Chairman : **(Dr.E.VADIVEL)**

Members : **(Dr.N.CHEZHIYAN)**

(Dr.R.GNANAM)

External Examiner: N. Vasundhara
Date: 8th Nov' 2001

ACKNOWLEDGEMENT

ACKNOWLEDGEMENT

With regardful memories.....

Would it be all envisaging to offer salutations at the feet of **The Lord**, who kindly imbued the energy and enthusiasm through ramifying paths of thick and thin of my efforts.

I have immense pleasure in expressing my deep sense of gratitude and indebtedness to **Dr.E.Vadivel**, Professor of Horticulture, Horticultural College and Research Institute, Coimbatore for having suggested this problem and for his guidance and the keen interest evinced by him throughout the progress of the work.

With deep respect and esteem regards, I owe my indebtedness to the members of the Advisory Committee, **Dr.N.Chezhiyan**, Professor and Head, Department of Spices and Plantation Crops and **Dr.R.Gnanam**, Assistant Professor, Centre for Plant Molecular Biology, for their valuable suggestions whenever and wherever approached for.

It gives me immense pleasure in extending my thanks to **Dr.R.S.Azhakiamanavalan**, Dean, Horticultural College and Research Institute, Coimbatore, **Dr.T.Thangaraj**, Dean, Horticultural College and Research Institute, Periyakulam, **Dr.D.Veeraragavathatham**, Professor and Head, Department of Vegetable Crops, **Dr.N.Kumar**, Professor and Head, Department of Fruit Crops, **Dr.M.Vijayakumar**, Professor and Head, Department of Floriculture and Landscaping, **Dr.K.Soorianathasundaram**, Associate Professor, **Dr.M.Jawaharlal, Dr.T.Arumugam, Dr.M.Kannan, Dr.Manian** and **Dr.Subramanian** for their constant help through out the course period.

Words fail to express my deep sense of gratitude to **Prof.Dr.S.Kannaiyan**, Vice Chancellor, TNAU and **Mrs.Banumathy Kannaiyan** for their moral support that has secured me a comfortable position in every step of my study.

I deem it a great pleasure to place my immense gratitude to **Mr.R.Venkatachalam**, Assistant Professor, Horticulture for his meticulous guidance in my research work.

I express my thanks to **Mrs.M.Ganga Sivakumar, Mr.G.Prabhakaran** and **Mrs.K.Shanthi** for their generous inspiration, help and guidance rendered during the conduct of experience.

I also record my warm gratefulness to **Mr.K.R.Rajadurai, Mrs.A.Beulah, L.Nalina, Dr.R.Kalaiyarasi, Bhuvana, Mrs.Beena, L.Suganthi, D.Sudha** and **S.Ananthi** who gave me their heartful and complete support during the investigation of the study.

I annex my gratitude to my beloved friend **S.Sahithya**. My thanks will be extended to **G.Radha, M.Deepa, R.Bharathi, A.Bhuvaneswari** and my classmates.

On a personal note, I wish to express my heartful thanks to my family friends **Mr.Balaiyan, Dr.M.R.Ravindran** and my beloved parents, brothers, sister, sister-in-law, my sweet kutti Dharani and Julie whose affection, love and care has helped me to succeed in all my endeavour.

I am much obliged to the TNCST fellowship for the financial assistance extended to me.

I am much thankful to the authorities who gave permitted me to utilise the computer facilities available to Department of Olericulture under the Centre of Excellence, Olericulture of World Bank aided AHRDP project.

My deep sense of reverence to **Mr.Augustine Jerard** and **Mr.Suriyanarayanan** for their excellent photographs.

To all others, who had helped me knowing and unknowing wherever they are, goes my thanks and with them the assurance that their assistance not been forgotten.

Finally I thank the Almighty who made everything a reality.

R. Chitra

(R.CHITRA)

ABSTRACT

ABSTRACT

In vitro STUDIES IN *Phyllanthus amarus* Schum and Thonn.

by

R. Chitra

Degree : MASTER OF SCIENCE IN HORTICULTURE

Chairman : **Dr.E.VADIVEL**

Professor of Horticulture
Horticultural College and Research Institute
Tamil Nadu Agricultural University
Coimbatore-641003

2001

Studies on standardisation of micropropagation techniques for *Phyllanthus amarus* were carried out at the Tissue Culture Laboratory of the Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore.

Mercuric chloride was tried as surface sterilant for sterilization of different explants at different concentration for different period of exposure. The contamination level was almost controlled when mercuric chloride was used at 1.0 % concentration for 3 minutes.

Explants taken during January-Febraury gave good response for survival of all the explants followed by collection during July-August. Among the different explants used, stem bit and nodal segment responded well for induction of white and green callus respectively and shoot tip and nodal segments responded well for multiple shoot induction.

Murashige and Skoog's medium at full strength proved to be best for callus induction and multiple shoot formation. MS + BAP ($3.0mgl^{-1}$) + Kin ($3.0mgl^{-1}$) recorded the maximum green callus from nodal segment and MS + 2,4-D ($4.0mgl^{-1}$) + NAA ($0.4mgl^{-1}$) induced more white callus from stem bit and leaf bit explants.

MS + BAP (4.0mgl^{-1}) + Kin (4.0mgl^{-1}) recorded maximum multiple shoot from shoot tip and nodal segments. Among the explants, nodal segments gave higher number of shoots (5.94 shoots/explant) than shoot tip (5.22 shoots/explant). The survival percentage of cultures was recorded more in shoot tip explants (95.00%) than nodal segments (88.00%). Addition of GA_3 to the medium, enhanced the length of multiple shoot.

On subculturing the multiple shoots in MS medium supplemented with BAP (2.0 mgl^{-1}) and GA_3 (0.5 mgl^{-1}) had resulted in 7.0 multiple shoots formation. Rooting (87.09%) of the shoots was best achieved in ½ MS + IBA (0.5mgl^{-1}) + IAA (0.5mgl^{-1}).

The positively polarised explants resulted in higher response.But the negatively polarised explants failed to show any response. For the estimation of phyllanthin and hypophyllanthin, in vitro grown plants recorded higher amount of phyllanthin (0.521%) and hypophyllanthin (0.296%) which was cultured in ½ MS medium supplemented with IBA (0.50mgl^{-1}) + IAA (0.50mgl^{-1}).

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS USED

BA	-	Benzyl adenine
BAP	-	6-benzyl amino purine
CD	-	Critical Difference
cm	-	Centimetre
DNA	-	Deoxyribo Nucleic Acid
2,4-D	-	2,4-dichlorophenoxy acetic acid
EDTA	-	Ethylene Diamine Tetra Acetic acid
Fe	-	Iron
g	-	Gram
GA_3	-	Gibberlic acid
gl^{-1}	-	Gram per litre
h	-	Hour
$HgCl_2$	-	Mercuric chloride
IAA	-	Indole, 3-acetic acid
IBA	-	3, indolebutryic acid
2-ip	-	N-isopentenylamino purine
KIN/KN/kinetin	-	6-fur furylamino purine
LAFC	-	Laminar Air-Flow Chamber
l or lit	-	litre
M	-	Molar
mg l^{-1} or mg/lit	-	Milligram per litre
min	-	Minute
ml	-	Milli litre
mM	-	Milli Molar
MS	-	Murashige and Skoog's medium
NAA	-	α-napthalene acetic acid
ppm	-	Parts per million
RH	-	Relative Humidity
SE	-	Standard Error
viz	-	Namely
v/v	-	Volume per volume
WP	-	Woody plant medium
w/w	-	Weight per weight
w/v	-	Weight per volume
µl	-	Microlitre
$\mu E/m^2/s$	-	MicroEquivalent per minute square per second
µm	-	Micro molar
%	-	Percentage

INTRODUCTION

CHAPTER I

INTRODUCTION

Plants, as a source of medicine, are of special importance in countries like India, Pakistan, Bangladesh and Sri Lanka which have well developed traditional systems of medicine called Ayurveda, Sidha and Unani, all of which derive more than 90 per cent of medicaments from higher plants. But the wealth of medicinal plants is getting depleted, and some ecotypes have already became extinct. Biotechnology with its apparently limitless potential, offers new and exciting opportunities to address the various problems in conservation and cultivation of medicinal plants.

Phyllanthus is a large and complex genus in the Euphorbiaceae, currently thought to contain between 550 and 750 species in 11 subgenera (Webster, 1970; Holm-Nielson, 1979). Herbaceous species of the subgenus *Phyllanthus*, including *Phyllanthus amarus*, predominate among records of usage in systems of traditional medicine as well as records of documented effects (Unander *et al.*, 1990).

P.amarus is commonly called as 'Bhumyamalaki' or 'Tamalaki'. It had many vernacular names to its credit in different parts of the country. In Kannada it is called as Nala nelli or Kiruneli, in Bengali, Orya and Marathi as Bhuiamla, in Hindi as Jangali amla, in Malayalam as Kizha velli, in Tamil as Keezha nelli, and in Telugu as Nelu usirika (Eranna and Suresh, 2001).

Though the plant is found to occur throughout India, it is believed to be a native of America. It is grown throughout the hotter parts of India from the Punjab to Assam and southwards to Travancore, ascending the hills up to 3,000 feet. It grows well under semi-temperate to tropical climatic conditions and even in high rainfall zones. It can be

grown on soils with wide pH range. However, it prefers calcareous well-drained and light textured soil.

The plant is an erect annual herb. It grows 10 to 60cm tall. Stem is erect, leaves elliptical oblong, obovate-oblong or minutely opiculate at apex. Flowers are axillary, unisexual and bisexual, cymes on deciduous branchlets. The main stem is profusely branched and the branches from the base are longer than at the top. Fruits (capsules) are trigonous and seeds triangular with 6-7 longitudinal ribs.

The plant is being used as one of the important ingredients in many indigenous poly herbal formulations and other ayurvedic preparations.It is used as a diuretic in dropsical affections, gonorrhoea and other troubles of genito-urinary tract. The herb is bitter, astringent, deobstruent, diuretic, febrifuge and antiseptic. It is used in stomach troubles such as dyspepsia, colic, diarrhoea and dysentery and also for dropsy and diseases of urinogenital system. Fresh root is a remedy for jaundice. Leaves are stomachic. Milky juice is used as application to offensive to sore. Powdered leaves and roots are pulverized and made into poultice with rice-water and used to lessen oedematous swellings and ulcers. Leaves are a popular remedy against fever and infusion of young shoots is given in dysentery.

Leaves contain bitter substances phyllanthin and hypophyllanthin.Three new lignans, *viz.*, niranthin, nitertralin and phyltertralin have been recently isolated from leaves.Two alkaloids, *viz.*, kaempferol-4-rhamnopyranoside and eriodic tyol-rhasmnopyranoside have been isolated from the roots. Stems contain saponin.

P.amarus also had *in vitro* activity against the Reverse Transcriptase of retroviruses (Yanagi *et al.*, 1989) and possibly against retroviruses *in vivo* (Millman *et al.*, 1987).

Seed production is difficult and many a times seeds do not show proper germination and seedling growth. The first step towards overcoming this problem is identifying a viable procedure for large-scale multiplication of superior strains. As in many other plant species, micropropagation through tissue culture could be the best alternative for the hard-to-propagate 'Bhumyamalaki'. The other alternative is the production of antiviral compounds (phyllanthin, hypophyllanthin etc.) from different types of cultures directly which are of commercial importance. Since not many attempts in this line have been made for the crop, the present study was initiated in Tissue Culture Laboratory of the Horticultural College and Research Institute, TamilNadu Agricultural University, Coimbatore with the following objectives:

1. To identify the ideal explants for *P.amarus* with high regeneration capacity in the *in vitro* culture system.
2. To optimize the surface sterilization procedures for the various explants.
3. To study the capability of the explants in terms of callogenesis and regeneration capacity from callus under different PGR fortification.
4. To test the effect of PGR for better organogenesis *viz.*, microshooting and microrooting.
5. To develop a protocol for rooting and hardening of *in vitro* derived *P.amarus* plantlets.
6. To analyse the antiviral compounds from different types of cultures.

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Plants have been the traditional source of raw materials and finished medicine since the dawn of civilization. Many medicinal plants have been recognized by rural folk and used in traditional medicine to cure various types of diseases. The earliest reference to the use of medicinal herbs as a cure for diseases is found in the manuscript of 'Eber Papyrus' which dates back to 16^{th} century B.C. Several examples of herbals with medicinal properties appear in the works of Charaka and Sushruta. The efficiency of medicinal herbs was recorded in Ayurveda, part of the Atharvana Veda and even in old Egyptian, Greek and European literature. Even today, about 75 per cent of the total population rely on medicinal plants in rural and remote area.

Efforts to domesticate and propagate the valuable medicinal plants economically face many hurdles. The amount and quality of active principles in a plant must be maintained at an optimal level by providing proper growth conditions, otherwise the end products will be sub-standard in quantity and quality, which would not be acceptable to the customers. Tissue culture techniques have been used in the horticulture industry to supply plants in large numbers, which are inconceivable, by conventional methods. These methods could be applied also to reintroduce in their native habitat or cultivated along with normal crops and conventional farming practices.

The foundation for plant tissue culture is the concept of totipotency, which is inherent in the cell as per the theory of Schleiden (1838) and Schwann (1839), Haberlandt (1902) was the first to establish the fact that an isolated fully differentiated cell is capable of resuming uninterrupted growth. Hanning (1904) excised nearly mature embryos of some

crucifers and successfully grew them to maturity on mineral salts and sugar solution. The first successful report of continuously growing culture of tomato root tips was made by White in 1934. Van Overbreak *et al.* (1941) reported for the first time, the stimulatory effect of coconut milk on embryo development and callus formation in *Datura.*

Skoog and Miller (1957) put forth the concept of hormonal control of organ formation and showed that the differentiation of roots and shoots in tobacco pith tissue culture was a function of the auxin-cytokinin ratio and that organ differentiation could be regulated by changing the relative concentrations of these two substances in the medium. Wickson and Thimmann (1958) showed that the growth of axillary buds, which remained dormant in the presence of terminal buds, could be initiated by exogenous application of cytokinin.

Morel (1960) applied the technique of shoot tip culture for a rapid propagation of orchids and it became possible to produce large numbers of genetically identical plants from a single bud within a short time. Murashige developed standard method for *in vitro* propagation of several species ranging from ferns to foliage, flower and fruit plants. He also developed the first completely defined nutrient medium along with Skoog in 1962. Since then plant tissue culture has evolved as a powerful research tool in the fundamental and applied aspects of agriculture, horticulture and forestry.

Several aspects of tissue culture were currently being applied to agriculture. The best commercial application so far has been in the production of clonal plants at a very rapid rate compared to the conventional methods through tissue culture technique. These plants were reported to grow faster and mature earlier than seed propagated plants (Vasil and Vasil, 1980).

General tissue culture techniques that were being increasingly used in crop improvement were the meristem or shoot apex culture for disease elimination, somatic embryogenesis for rapid propagation of selected genotypes, cell culture for the recovery of useful variants, cell line screening for generating, selecting and propagating environmental stress tolerant lines, *in vitro* mutagenesis for obtaining desirable mutants, anther culture and *in vitro* pollination or fertilization or somatic hybridization and introduction of new genetic material into plant cells; and cryopreservation for long term storage of germplasm (Rajmohan *et al.*, 1989). Success achieved in plant tissue culture has been the greatest in many horticultural species (Hu and Wang, 1983).

Published literature, with relevance to the present study has been organized under the following major titles.

1. Factors influencing the success of *in vitro* culture.
2. *In vitro* regeneration of plantlets.
3. Hardening of *in vitro* derived plantlets.
4. Estimation of antiviral compounds (phyllanthin, hypophyllanthin).

2.1. Factors influencing the success of *in vitro* culture

2.1.1. Selection of explant

Most living plant cells can be induced to divide *in vitro* and a wide variety of plant parts have been used as the tissue source for cultures in which shoot organogenesis has occurred (Murashige, 1974; George and Sherrington, 1984). Murashige (1974) recognized several factors that could be considered in explant selection. These include (i) the organ serve as tissue source, (ii) the physiological and ontogenetic age of the organ, (iii) the season in which the explants are taken from the mother plant, (iv) the size

of the explant and (v) the overall quality of the plant from which the explants obtained. George and Sherrington (1984) have added (vi) genotype, (vii) explant orientation, (viii) pre-treatment and (ix) inoculation density to this test, but it was clear from more recent studies that two factors, *viz.*, pre-treatment and genotype, can be critical in determining the organogenetic response of a given species.

2.1.2. Source of the explant

The morphogenetic potential of various plant varies depending on the explant used. The use of both vegetative and reproductive plant parts to initiate cultures in several species of medicinal importance has been reported by many scientists.

Explants of nodal segments of *Rauwolfia micrantha* (Sudha and Seeni, 1996), axillary nodes of *Gymnema elegans* (Komalavalli and Rao, 1997), shoot tips of *Hedeoma multiflorum* (Adolfina *et al.*,1997) and *Withania somnifera* (Teli *et al.*, 1999) have responded accountably to direct organogenesis.

Stem bits of *Phyllanthus amarus* (Unander, 1991), petioles of *Viola patrinii* (Tadahikosato et al., 1993), leaf segments of *Hemidesmus indicus* (Malathy and Pal, 1998), protoplast of peas (Petra Bohmer *et al.*, 1996) and shoot tips of *Piper betel* (Aminuddin *et al.*, 1996) and *Phyllanthus emblica* (Haicour, 1997) have shown considerable response to callus initiation and organogenesis.

2.1.3. Size of the explant

The size of the explant used varies with the plant species and influences the survival of the culture. Optimum size of explants has been prescribed for use in tissue culture. Very small explants, whether they are shoot tips, meristems, fragments of whole

plant or pieces of callus could not survive well in culture, whereas, very large explants are difficult to be decontaminated effectively and are difficult to be manipulated.

In general, optimal size of shoot bud explant ranging from 0.5 to 0.3mm in size with 2 to 3 leaf primordia covering the apical clone (Vuylsteke and De Langhe, 1985). In *Ocimum americanum* and *Ocimum sanctum*, axillary shoot buds of 3.0 to 4.0mm in size were effective for multiple shoot induction (Sitakanta Pattnaik *et al.*, 1996). Komalavalli and Rao (1997) reported that 7.0 to 10.0mm sized axillary nodes as explants gave better multiple shoot induction in *Gymnema elegans*. Shoot tips of 0.3 to 0.5cm and nodal segments of 1.0 to 2.0cm length proved to be effective in induction of multiple shoots in *Aristolachia indica* (Manjula *et al.*, 1997).

In *Phyllanthus amarus*, stem bits of 5.0 to 10.0mm length had been used as explants for callus induction (Unander, 1991). Shoot segments of 1.0cm length of *Catharanthus roseus* produced dedifferentiated callus (Jin Yuan *et al.*,1994). Leaf segments of 1.0cm^2 area of *Hemidesmus indicus* produced green nodular callus (Sarasan *et al.*, 1994). Leaf discs of 1.0cm diameter and internodal segments of 1.0 to 1.5cm length were effective in callus initiation and regeneration in *Aristolachia indica* (Manjula *et al.*, 1997). Stem explants of 1.0cm length were also effective in callus induction in *Aristolachia indica* (Remashree *et al.*, 1997).

2.1.4. Physiological age of the explant

Several physiological changes took place during the maturation process of plants that influence the *in vitro* behaviour of the explants (David, 1982). This was manifested in the differential ability of the explants to form adventitious and axillary buds and in the

rate of shoot elongation and rooting. It has not been possible to apply the techniques developed for juvenile materials to mature plants.

Organogenesis depends on the age of the tissue used as explants. The juvenile tissues generally respond better than the older tissues in *in vitro* systems (Murashige, 1974; Rajmohan *et al.*, 1989). The youngest and less differentiated tissues respond better in cultures in a wide range of species (Hughes, 1981). Explants taken from newly originated organs are almost likely to interfere with organogenesis directly. Seven to ten days old leaves and nodes of *Aristolachia bracteolata* were inoculated in medium showed better survival of plantlets (Remashree *et al.*, 1994). Of the varies explants used leaf explants of *Centella asiatica*, that were collected from five to six months old glass house grown plants gave better callusing and regeneration response (Suchitra Banerjee *et al.*, 1999). Nodal segments taken from tender twigs from five to six year old matured plants of *Gymnema sylvestre* gave good response for multiplication of shoots (Sairam Reddy *et al.*, 1999).

The response of the explant was also influenced by season. The physiological state of the stock plant was influenced the explant behaviour *in vitro*. Sitakanta Pattnaik *et al.* (1996) reported that the multiplication and growth of shoot buds of *Ocimum sanctum* was greatly enhanced when the buds were collected during September through December. John (1996) reported that in *Holostemma annulare*, the explants collected in the months of January to April gave lower contamination and higher survival percentages with the contamination rate lowest in the months of January and February. Similar effects of season on bud sprouting and explant contamination had also been noticed in *Trichopus zeylanicus* (Seema, 1997) and *Gymnema sylvestre* (Anu, 1993). In *Tridax procumbens*, high frequency bud break

(83%) and multiple shoot formation was induced from nodal segments collected between September and November (Sahoo and Chand, 1998).

2.1.5. Position of the explant

In general explants taken from the tip of a shoot were in a young stage of development and respond better than explants taken from the base.Roest and Bokelmann (1989) reported that in Carnation nodal segment cultures, the percentage of shoot developed between, explants taken from the top and the base of the shoot were 88.6 and 69.81 respectively.

2.1.6. Placement of the explant on the culture medium

The placement of the explant on the medium varies with the plant species and has been found to influence the survival of the culture. Custers and Bergervoet (1980) noted that adventitious shoots from hypocotyls of seedlings were obtained on the proximal end more frequently than the distal end. The basal end of the section and the abaxial side of the leaf when placed in contact with the medium has shown better organogenesis and callus induction in chrysanthemum (Chin-Yi Lu *et al.*, 1990).

2.1.7. Surface sterilization

In both scientific and commercial micropropagation laboratories, microbial contamination was considered the single most important reason for plant losses *in vitro* (Leifert *et al.*, 1992). When tissue cultures were tested by microbiological indexing methods after initiation, most contaminants found during later stages of the micropropagation process were introduced accidently in the laboratory, during media preparation and/or sub-culturing of plants, when the culture containers were opened and when plants and growth media exposed (Singh *et al.*, 1987). Boxus and Terzi, (1987) also

showed that contaminants can be rapidly spread during the handling of plant cultures by operators using inefficient aspectic techniques.

Most fungal, yeast and some bacterial contaminants killed the plant material soon after they have been introduced (Leifert *et al.*, 1990; Danby *et al.*, 1993). Bacterial contaminants such as *Staphylococcus, Micrococcus, Lactobacillus, Pseudomans* spp., on the other hand, can remain latent (not producing disease symptoms on the plant or visible growth on the medium) after they have been introduced into cultures of certain plant species (Leifert *et al.*, 1992).

In *in vitro* systems, surface sterilization was essential to obtain successful tissue culture plantlets. The efficiency of the sterilants varies depending upon the type of chemical, concentration used, time of exposure, etc. Selection of a suitable surface sterilant and the time required for exposure of explants were of crucial importance (Murashige and Skoog , 1962).

Various explants seemed to have a strong interaction effect with the surface sterilants, concentration and time of exposure on callus formation (Kartha *et al.*, 1977; Guney and Rao, 1980; Sukumar and Sree Rangaswamy, 1988; Karuppaiah and Rajasekaran, 1993; Chandrasekaran and Narayanaswami, 1993). Presently, mercuric chloride (0.1 to 2.0 per cent w/v), sodium hypochlorite (0.5 to 2.0 per cent w/v) or calcium hypochlorite (5.0 to 10.0 per cent w/v) were the most commonly used surface sterilants. As sodium hypochlorite and calcium hypochlorite are toxic to plant cells (Hu and Wang , 1983) it is necessary to wash the explants twice or thrice with sterile distilled water to ensure complete removal of the surface sterilants, or a short time of exposure

was advised (Sommer and Caldas, 1981). But the concentration below 0.1 per cent proved to be ineffective.

The concentrations of surface sterilants used to seem to differ, depending on crop species and plant parts (Nekrosova, 1964). The duration of soaking the tissues in the disinfectant was also important. Maroti and Levi (1977) reported to rinse first with ethanol (45%) for 3 minutes followed by a 10minutes bleach treatment (5-10%) and finally three rinses with sterile water. Alcohol alone or in combination with other surface sterilants is also an effective disinfectant (Bonga, 1982). A drop of detergent solution such as Teepol, Tween-20 or Tween-80 is added to the surface sterilant to reduce surface tension and to increase the wettability. Magnetic stirring , ultrasonic vibration or vacuum may be applied during soaking in order to reduce the possibility of trapping air bubbles on the explant surface (Hu and Wang , 1983).

Unander (1991) suggested a sterilization procedure for explants of *Phyllanthus amarus*. In this study, small section of stems from young, potted plants or from the new growth of older plants were first washed with soap and water to remove any superficial dirt, then thoroughly rinsed with tap water and presoaked for 30 to 60 minutes in distilled water with Tween-20 added at 1 drop/100 ml. Tissue sections were then immersed in 0.5% sodium hypochlorite with the same amount of Tween-20 and rinsed three times in sterile, distilled water.

In *Crocus sativas*, the sprouts containing floral buds, were separated from the corm and sterilized by dipping them in 70 per cent ethanol for a minute and in sodium hypochlorite (2.5% available Chlorine) for 8minutes followed by thorough washings thrice with sterile distilled water (Sharma *et al.*, 1990). In *Piper longum*, Bhat *et al.* (1992) reported that

in water for 5-10 minutes followed by treatment with 0.1 per cent mercuric chloride for 20 minutes was an effective sterilization method. Rajasubramaniam and Pardha Saradhi (1994) described sterilization procedure for explants of *Phyllanthus fraternus*. Seeds were treated with 0.5% Teepol B-300 for 2 minutes and washed with distilled water. They were then surface disinfected with 0.1% mercuric chloride for 3 minutes and washed thoroughly with sterile distilled water.

2.1.8. Culture medium

The success of plant tissue culture as means of plant propagation and plant improvement was greatly influenced by the nature of the culture medium used. For healthy and vigorous growth, intact plant needs to take up from the soil relatively large amounts of some inorganic ions (major plant nutrients) and small quantities of other ions (minor plant nutrients or trace elements). Plant tissue culture media provides not only these major and minor nutrients but also carbohydrates, usually sucrose, as substrate for the carbon, which a plant normally fixed from the atmosphere by photosynthesis. Trace amount of certain organic compounds, notably vitamins, aminoacids and plant growth regulators improved the success. Although attempts have been made to avoid the use of undefined compounds such as fruit juice, yeast extract and caesin hydrolysate, improved results have sometimes been reported by their addition (George and Sherrington, 1984).

Coconut milk for instance, was often employed by research laboratories and banana homogenate was still a popular addition to media for orchid culture. Since 1962, most researchers have been using Murashige and Skoog's (1962) medium. A variety of plant tissue and cell culture media now in practice has been reviewed by Gamborg *et al.*(1976) and Huang and Murashige (1977). Other derivatives of medium include Lin and Staba

(1961) medium, B5 medium developed by Gamborg *et al.* (1976) and WPM developed by Lloyd and McCown (1980) which has a lower salt content.

The major differences among these high salt media are the amounts and forms of nitrogen and the relative amounts of some of the micro-nutrients contained in it (Gamborg *et al.*, 1976). The MS medium had the highest salt content and some workers found it beneficial to reduce its strength by half (Skirvin, 1980). Skirvin (1980) reported that more than 60 different media have been used for tissues of various woody species.

For most of the medicinal and aromatic plants, *in vitro* research workers have reported desired success only in Murashige and Skoog (1962) medium, indicating the superiority of the same over the other media. However, on many occasions, the reported combinations, especially the trace elements, vitamins and growth regulators are results in poor success, depicting the requirement for standardization of specified media for the crop species or varieties and plant part used as explant.

2.1.8.1. Mineral salts

The salt composition of several media has been reviewed by Gamborg *et al.* (1976). The Murashige and Skoog (1962) medium is the most widely used plant medium for tissue culture of medicinal plants. It was high in nitrates, potassium and ammonia. Although MS medium has been found to be specifically developed for tobacco tissue culture, it has been successfully used in culturing a variety of plants.

In *Asparagus cooperi*, Biswajit Ghosh and Sumitra Sen (1992) reported that macrosalt of 100 mgl^{-1} of $Ca(No_3)_2.4H_2O$ in the MS medium was essential for shoot growth.

2.1.8.2. Carbon and energy source

A carbon energy source was inevitable in any tissue culture media. Sucrose is probably the most widely used carbohydrate source in plant tissue culture media and many and earlier reports identified sucrose as the optimum carbon source (Evans *et al.*, 1981; Dunwell, 1985). It has also been reported that maltose (Strickland *et al.*, 1987) is capable of sustaining and promoting growth and morphogenetic development of certain cell lines. There has been recent interest in the use of maltose in tissue culture media and significant findings related to *in vitro* development of anthers and cell lines (Verma and Dougall, 1977).

Tissue culture growth was not only affected by the type of carbon source but also the concentration of particular sugars used in the medium. Thorpe (1978) showed that in tobacco, atleast part of the sucrose requirement was osmotic. Organogenesis in the Liliaceae family was inhibited at high concentrations of sucrose. The 100 per cent regeneration was obtained at $30gl^{-1}$ but at $90gl^{-1}$ only 15 per cent regeneration has been reported (Takayama and Misawa, 1979).

2.1.8.3. Vitamins

The vitamins mostly used in plant tissue culture media are thiamine, nicotinic acid and pyridoxine (Linsmaier and Skoog, 1965). However, Murashige and Skoog (1962) vitamin mixture contains myo-inositol ($100.0mgl^{-1}$), nicotinic acid ($0.5mgl^{-1}$), pyridoxine ($0.5mgl^{-1}$) with additions like glycine ($0.2mgl^{-1}$) and ascorbic acid ($100.0mgl^{-1}$) (Banerjee *et al.*, 1986) and thiamine HCl ($0.4mgl^{-1}$) (Vuylsteke and Delanghe, 1985) which are being used for tissue culture of several crop species.

The vitamins used in most of the *in vitro* culture media were myo-inositol (100-200mgl^{-1}), thiamine (0.1-5.0mgl^{-1}), calcium pentothenate (0.5-2.5mgl^{-1}), folic acid (0.1-0.5mgl^{-1}), riboflavin (0.1-10.0mgl^{-1}), ascorbic acid (100mgl^{-1}), nicotinic acid (0.1-5.0mgl^{-1}), pyridoxine (0.1-1.0mgl^{-1}) and biotin (0.1-1.0mgl^{-1}) (Perik, 1987). The various basal media used contain different proportions of these vitamins.

2.1.8.4. Growth regulators

The success of an *in vitro* system was much dependent upon the use of correct growth regulators in optimum concentrations for the desired effect (Krikorian, 1982). Skoog and Miller (1957) reported that when the concentration of cytokinin, relative to auxin was high, shoots were induced and when the concentration of cytokinin relative to auxin was low, roots were induced and at intermediate concentrations, the tissue grew as an unorganized callus.

Although the concentrations and types of auxins and cytokinins used vary. In general, the concentrations of these two have been found to influence regeneration in a wide variety of dicotyledenous and monocotyledenous plants (Murashige, 1974; Narayanaswamy, 1977). Two principle classes of growth regulators are used in tissue culture studies, namely auxins and cytokinins. Most commonly used auxins are 2,4–D, IBA, IAA, NAA and 2,4,5–T. The most commonly used cytokinins were Benzyl adenine, Kinetine, BAP and IP.

The auxins most frequently used to induce *in vitro* rooting were IAA, NAA and IBA (Murashige, 1974) and to initiate callus growth, 2,4–D was mostly used. Addition of traces of 2,4–D in the medium leading to initiation of callus from tissues of several crops

have been demonstrated by many scientists (Nataraj, 1975; Chua *et al.*, 1981). So far no universal ratio of auxin and cytokinin has been reported for root and shoot induction.

Hu and Wang (1983) identified the young shoot apex as an active site for auxin biosynthesis. Too higher concentrations of auxin, however, not only inhibits axillary bud breaking but also induces callus formation (Hasegava, 1980).

Pre-treating the vegetative buds with cytokinins has been shown to have a favourable effect in overcoming apical dominance (Pillai and Hildebrandt, 1969) under *in vitro* culture conditions. For axillary shoot proliferation, cytokinin has been utilized to overcome the apical dominance of shoots and to enhance the branching of lateral buds from leaf axils (Murashige, 1974). High cytokinin content has been found to be deleterious to the initiation and elongation process of roots of both monocots and dicots. A kind of synergism between two cytokinins namely, kinetin and BA has been reported in certain cases of axillary bud proliferation under *in vitro* conditions (Gupta *et al.*, 1981).

2.1.8.5. Other compounds

2.1.8.5.1. Coconut water

The liquid endosperm of coconut promotes growth and differentiation in a wide variety of excised plant tissues. Coconut water has been reported to be used first in tissue culture by van Overbreak *et al.* (1941) who found that it was necessary for the development of young embryos of *Datura*. Favourable effects of coconut water in *in vitro* cultures have since been reported by many workers (Schenk and Hildebrandt, 1972; Mathur *et al.*, 1989).

A number of cell division factors are present in coconut water, including diphenyl urea (Shantz and Steward, 1952). 9, B–D ribo-furanasyl zeatin (Letham, 1954) and a

compound which co-chryomatographs with zeatin riboside (Vanstaden and Drewes, 1974). It also contains a large number of free amino acids, including phenyalanine which has cell division activitiy in soybean assays (Raghavan, 1977).

The enhancing effect of coconut water in the promotion of growth and differentiation of excised tissues and organs have been attributed to the presence of cytokinin and GA like substances in it (Stratus and Rodney, 1960). A slight enhancement in sprouting was observed with the addition of 30% coconut water in *Gymnema sylvestre* (Anu *et al.*, 1994). The coconut water (15%) enhanced the axillary shoot development and also maintained the greenness of leaves in *Gymnema sylvestre* (Surya Narmada, 2000).

2.1.8.5.2. Activated charcoal

Activated charcoal is generally added to the media to prevent phenol exudation from micro-cuttings and explants and to induce rooting of cuttings. Since the accumulation of free phenols in the medium could be toxic for the explant tissues, the chelating effect of activated charcoals with phenols was beneficial for the physiological state of the cuttings and their propagating abilities. Activated charcoal also binds hormones and other metabolites (Weatherhead *et al.*, 1978) and removes auxins and polyphenols from the explant (Mission *et al.*, 1983). The effects of activated charcoal can be attributed to four factors such as darkening of the medium which favours rooting irreversible, adsorption of inhibitory compounds, growth hormones and other organic compounds from the medium (Weatherhead *et al.*, 1978; Arditti, 1982; Johansson, 1983). When added to a liquid medium at concentrations ranging from 0.1 to 5.0 per cent, activated charcoal reduced IAA and IBA concentration by more than 97 per cent (Scott and Ellen, 1990).

2.1.9. Agar

Agar is used as the carrier material to solidify the medium for supporting root growth. Agar may contain certain growth inhibiting substances which may result in rooting inhibition in certain sensitive species which may be due to poor aeration and a slow rate of diffusion of toxic metabolic wastes released by growing tissues (Hu and Wang, 1983). To circumvent the inhibitory effect of agar medium, activated charcoal may be added to the agar medium or liquid medium may be used with filter paper bridge system which provides excellent aeration for root development (Hu and Wang , 1983). The normal concentration of agar used for rooting varies from 0.0 (liquid medium) to 0.9%.

2.1.10. pH

pH of the culture medium affects nutrient availability and uptake (Minochae, 1987) and has been shown to influcence a number of plant developmental processes, micropropagation rate (Reeves *et al.*, 1983) and cell division *in vitro* (Basu *et al.*, 1988), including organogenesis (Zhang and Stoltz, 1989) and floral differentiation (Cousson *et al.*, 1989). Between pH 5.5 and 6.0 significant differences have been demonstrated for the uptake of plant growth regulators (Kaiser and Hartung , 1981).

Most plant tissue culture media, however are poorly buffered (Martin, 1980) and as such are subjected to significant changes in pH, depending on the specific medium formulation, medium sterilization method and type of plant material cultured. Heat sterilization can significantly alter medium pH by denaturation of proteins, dissolution of salts (Behagel, 1971) and hydrolysis of carbohydrates (Schenk *et al.*, 1991).

However, little work has been carried out to determine the effect of pH of the medium on plant growth *in vitro* although different plant species growing *in vitro* were

shown to have distinct pH optima for growth ranging from 4.2 to 6.5 (Scheffer and Schachtschabel, 1979). *In vivo,* the pH affected availability of nutrients by the plant and the survival of soil borne pathogens in the rhizosphere (Scheffer and Schachtschabel, 1979; Mengal, 1984).

Plant tissue culture media were usually adjusted before autoclaving to pH values betwen 5.5 and 5.9, since lower pH values during autoclaving prevent the gelling agent from solidifying (George and Sherrington, 1984). However, in liquid *in vitro* systems such as plant cell cultures, the pH has also been observed to affect the uptake of nutrients (such as nitrogen) and growth rates of plants (Martin and Rose, 1975; Hunault, 1985). Decreasing the pH of plant tissue culture media below 4.5 has been found to affect the morphogenesis of plant tissue cultures (Rastogi and Sawhney, 1986; Smith and Krikorian, 1990) to suppress bacterial contaminants (Leifert *et al.*, 1992) and to affect the activity of antibiotics added to the plant tissue culture media to suppress contaminants (Leifert *et al.*, 1991). A culture medium pH of 5.8 was found optimum for *Phyllanthus fraternus* (Rajasubramaniam and Pardha Saradhi, 1994).

2.1.11. Culture environment

2.1.11.1. Light

Light intensity, quality and duration were the three major factors affecting the growth of *in vitro* culture (Murashige, 1974; 1977). The optimum light intensity for shoot formation in a large number of herbaceous species has been found to be 1000 to 2000 lux. Growth reduces at intensities as high as 3000 lux and as low as 300 lux (Murashige, 1974).

In *Phyllanthus amarus*, cultures kept in the growth chamber under continuous fluorescent light at an average light intensity of 30$\mu E/m^2/s$ gave better results (Unander, 1991).

In *Phyllanthus fraternus*, cultures maintained under 14h/10 h daily illumination with white, cool fluorescent light of photon flux 100μmol/m^2/s was effective for regeneration of shoots (Rajasubramaniam and Pardha Saradhi, 1994). Cultures of *Plumbago indica* maintained under 2000 lux light intensity provided by a white fluorescent lamp for 16 h photoperiod showed better response to *in vitro* regeneration (Smita Chetia and Handique, 2000).

2.1.11.2. Temperature

Most tissue cultures were maintained in growth rooms at the same temperatures over day and night. Yeomen (1986) reported that the usual environment temperature of a species concerned should be taken into account for its better performance under *in vitro* conditions. However, most *in vitro* cultures were grown successfully at temperature around 25±2^{0}C.

In *Phyllanthus amarus*, cultures maintained at a temperature of 28^{0}C gave better response to callus induction (Unander, 1991) and cultures of *Phyllanthus fraternus* maintained at 25±2^{0}C performed better (Rajasubramaniam and Pardha Saradhi, 1994). Cultures of *Centella asiatica* maintained at a temperature of 25±2^{0}C gave better response for callusing and regeneration (Suchitra Banerjee *et al.*, 1999).

2.1.11.3. Humidity

Relative humidity is an important factor in hardening and planting out of *in vitro* raised cultures. Hu and Wang (1983) reported that air humidity was not often controlled and when it was controlled, 70 per cent had been found to be the most frequent setting. Relative humidity of 55-69% was found optimum to induce good response in cultures of *Centella asiatica* (Suchitra Banerjee *et al.*, 1999).

2.2. *In vitro* regeneration

2.2.1. Indirect organogenesis

Levels of growth regulating substances, particularly auxins, higher than those necessary to stimulate the direct formation of adventitious shoots generally gave rise to the proliferation of callus from the explants. Callus may be obtained from different parts of the plant. The callus formation and regeneration capacity of explants *in vitro* is influenced by various factors such as cultivars, species, explant tissues and nature, concentration of growth substances and additives etc. (Padmanabhan *et al.*, 1974; Yeoman and Mc Cold, 1977; Evans *et al.*, 1981 and Zapata *et al.*, 1981).

2.2.1.1. Callus Induction

For induction of callus from leaf bit and stem bit explants, the growth regulators auxins and cytokinins have been used. The nature of auxins or cytokinins however differed among the users.

The combination of 2,4-D ($1.0mgl^{-1}$) and BA ($0.5mgl^{-1}$) produced the maximum callus induction from the leaf segments of *Phyllanthus amarus* (Unander, 1991). In *Catharanthus roseus*, the hypocotyl explants cultured in MS medium supplemented with $1.0mgl^{-1}$ NAA and $0.5mgl^{-1}$ Kinetin gave the highest percentage of callus induction under both light (83.9%) and dark (81.2%) conditions (Sehrawat *et al.*, 1999).

In *Hemidesmus indicus*, callus raised in 2,4–D containing medium was yellowish and friable, while that in NAA containing medium was green, nodular, compact calli (Sarasan *et al.*, 1994). Green and compact calli were obtained from internodal explants of *Datura metel* cultured on MS medium supplemented with $0.5\text{-}3.0mgl^{-1}$ of BAP (Arockiaswamy *et al.*, 1999).

2.2.1.2. Callus differentiation

The requirement of optimal concentration of auxins and cytokinins for better regeneration from explant tissues has been reported by many scientists.

In *Kaempferia galanga*, callus derived from rhizome buds gave maximum number of shoots when MS media was supplemented with 1.0mgl^{-1} NAA and 0.5mgl^{-1} BAP (Remasree *et al.*,1997). Well-established callus tissue of *Datura metel* differentiated into shoot as well as root in MS medium containing 2.0mgl^{-1} BAP and 0.5-3.0mgl^{-1} IBA (Arockiaswamy *et al.*, 1999).

Green nodular callus of *Hemidesmus indicus* turned morphogenetic upon subculturing to MS with 1.5-2.0mgl^{-1} Kinetin along with 10% coconut milk. Small shoots developed from the compact regions of the callus within 20 days (Sarasan *et al.*, 1994). MS medium supplemented with 1.5mgl^{-1} BAP and 0.25mgl^{-1} 2,4–D produced a single shoot along with 4-6 shoot primordia and numerous meristematic protruberances from callus initiated from young rhizomes of *Alpinia calcarata* (Martin *et al.*, 1997).

2.2.2. Direct organogenesis

The direct regeneration of plantlets in tissue culture depend on the choice of explant, media, growth regulators and environment. Several workers have standardized the methodology for direct organogenesis in different species.

MS medium supplemented with 0.54μM NAA and 13.31μM BA induced maximum number of shoots from shoot tip and nodal segments culture in *Aristolachia indica* (Manjula *et al.*, 1997). Microshoot formation was observed from the tuber nodes of *Gloriosa superba*, in the modified MS medium, fortified with BAP 3.0mgl^{-1} alone or in combination with 0.6mgl^{-1} NAA (Munavarjan, 2000). In *Plumbago indica*, the nodal explants

cultured on MS medium supplemented with BA (0.25-3.0mgl^{-1}) and IAA (0.05-0.2mgl^{-1}) or adenine sulfate produced multiple shoots (Smitha Chetia and Handique, 2000).

Maximum number of shoots (4-5) was obtained from young rhizome buds of *Acorus calamus* on medium fortified with 0.5mgl^{-1} BAP and 0.5mgl^{-1} NAA (Harikrishnan and Molly Hariharan, 1997). The highest number of shoots (6.1 microshoots/explant) from nodal segments of *Atropa belladona* was obtained on a modified MS medium supplemented with 1.5mgl^{-1} BA and 0.5mgl^{-1} IAA (Al-Wasel, 1999).

The combination of 0.4ppm Kinetin with IAA 2.5ppm and BAP 4.0ppm with NAA 0.8ppm gave better response in half MS medium in *Gymnema sylvestre* (Anu *et al.*, 1994). Multiple shoot formation was induced from shoot bud explants on MS medium supplemented with BA 0.25mgl^{-1} in *Ocimum americanum* and 1.0mgl^{-1} BA and 0.5mgl^{-1} GA_3 in *O.sanctum* (Sitakanta Pattnaik and Pradeep Chand, 1996).

2.2.3. Rooting

In vitro plants must possess a strong and functional root system. Hu and Wang (1983) observed that three phases were involved in rhizogenesis, *viz.*, induction and elongation. Among the auxins, IBA and NAA have been most effective for root induction and BA which was widely used for shoot multiplication does so particularly strongly that roots were delayed even after transferring to cytokinin-free medium (Yeoman,1986).The root elongation phase was very sensitive to auxin concentration. High concentrations of auxin inhibited rooting in some species.

Efficient rooting in *Pogoestemon patchouli* could be obtained in rooting medium supplemented with 5.0mgl^{-1} NAA (Baskarrajan, 1994). In *Aritolachia indica*, microshoots were rooted in White's medium supplemented with 2.46μM IBA (Manjula *et al.*, 1997). In

Datura metel, the best response to rooting was observed in the presence of IBA at 2.0mgl^{-1} (Arockiasamy *et al.*, 1999). Rooting of *in vitro* differentiated shoots of *Plumbago zeylanica* was achieved within 10 days of culture in media containing 0.57μM IAA with 2% (w/v) sucrose (Rout *et al.*, 1999). In *Centella asiatica*, root induction was achieved on half MS medium supplemented with 1.0mgl^{-1} IBA (Suchitra Banerjee *et al.*, 1999).

2.3. Hardening

The fully regenerated plantlets in a tissue culture vessel enjoys on environment of very high humidity, low light level and usually a constant temperature. But plantlets leaving this environment were consequently very poorly adapted to resist low relative humidity, higher light levels and more variable temperatures under *in vivo* conditions (Wainwright, 1988). Physiologically, the leaves grown in tissue cultures have been shown to be incapable of significant photosynthesis (Langford and Wainwright, 1987). As cuticular wax on leaf surface is minimal and the stomata are unable to close, the plantlets are unable to control water loss (Grout and Aston, 1978).

The success in acclimatization of micro propagated plant is largely dependent on not only the post-transfer growth conditions, but also the pre-transfer culture conditions (Ziv, 1986). Raised the light level in culture, just prior to transfer to soil has also been recommended (Murashige,1974). Reduced the sucrose levels or even increasing these levels in the culture, prior to weaning has been in practice, for optimizing microplant size after hardening off (Wainwright, 1988).

Light, temperature and relative humidity were the three major factors to be controlled during acclimatization. Hu and Wang (1983) suggested a sufficient period of humidity acclimatization for the newly transferred plantlets. Methods of controlling

relative humidity have been accomplished through establishing polythene tent, misting and fogging. In polythene tanks, as aerial weaning environment was closed, it was possible to take advantage of CO_2 enrichment during hardening off (Lakso *et al*, 1986).

Kar and Sen (1985) have described a procedure for hardening of *Asparagus racemosus* plantlets. Prior to transfer to the potted soil, all plantlets were maintained in half strength liquid MS medium with 1 per cent sucrose. After two to three weeks the plants were transferred to the same basal medium without sucrose. Two weeks later the plants were washed with water, planted into pots containing sandy soil and humus (3:1) and kept in growth chamber (temperature 25°C, humidity 55 per cent, light 30 x 10^8 $\mu M/m^2/s$ for 16 h/8 h light/dark period). After 4 weeks the pots were transferred to the field and 70 per cent of the plants survived.

Well rooted plantlets of *Ocimum sanctum* were transferred to plastic pots (5cm diameter) containing an artificial soil (soilrite mix) and maintained inside a plant growth chamber set at 26±1°C, 16 h day length (35-50$\mu E/m^2/s$) and 75-80% relative humidity. The potted plantlets were irrigated with 1/8-MS basal salts solution devoid of sucrose and inositol, every 4 days for a period of 2 weeks. The plants were then transplanted to earthern pots (10cm diameter) containing natural soil and kept under in the glasshouse for another 2 weeks. The potted plants were then placed outdoors under full sun (Sitakanta Pattnaik and Pradeep Chand, 1996).

Maria Cristina *et al.* (1990) suggested a hardening procedure for *Datura insignis*. Rooted plantlets were removed from culture, transferred to pots containing a sterile mixture of soil: vermiculite (2:1) and placed in the culture room for 2 months before transplanting to the field.

2.4. Estimation of antiviral compounds

A large number of chemical compounds including fragrance, flavours, pigments, natural sweeteners, antimicrobials and pharmaceuticals were obtained from plants. In most cases, these compounds belong to a broad metabolic group, collectively referred to as secondary products. Plant cell cultures could be established from a large array of plant species, including most of those that produce secondary products of commercial interest (Berlin, 1984).

The economic impact of utilizing specifically selected tissue cultures that yield increased levels of a secondary metabolite over the intact plant would be high. The other compounds are the possibility of increased purity of the resultant product, the conversion of inexpensive precursors into expensive end products by biotransformation and the potential for feeding substrate analogues to the culture to create novel compounds.

In *P. amarus*, isolation of antiviral compounds (phyllanthin, hypophyllanthin etc) and elucidation of their action on the hepatitis virus have been achieved. These two compounds are the major lignans (Row *et al.*, 1966). *P. amarus* have shown to exhibit antihepatotoxic activity against carbon tetrachloride and galactosamine in primary cultured rat hepatocytes (Syamasunder *et al.*, 1985).The identity of the lignans was confirmed by comparing the optical rotation, UV, IR and HNMR spectro of the isolates with those reported in the literature (Anjaneyulu *et al.*, 1973).

Various factors affect the production of secondary metabolites from callus. In *Phyllanthus amarus*, callus derived on MS medium supplemented with 0.5mgl^{-1} or 1.0mgl^{-1} of either 2,4–D or IBA to produce maximum quantity of phyllanthin and hypophyllanthin (Unander, 1991). Cell suspensions of *Morinda citrifolia* produced large amounts of

anthraquinones when cultivated on B5 medium containing NAA at $1.0mgl^{-1}$, the production of which is inhibited by addition of 2,4–D at $2mgl^{-1}$ (Plas-van-der *et al.*, 1995). Alkaloid production was found to be higher rate at $1.0mgl^{-1}$ 2,4-D in *Ipomea batatas* (Kavikishore and Mehta, 1987).

Levels of alkaloid accumulation in hairy root cultures of *Catharanthus roseus* showed a clear increase with lowering of temperature (Toivonen *et al.*, 1992).UV light act as a stimulus through a phytochrome receptor for a series of enzymes involved in phenyl propanoid metabolism (Duell-Pfaff Wellman, 1982).

. Punarnavine profile of regenerated roots of *Boerhavia diffusa* was studied by Srivastava and Padhya (1995). In the presence of 2,4–D, leaf segments produced callus which regenerated roots that contained trace amounts of punarnavine.

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

This investigation of " *In vitro* studies in *Phyllanthus amarus* Schum and Thonn" was carried out at the Tissue Culture Laboratory of the Horticultural College and Research Institute, Tamilnadu Agricultural University, Coimbatore, during 1999-2001.

Optimization of explants, culture media, combinations of growth regulators and culture conditions for enhancing callus induction, callus regeneration and for rapid multiplication through direct organogenesis were carried out. Biochemical studies were also carried out to estimate phyllanthin and hypophyllanthin content of different types of cultures.

3.1. Materials

3.1.1. Glasswares

Borosilicate glasswares were used for the experiments. Prior to use, glasswares were soaked in a detergent solution (Teepol 0.10 per cent) and washed thoroughly in tap water and rinsed twice in double distilled water. The glasswares were then dried in a hot air oven at 100°C for 24 hours and stored until use.

3.1.2. Nutrient medium

Major and minor nutrients used in the study were as per Murashige and Skoog (1962). The composition of media is presented in Annexure I (The nutrients and chemicals used were obtained from M/s. Sigma chemicals, USA).

3.1.3. Other compounds

Other compounds such as agar, sucrose and plant growth regulators, namely auxins (IAA, NAA, 2,4 – D) and cytokinins (Kinetin and BAP) and the surface sterilant mercuric chloride were used in this study.

3.1.4. Plant materials

Plant materials were collected from the Botanical Garden of the Horticultural College and Research Institute, Coimbatore. For continued supply of explants, stock plants were raised through seeds in the Medicinal Plant Conservatory of the Botanical Garden, Horticultural College and Research Institute in Coimbatore.

3.2. Methods

3.2.1. Preparation of nutrient media

3.2.1.1. Preparation of stock solutions

The medium consisted of macronutrients, micronutrients, iron EDTA, vitamins, amino acids, sucrose, agar and plant growth regulators. All the stock solutions and final media were prepared by following the procedure of Bhojwani and Razdan (1983).

For Murashige and Skoog's basal medium different stock solutions were prepared and used.

3.2.1.1.1. Macronutrients

Each salt was weighed exactly (as given in Annexure I) and dissolved separately in a small amount of distilled water. Finally all the salt solutions were mixed together and volume made up with distilled water. Calcium chloride was added finally in order to prevent precipitation.

3.2.1.1.2. Micronutrients

Each chemical was weighed exactly (as given in Annexure I), dissolved separately, mixed together and finally the volume was made up with distilled water.

3.2.1.1.3. Iron EDTA

Required quantity of $FeSo_4.7H_2O$ and $Na_2.EDTA.2H_2O$ (as given in Annexure I) were dissolved separately in 200ml distilled water. $Na_2.EDTA$ solution was boiled, added gently to $FeSo_4.7H_2O$ and then the volume made upto 500ml.

3.2.1.1.4. Potassium iodide

Required quantity of Potassium iodide (as given in Annexure I) salt was dissolved in distilled water and finally the volume was made up using distilled water and the stock solution was stored in amber coloured bottle.

3.2.1.1.5. Vitamins and Amino acid

The components (as given in Annexure I) were weighed separately dissolved in distilled water and finally the volume was made up.

3.2.1.2. Preparation of growth regulators

Separate stock solutions were prepared for each growth hormone by dissolving it in a very minute quantity of the appropriate solvent (1N NaOH or HCl) and making upto the final volume with distilled water.

3.2.1.2.1. Auxins

100 mg each of NAA, IAA, IBA, 2,4 – D were dissolving in 2 to 3 ml of ethanol, warmed and gradually diluted to 100 ml, using glass double distilled water.

3.2.1.2.2. Cytokinins

100 mg each of kinetin and BAP were diluted in a small volume of 0.1N HCl, heated and gradually diluted to 100 ml, using glass double distilled water.

3.2.1.3. Preparation of sucrose and myo-inositol

Sucrose was added at the concentration of 3 per cent (w/v) and myo-inositol at the concentration of 100 mgl^{-1}.

3.2.1.4. Preparation of agar medium

The basal nutrient medium comprised the stock solutions mixed in accordance with the composition of the basal medium requirements, sucrose and myo-inositol. Agar (0.8 per cent w/v) was dissolved in boiling water and added to the basal medium, and the pH of the medium was adjusted to 5.6 to 5.8 using 0.1N HCl or 0.1N NaOH. The growth regulators at required concentrations were added, homogenised and the medium was then distributed to clean culture tubes of 25 x 150mm size. The culture tubes were plugged with sterile non-absorbant cotton and sterilized in an autoclave at a temperature of 121°C and a pressure of 15 pounds per square inch for 20minutes (Dodds and Roberts, 1982).

3.2.2. Collection and preparation of explant

3.2.2.1. Explant collection

The explants were collected from healthy mother plants.

The explants taken for study were leaf bits, stem bits, shoot bits, nodal segments, root bits, fruit and seed.

3.2.2.2. Size of the explant

Appropriate sizes of the explant as detailed below were used for inoculation. Explants were made to a considerable size outside the "Thermodyne Laminar Air-Flow

Chamber", but in aseptic condition. However, the required size for inoculation was made in side the laminar airflow chamber in sterile conditions. The explants size taken for study were:

i. Leaf bits (area) - 0.5-1.0 cm^2

ii. Stem bits (length) - 1.0-1.5 cm

iii. Shoot tips (length) - 0.5-1.0 cm

iv. Nodal segments (length) - 1.0-1.5 cm

v. Root bits (length) - 1.0-1.5 cm

vi. Fruit - Whole and half size

vii.Seed - Fresh and dried

While taking shoot tips, nodal segment and stem bits, a slant cut was given to one end to ensure the polarity.

3.2.2.3. Sterilization of explants

The explants were washed in tap water and with teepol. They were excised aseptically and kept in sterilized petridishes containing sterile distilled water. The explants were surface sterilized with 0.1% mercuric chloride solution for 2-3minutes followed by rinsing with sterile double distilled water for 3-5 times and kept in a petridish containing sterile distilled water.

3.2.3. Isolation and transfer of explants

Isolation and transfer of explants to culture tubes were carried out inside a LAFC under aseptic conditions. The chamber was earlier sterilized with absolute alcohol and ultra violet radiation (253.7^{0}A). The instruments used for inoculation were autoclaved at 15 psi at 120^{0}C for 20minutes and sterilized with 70% alcohol, followed by sterilization

with 0.1% mercuric chloride solution. Hands were also swabbed with 70% alcohol before carrying out operation in order to ensure aseptic conditions. The sterilized explants were inoculated onto the media contained culture tubes. About 20 tubes were inoculated for each treatment.

3.2.4. Culture incubation

The inoculated tubes were kept in the culture room with the temperature maintained at 25 ± 2^0 C and humidity at 70%. The light and dark cycle of 16 hours and 8 hours respectively was maintained with 2000-3000 lux intensity.

3.3. Experiment details

The experiment was carried out with Factorial Completely Randomised Design (FCRD) with 5 treatments and 3 replications. For each replication 20 test tubes were inoculated.

3.3.1. Seasonal effect of explant

The inoculation of explants was carried out under different months (Jan-Feb, Mar-April, May-June, July-Aug, Sep-Oct and Nov-Dec). To record conducive season for better survival percentage and contamination percentage.

3.3.2. Identification of suitable explants

By using various explants, observations were made to identify explants based on their culture response/survival percentage, contamination percentage and mortality percentage. These were taken based on visual count.

3.3.3. Standardization of surface sterilization methods

An experiment was carried out to standardize the optimum concentration of the sterilant mercuric chloride and time of exposure of different explants to the surface sterilant at the concentrations of 0.1, 0.5 and 1.0 per cent for 1.0, 3.0 and 5.0minutes. The observation on culture survival and extent of contamination were recorded and expressed as percentage.

3.3.4. Direct organogenesis

For direct organogenesis either by axillary shoot proliferation or by adventitious shoot formation, the explants, viz., shoot tip and nodal segments were inoculated in MS basal medium supplemented with BAP (1.0–5.0mgl^{-1}) along with GA_3 (0.5mgl^{-1}) and BAP (1.0-5.0mgl^{-1}) along with kinetin (1.0-5.0mgl^{-1}). Basal medium without any supplements served as the control.

Each treatment consisted of twenty tubes. The cultures were exposed to a light of 1000 lux and darkness cycle of 16 hours and 8 hours respectively. The culture room temperature was maintained at 25±2°C. subculturing was done at an interval of 3-4 weeks.

The following observations were recorded at regular intervals:

1. Percentage of responding cultures.

2. Number of multiple shoots regenerated per explant.

Visual counts were taken 4 weeks after inoculation.

3. Shoot length (cm).

Shoots were removed from the culture medium and the mean shoot length was measured in centimeters from the base to the tip.

3.3.5. Indirect organogenesis

3.3.5.1. Standardization of culture medium for callus induction

Murashige and Skoog (1962) medium was used for the induction of callus from leaf bits, stem bits, shoot tips and nodal segments. Sucrose (3.0 per cent), agar (0.8 per cent),the cytokinins Kinetin (1.0-5.0mgl^{-1}) and BAP (1.0-5.0mgl^{-1}) and the auxins 2,4–D (1.0-5.0mgl^{-1}) and NAA (0.1-0.5mgl^{-1}) were added to the medium.

The cultures containing 2,4–D were kept in darkness by covering them with a black cloth and the remaining cultures were incubated at 25±2^{0}C in light and darkness cycle of 16 hours and 8 hours respectively.

The following observations were recorded.

1. Percentage response to callusing.
2. Days taken for callus initiation.
3. Relative growth of callus.

Callus developed in the primary culture medium was scored after 4 weeks using the following scale.

a. Poor callusing - 1

b. Slight callusing - 2

c. Moderate callusing - 3

d. Profuse callusing - 4

4. Callus index

Callus index was calculated in the following way.

CI = Percentage culture forming callus x Relative growth of callus.

5. Weight of callus (g).

After completion of callus formation, the callus was removed from the culture medium washed completely free of agar and the mean weight in grams was record.

3.3.5.2. Callus regeneration

After 2 weeks of callus initiation, callus clumps were transferred to regeneration medium. The regeneration medium consisted of MS basal medium supplemented with BAP (1.0-5.0mgl^{-1}) and GA_3 (1.0mgl^{-1}) or BAP (1.0-5.0mgl^{-1}) and Glycine (50.0mgl^{-1}).

The cultures were exposed to a light (1000 lux approximately) and darkness cycle of 16 hours and 8 hours respectively. The culture room temperature was maintained at 25 ± 2^0 C. Subculturing was done at an interval of 4 weeks.

Observations on percentage response to organogenesis were recorded.

3.3.6. Rooting

After separating the multiple shoots, each individual shoot was subcultured onto half MS medium containing various concentration of IAA (0.25,0.50, 0.75, 1.00 and 1.25 mgl^{-1}) and IBA (0.25,0.50, 0.75, 1.00 and 1.25mgl^{-1}) were combined to used for the development of adventitious roots. The cultures were exposed to the same physical environment as that for shoot induction.

The following observations were recorded.

1. Days taken for root initiation.
2. Percentage of cultures rooted.
3. Number of roots per culture.

Visual counts were taken 15 days after rooting.

4. Root length (cm)

Seedlings were carefully removed from the culture tubes and placed over a sterile petridish. Root length was measured in centimetres and recorded.

3.3.7. Hardening, *ex vitro* planting and establishment of the *in vitro* derived plants

Fully developed plantlets were removed from the culture tubes and made free from agar by washing gently with glass double distilled water and then transferred to small perforated plastic containers containing sterilized mixture of soil, sand and organic manure (1:1:1) or sand and vermiculite (1:1) mixture. The transferred plantlets were kept in the culture room for 15 to 20 days for the purpose of hardening. Sterilized MS plant salt mixture solution (half strength) was used as a nutrient source for the transferred plantlets. After hardening, the plantlets were transferred to bigger pots containing red earth, sand and farmyard manure (1:1:1) and maintained in the green house.

3.4. Biochemical studies

3.4.1. Estimation of phyllanthin and hypophyllanthin

For estimation of phyllanthin and hypophyllanthin, different types of cultures were used, as mentioned below.

Cultures used for estimation of phyllanthin and hypophyllanthin

S. No	Nature of culture	Source of culture	Culture medium on which the culture was initiated
1.	Multiple shoot clumbs with basal callus	Shoot tip	MS + BAP ($2.0mgl^{-1}$) + GA_3 ($1.0mgl^{-1}$)
2.	Multiple shoot clumbs with basal callus	Nodal segment	MS + BAP ($3.0mgl^{-1}$) + GA_3 ($1.0mgl^{-1}$)

3.	Microshoots without roots	Nodal segment	MS + BAP ($2.0mgl^{-1}$) + GA_3 ($1.0mgl^{-1}$)
4.	Microshoots with roots	Nodal segment	MS + IBA ($0.5mgl^{-1}$) + IAA ($0.5mgl^{-1}$)
5.	Green callus	Shoot tip	MS + BAP ($3.0mgl^{-1}$) + Kin ($3.0mgl^{-1}$)
6.	Green callus	Nodal segment	MS + BAP ($3.0mgl^{-1}$) + Kin ($3.0mgl^{-1}$)
7.	White callus	Stem bit	MS + 2,4-D ($4.0mgl^{-1}$) + NAA ($0.4mgl^{-1}$)
8.	White callus	Leaf bit	MS + 2,4-D ($4.0mgl^{-1}$) + NAA ($0.4mgl^{-1}$)

The two lignans were isolated from *P. amarus* following the method reported by Row *et al.* (1966), and the identity of the lignans was confirmed by comparing the optical rotation, UV, IR and HNMR spectra of the isolates with those reported in the literature (Anjaneyulu *et al.*, 1973). These compounds were used as external standards for their estimation in the plant samples. All analyses were run in triplicate.

3.4.1.1. Extraction procedure

Sample (1g) was mixed with lime (300mg) and water (2.5ml), and macerated at room temperature for 18 hours. The macerated material was refluxed at 60 minutes in a boiling water bath with methanol (30ml) containing 3.0% potassium hydroxide.The reflux material was filtered, the residue washed with methanol (3 x 5ml), and volume of the

combined filtrate and washings was made up to 50 ml. The sample was subjected to spectrophotometer and the lignans were estimated at 230 nm.

3.4.1.2. Preparation of working stock solution

Phyllanthin and hypophyllanthin were dissolved separately in methanol to obtain solutions containing 2.0mgml^{-1} of each. Three separate sets of such stock solutions were prepared. The solutions of that were mixed in equal proportions to obtain a working stock solution of standards containing 0.05, 0.10. 0.20, 0.40, 0.80, 1.00, 2.00, 3.00 and 4.00 µgml^{-1} each of phyllanthin and hypophyllanthin. Aliquot of each dilution were subjected to spectrophotometer and the readings were recorded at 230 nm.

3.5.Statistical analysis

The observations for various morphological characters were taken from 3 replications and were tabulated. Statistical analysis was carried in a Factorial Completely Randomized Design, as per Panse and Sukhatme (1978) and the results were interpreted.

CHAPTER IV

RESULTS

The results of the present study on standardization of protocol for mass multiplication of *Phyllanthus amarus* are presented in this chapter.

4.1. Explant standardization

4.1.1. Culture response

The explants like shot tips, leaf bits, stem bits, nodal segments, root bits, fruits and seeds were sourced from the stock plant that were maintained for the purpose of micropropagation. Among the various explants used, nodal segments gave significantly highest response (87.25%) followed by shoot tip (86.63%), stem bit (76.61%) and leaf bit (61.28%). The remaining explants did not express any response (Table 1).

4.1.2. Contamination

The nodal segments were significantly superior in combating contamination, wherein the least rate of contamination (19.10%) was recorded, followed by the shoot tips(19.97%), stem bits (30.33%) and leaf bits (41.54%) (Table 1).

4.1.3. Culture survival

The data on per cent survival showed highly significant variations with 65.43% in nodal segments, 63.61% in shoot tips, followed by the stem bits (46.55%) and leaf bits (40.55%). The other plants were futile to *in vitro* culture (Table 1)

4.1.4. Explant mortality

Nodal segments were observed to give the significantly least per cent mortality (12.26%).

Table-1 Standardisation of explants for micro propagation

Explants	Culture response (%)	Contamination (%)	Culture survival (%)	Dead explants (%)
Shoot tip	86.63(52.90)	19.97(26.54)	63.61(52.90)	15.15(22.85)
Leaf bit	61.28(39.55)	41.54(40.13)	40.55(39.55)	31.45(34.11)
Stem bit	76.61(43.02)	30.33(33.41)	46.55(43.02)	21.58(27.68)
Nodal segment	87.25(53.99)	19.10(25.91)	65.43(53.99)	12.26(20.46)
Root bits	00.00	62.05(51.97)	00.00	37.95(38.02)
Fruit	00.00	63.42(52.79)	00.00	36.57(37.21)
Seed	00.00	69.24(56.32)	00.00	30.75(33.67)
Mean	**44.545(27.313)**	**43.666(41.012)**	**30.883(27.314)**	**26.533(30.576)**
SEd	0.814(0.416)	1.101(0.689)	0.704(0.416)	1.313(0.983)
CD (0.05)	1.747(0.892)**	2.361(1.478)**	1.510(0.892)**	2.819(2.108)**

Values in paranthesis are arcsine-transformed values

This was followed by shoot tip (15.15%), stem bit (21.58%) and leaf bit with 31.45% fatality (Table 1).

4.2.Effect of season on explant growth

4.2.1. Culture survival

Explants inoculated in the month of January-Febraury gave significantly higher per cent survival (83.43%), followed by July-August (81.79%). Whereas the survival per cent in monsoon months, *viz.*, November-December, showed significantly least survival (19.76%) (Table 2, Fig.1))

4.2.2. Contamination

Significantly superior result was obtained with least contamination in January-Febraury (16.56%), followed by July-August (18.20%). Significantly more susceptibility for contamination was noticed in November-December (80.23%) (Table 2, Fig.1).

4.3. Sterilization of explants

4.3.1. Contamination

Among the explants responded, the lowest per cent contamination was recorded in stem bits (20.00%) in 1.0% concentration for 3minute.The contamination per cent was significantly higher in stem bits (85.00%) in 0.1% concentration for 1 minute. However, shoot tips and nodal segments treated with 1.0% $HgCl_2$ with the duration of 3 minutes, showed significantly low contamination with 30.00% and 35.00% respectively, followed by the leaf bit treated with 1.0% $HgCl_2$ with duration of 1 minute showed 36.00% contamination (Table 3).

4.3.2. Explant mortality

All explants except shoot tips, leaf bits, stem bits and nodal segments showed higher per cent of fatality. The mortality of shoot tip was recorded to be significantly low (10.00%) at 1.0%

Table-2 Effect of season on explant growth

Season	Culture survival (%)	Contamination (%)
Jan-Feb	83.43 (65.99)	16.56 (24.01)
Mar-April	77.26 (61.52)	22.74 (28.47)
May-June	79.67 (63.20)	20.32 (26.79)
July-Aug	81.79 (64.74)	18.20 (25.25)
Sep-Oct	22.08 (28.02)	77.91 (61.98)
Nov-Dec	19.76 (26.39)	80.23 (63.60)
Mean	**60.669 (51.647)**	**39.330 (38.354)**
SEd	0.758 (0.533)	0.758 (0.533)
CD (0.05)	1.651 (1.162)**	1.651 (1.162)**

Values in paranthesis are arcsine-transformed values

Fig.1. Effect of season on explant growth

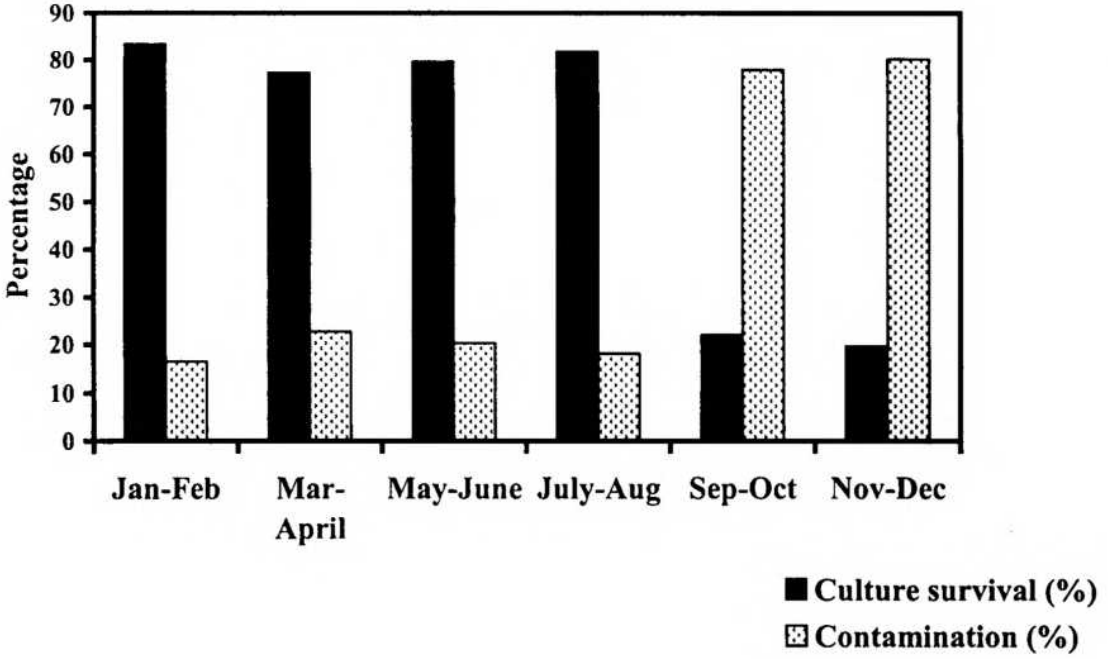


Table-3 Sterilisation fixation of various explants

Ex plants	Duration of Exposure(min)	Contamination (%)			Ex plant Mortality (%)			Culture survival (%)		
		$HgCl_2$ Concentration (%)								
		0.10	0.50	1.00	0.10	0.50	1.00	0.10	0.50	1.00
Shoot tip	1	55.00	55.00	65.00	25.00	19.00	16.00	10.00	26.00	29.00
	3	59.00	50.00	30.00	22.00	12.00	10.00	19.00	35.00	60.00
	5	62.00	53.00	50.00	23.00	13.00	12.00	15.00	28.00	38.00
Leaf bit	1	45.00	40.00	36.00	18.00	16.00	15.00	13.00	40.00	53.00
	3	48.00	46.00	45.00	32.00	30.00	28.00	15.00	20.00	20.00
	5	49.00	43.00	41.00	38.00	36.00	34.00	8.00	10.00	11.00
Stem bit	1	85.00	83.00	80.00	17.00	15.00	14.00	10.00	11.00	12.00
	3	30.00	25.00	20.00	18.00	16.00	12.00	62.00	63.00	65.00
	5	37.00	37.00	27.00	18.00	15.00	16.00	19.00	20.00	22.00
Nodal segment	1	62.00	51.00	45.00	45.00	42.00	38.00	10.00	10.00	13.00
	3	59.00	52.00	35.00	23.00	20.00	19.00	54.00	56.00	58.00
	5	55.00	54.00	43.00	51.00	49.00	48.00	21.00	22.00	23.00

Statistically not analysed.

$HgCl_2$ concentration in 3 minutes exposure. When a similar pattern of treatment was followed in nodal segment, an increased mortality per cent was noticed (Table 3).

4.3.3. Culture survival

Except nodal segment, shoot tip, leaf bit and stem bit, others almost gave a futile response. In case of stem bits the survival was significantly enhanced (65.00%) at 1.0% $HgCl_2$, exposed for 3 minutes. This was followed by shoot tip, which responded upto 60.00% at 1.0% $HgCl_2$ in 3 minutes time (Table 3).

4.4. Indirect organogenesis

4.4.1. Standardization of explants

The experiment conducted to standardize the explants for callusability revealed that the nodal segment (383.95) and shoot tip (379.80) recorded higher callus indices than the leaf bit (325.65) and stem bit (320.90). Though even nodal segments showed high callusability (383.95), the calli were green in colour (Table 6,7,10 &11). However, the stem bit and leaf bit derived calli were brown or white in colour. The various types of calli derived from various explants used on different culture environment are furnished in table.

Types of calli derived from different explants

S. No	Nature of callus	Explant	Growth regulators combination
1.	Green callus	Shoot tip and nodal segment	BAP + GA_3
2.	White callus	Stem bit and leaf bit	2,4-D + NAA
3.	Brown callus	Stem bit and leaf bit	2,4-D
4.	Rhizogenic, brown callus	Stem bit and leaf bit	2,4-D + NAA
5.	Green callus and callus with phenolic exudation	Nodal segment	BAP + kinetin

4.4.2. Effect of various growth regulators on callusability

The various explants were cultured in MS media supplemented with auxins and cytokinins, alone or in combination and kept under dark condition.

4.4.2.1. Effect of 2,4-D on callus induction from stem bit

Various concentrations of 2,4-D (1.0-5.0mgl^{-1}) were tried and cultures were maintained in darkness. The results indicated that, 2,4-D at 4.0mgl^{-1} gave good callusability for the explant stembit (304.09) (Table 4). Decreasing the concentration of 2,4-D recorded lower callusability.

The duration of callusing was also observed for the cultures. Among all the treatments, early (48.97 days) callusing was observed in stem bit which was supplemented with 2,4-D (4.0mgl^{-1}) and delayed (60.17 days) callusing was recorded with 2,4-D (1.0mgl^{-1}) (Table 4, Fig.2).

4.4.2.2. Effect of 2,4-D on callus induction from leaf bit

Various concentrations of 2,4-D (1.0-5.0mgl^{-1}) were tried and cultures were maintained in darkness. High callusability (104.97) in 55.52 days was recorded with 2,4-D (4.0mgl^{-1}) and the low callusability (19.75) in 62.06 days was recorded with 2,4-D (2.0mgl^{-1}) (Table 5, Fig.2).

With respect to the nature of callus, brown callus was observed at lower concentration and white friable callus at higher concentration of 2,4-D in stem bit and leaf bit explants.

4.4.2.3. Combined effect of 2,4-D and NAA on callus induction from stem bit

Combinations of two different auxin at different concentrations were tried. 2,4-D (1.0-5.0mgl^{-1}) and NAA (0.1-0.5mgl^{-1}) were supplemented to the MS medium. High callusability was observed with stem bit explant (325.62) at a concentration of 4.0mgl^{-1} 2,4-D and 0.4mgl^{-1} NAA and the low callusability (23.14) was recorded with 5.0mgl^{-1} 2,4-D and 0.5mgl^{-1} NAA. Early callusing (46.66 days) was associated with good callusability (Table 6, Fig.3).

Table-4 Effect of 2,4-D on callus induction from stem bit

Treatment (Concentration of 2,4-D) (mgl^{-1})	Percentage of forming callus	Relative growth	Callus index	Days taken for callusing
T_0 (0.0)	---	---	---	---
T_1 (1.0)	20.35 (20.57)	1.99	40.58	60.17
T_2 (2.0)	23.28 (23.93)	2.31	54.01	58.89
T_3 (3.0)	34.82 (30.92)	3.51	122.14	55.99
T_4 (4.0)	75.09 (35.62)	4.05	304.09	48.97
T_5 (5.0)	59.62 (38.24)	3.64	217.05	52.88
Mean	**42.633(25.858)**	**3.104**	**147.577**	**55.382**
SEd	0.885(1.779)	0.340	11.863	0.676
CD (0.05)	1.971(3.963)**	0.758**	26.432**	1.507**

Values in parenthesis are arcsine-transformed values.

Table-5 Effect of 2,4-D on callus induction from leaf bit

Treatment (Concentration of 2,4-D) (mgl^{-1})	Percentage of forming callus	Relative growth	Callus index	Days taken for callusing
T_0 (0.0)	---	---	---	---
T_1 (1.0)	00.00 (00.00)	00.00	00.00	00.00
T_2 (2.0)	16.49 (28.84)	2.03	19.75	62.06
T_3 (3.0)	26.58 (36.16)	4.32	43.47	60.63
T_4 (4.0)	38.32 (50.54)	3.49	104.97	55.52
T_5 (5.0)	33.92 (60.06)	2.72	82.79	58.49
Mean	**23.006(40.486)**	**1.627**	**50.202**	**47.345**
SEd	2.702(0.594)	0.193	7.234	0.747
CD (0.05)	6.021 (1.323)**	0.430**	16.117**	1.664**

Values in parenthesis are arcsine-transformed values.

Fig.2. Effect of 2,4-D (4.0mgl^{-1}) 0n callus induction from stem bits and leaf bits

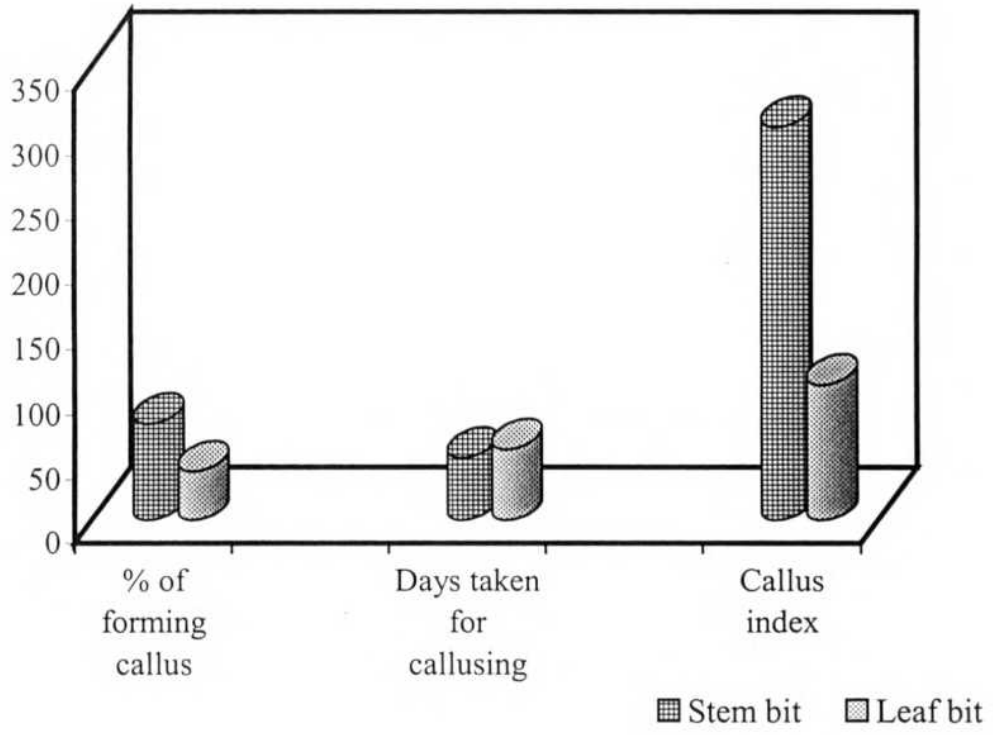


Table-6 Effect of 2,4-D and NAA on callus induction from stem bit

Treatment (Concentration of 2,4-D + NAA) (mgl^{-1})	Percentage of forming callus	Relative growth	Callus index	Days taken for callusing
T_0 (0.0 + 0.0)	---	---	---	---
T_1 (1.0 + 0.1)	29.97 (32.84)	1.33	40.24	56.33
T_2 (2.0 + 0.2)	21.66 (27.09)	1.65	37.33	58.16
T_3 (3.0 + 0.3)	73.33 (59.02)	2.88	211.81	52.00
T_4 (4.0 + 0.4)	84.99 (67.23)	3.83	325.62	46.66
T_5 (5.0 + 0.5)	20.25 (26.68)	1.16	23.14	56.83
Mean	**46.044(42.576)**	**2.172**	**127.631**	**54.000**
SEd	6.978(4.758)	0.191	17.977	1.952
CD (0.05)	15.549(10.602)**	0.425**	40.056**	4.350**

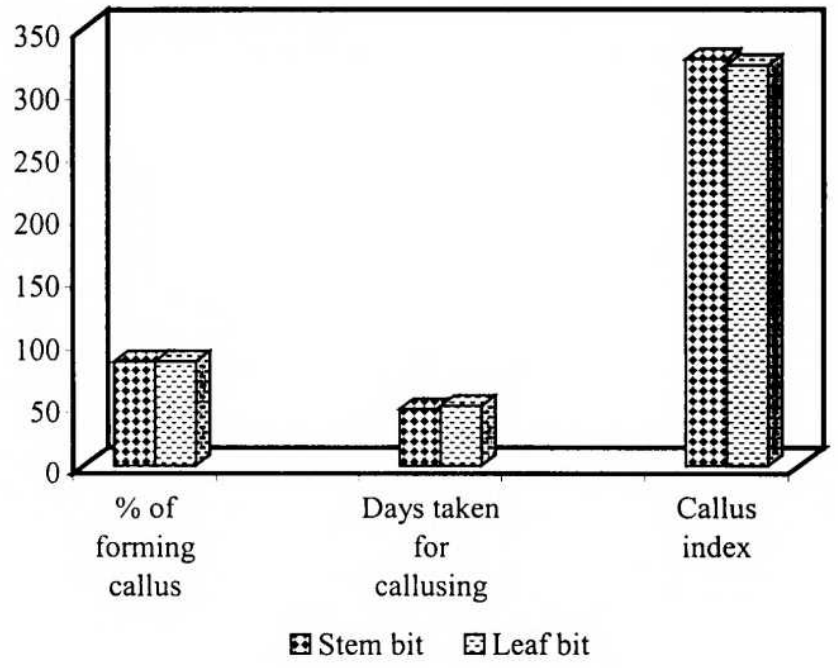
Values in parenthesis are arcsine-transformed values

Table-7 Effect of 2,4-D and NAA on callus induction from leaf bit

Treatment (Concentration of 2,4-D + NAA) (mgl^{-1})	Percentage of forming callus	Relative growth	Callus index	Days taken for callusing
T_0 (0.0 + 0.0)	---	---	---	---
T_1 (1.0 + 0.1)	20.00 (26.45)	1.21	24.23	59.16
T_2 (2.0 + 0.2)	40.00 (49.21)	1.20	48.90	57.66
T_3 (3.0 + 0.3)	62.22 (52.17)	2.60	166.72	51.77
T_4 (4.0 + 0.4)	84.99 (67.23)	3.77	320.90	49.16
T_5 (5.0 + 0.5)	21.10 (27.24)	1.43	30.14	58.50
Mean	**45.665(42.464)**	**2.047**	**118.183**	**55.253**
SEd	5.079(3.176)	0.375	31.149	0.889
CD (0.05)	11.317(7.076)**	0.835**	69.404**	1.982**

Values in parenthesis are arcsine-transformed values.

Fig. 3. Effect of 2,4-D (4.0 mg/l) and NAA (0.4 mg/l) on callus induction from stem bit and leaf bit



4.4.2.4. Combined effect of 2,4-D and NAA on callus induction from leaf bit

Leaf bits also responded to all the concentrations. Higher callusability (320.90) in 49.16 days was recorded with 4.0mgl^{-1} 2,4-D and 0.4mgl^{-1} NAA and lower callusability (24.23) in 59.16 days was observed with 1.0mgl^{-1} 2,4-D and 0.1mgl^{-1} NAA (Table 7, Fig.3).

Irrespective of the explant type and the concentration and combination of growth regulators, most of the calli produced were white and globular.

4.4.2.5. Combined effect of BAP and GA_3 on callus induction from shoot tip

Combinations of cytokinin and gibberellin at different concentrations were tried and cultures were maintained under light condition for callusability. The combination of BAP (1.0-5.0mgl^{-1}) and GA_3 (0.5mgl^{-1}) recorded good response for callusability in shoot tips. The highest (332.60) and earliest (28.99 days) callusability was recorded in shoot tip explant cultured in MS medium supplemented with 2.0mgl^{-1} BAP and 0.5mgl^{-1} GA_3.The lowest (88.36) and delayed (30.75 days) callusability was occurred at a concentration of BAP (5.0mgl^{-1}) and GA_3 (0.5mgl^{-1}) (Table 8, Fig.4).

4.4.2.6. Combined effect of BAP and GA_3 on callus induction from nodal segment

Nodal segments responded more to callusing (334.28) when compared to shoot tip explant cultured in MS medium supplemented with 2.0mgl^{-1} BAP and 0.5mgl^{-1} GA_3. The least callusability (63.72) was observed with BAP (5.0mgl^{-1}) and GA_3 (0.5mgl^{-1}). Early (27.74 days) callusing was observed in nodal segment which was supplemented with 2.0mgl^{-1} BAP and 0.5mgl^{-1} GA_3 and delayed (30.66 days) callusing was recorded with BAP (5.0mgl^{-1}) and GA_3 (0.5mgl^{-1}) (Table 9, Fig.4). All the treatments resulted in the production of green calli.

4.4.2.7. Combined effect of BAP and Kinetin on callus induction from shoot tip

The combination of two cytokinins were also tried for callusability. The combination of BAP (1.0-5.0mgl^{-1}) and GA_3 (0.5mgl^{-1}) was not satisfactory with respect to callus induction

Table-8 Effect of BAP and GA_3 on callus induction from shoot tip

Treatment (Concentration of BAP + GA_3) (mgl^{-1})	Percentage of forming callus	Relative growth	Callus index	Days taken for callusing
T_0 (0.0 + 0.0)	---	---	---	---
T_1 (1.0 + 0.5)	55.99 (48.49)	3.33	185.52	29.13
T_2 (2.0 + 0.5)	84.35 (66.71)	3.94	332.60	28.99
T_3 (3.0 + 0.5)	68.18 (55.69)	2.94	132.81	29.06
T_4 (4.0 + 0.5)	49.88 (44.93)	2.88	144.04	29.08
T_5 (5.0 + 0.5)	28.89 (32.50)	3.05	88.36	30.75
Mean	**57.462(49.667)**	**3.230**	**176.673**	**29.404**
SEd	3.886(2.303)	0.276	38.942	0.557
CD (0.05)	8.651(5.131)**	0.614*	86.768**	1.240*

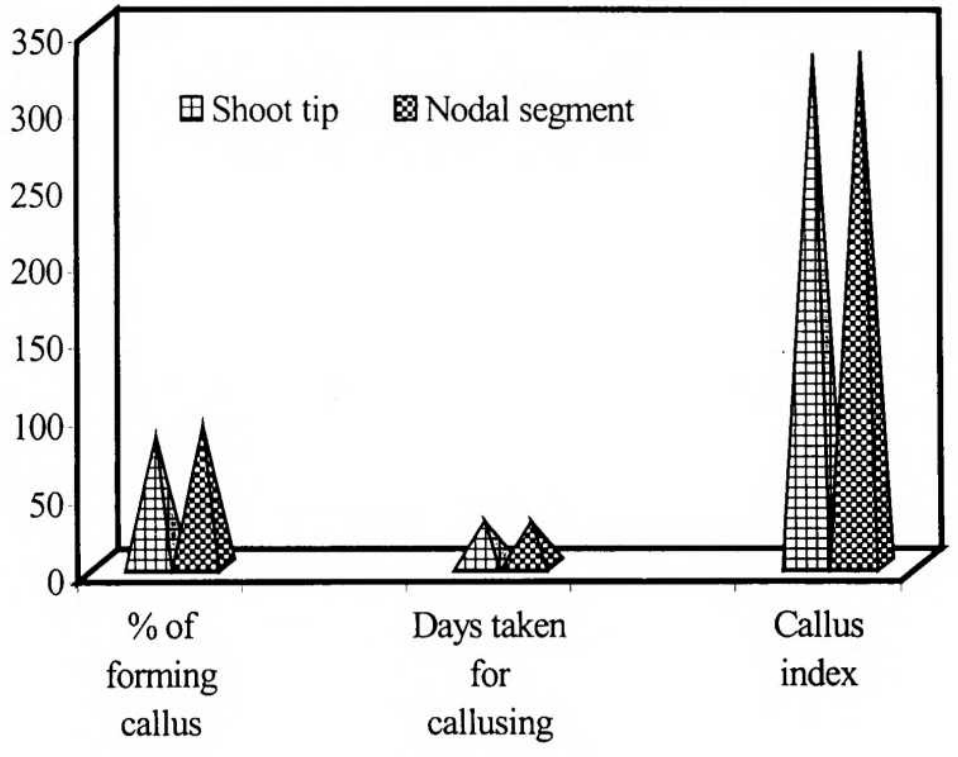
Values in parenthesis are arcsine-transformed values.

Table-9 Effect of BAP and GA_3 on callus induction from nodal segment

Treatment (Concentration of BAP + GA_3) (mgl^{-1})	Percentage of forming callus	Relative growth	Callus index	Days taken for callusing
T_0 (0.0 + 0.0)	---	---	---	---
T_1 (1.0 + 0.5)	56.66(48.88)	2.33	131.07	28.75
T_2 (2.0 + 0.5)	91.36(73.19)	3.66	334.28	27.74
T_3 (3.0 + 0.5)	64.85(53.69)	2.61	170.46	29.30
T_4 (4.0 + 0.5)	49.77(44.87)	2.38	118.91	29.08
T_5 (5.0 + 0.5)	28.88(32.49)	2.22	63.72	30.66
Mean	**58.308(50.627)**	**2.641**	**163.693**	**29.107**
SEd	4.370(2.846)	0.202	16.111	0.888
CD (0.05)	9.737(6.340)**	0.451**	35.897**	1.980 NS

Values in parenthesis are arcsine-transformed values.

Fig.4. Effect of BAP (2.0 mg/l) and GA_3 (0.5 mg/l) on callus induction from shoot tip and nodal segment



compared to BAP-kinetin combination. BAP (1.0-5.0mgl^{-1}) and kinetin (1.0-5.0mgl^{-1}) were tried for callusability in shoot tip explants.

Among all the concentrations, high (379.80) callusability was observed in shoot tip explants at an early period (34.72 days) cultured in MS medium supplemented with BAP (3.0mgl^{-1}) and kinetin (3.0mgl^{-1}). The least (26.41) and delayed (39.66 days) callusability was observed with BAP (1.0mgl^{-1}) and kinetin (1.0mgl^{-1}). Increasing the concentration of BAP and kinetin beyond 3.0mgl^{-1} reduced the callusability (Table 10, Fig.5).

4.4.2.8. Combined effect of BAP and Kinetin on callus induction from nodal segment

Combination of BAP (1.0-5.0mgl^{-1}) and kinetin (1.0-5.0mgl^{-1}) were tried for callusability in shoot tip explants. The highest (383.95) and earliest (34.16 days) callusability was observed with BAP (3.0mgl^{-1}) and kinetin (3.0mgl^{-1}) using nodal segment as explant. The least (40.65) and delayed (40.51 days) callusability in nodal segment was observed at very low concentration of BAP (1.0mgl^{-1}) and kinetin (1.0mgl^{-1}). Green globular callus was seen in all cultures (Table 11, Fig.5).

Among various treatments, using various growth regulators tried, BAP 3.0mgl^{-1} and kinetin 3.0mgl^{-1} showed higher and early callusability for nodal segment explants compared to all the treatments.

4.4.2.9. Combined effect of BAP and GA_3 on weight of callus

The weight of callus was recorded 30 days after inoculation. The combination of BAP (1.0-5.0mgl^{-1}) and GA_3 (0.5mgl^{-1}) were tried. The highest callus weight (1.48 g/tube) was obtained from shoot tip at low concentration of BAP (2.0mgl^{-1}) and GA_3 (0.5mgl^{-1}). The least callus weight (0.62 g/tube) was observed at BAP (5.0mgl^{-1}) and GA_3 (0.5mgl^{-1}).

Nodal segment showed least callus weight (1.04 g/tube) as compared to shoot tip explant. The highest callus weight (1.04 g/tube) was observed with BAP (2.0mgl^{-1}) + GA_3 (0.5mgl^{-1})

Table-10 Effect of BAP and Kinetin on callus induction from shoot tip

Treatment (Concentration of BAP + Kin) (mgl^{-1})	Percentage of forming callus	Relative growth	Callus index	Days taken for callusing
T_0 (0.0 + 0.0)	---	---	---	---
T_1 (1.0 + 1.0)	23.33(28.64)	1.16	26.41	39.66
T_2 (2.0 + 2.0)	75.00(60.07)	3.55	265.15	34.94
T_3 (3.0 + 3.0)	95.00(79.12)	4.00	379.80	34.72
T_4 (4.0 + 4.0)	70.55(57.17)	2.21	157.14	38.33
T_5 (5.0 + 5.0)	55.00(47.88)	2.01	111.91	38.50
Mean	**63.777(54.579)**	**2.589**	**188.085**	**37.232**
SEd	4.767(4.282)	0.285	21.602	0.717
CD (0.05)	10.621(9.542)**	0.635**	48.132**	1.598**

Values in parenthesis are arcsine-transformed values.

Table-11 Effect of BAP and Kinetin on callus induction from nodal segment

Treatment (Concentration of BAP + Kin) (mgl^{-1})	Percentage of forming callus	Relative growth	Callus index	Days taken for callusing
T_0 (0.0 + 0.0)	---	---	---	---
T_1 (1.0 + 1.0)	33.32(35.21)	1.22	40.65	40.51
T_2 (2.0 + 2.0)	65.59(54.15)	3.49	231.26	35.25
T_3 (3.0 + 3.0)	88.88(70.93)	4.32	383.95	34.16
T_4 (4.0 + 4.0)	55.55(48.20)	2.03	112.81	39.32
T_5 (5.0 + 5.0)	38.59(38.26)	2.72	103.13	37.40
Mean	**56.389(49.345)**	**2.761**	**174.363**	**37.333**
SEd	5.942(3.856)	0.232	23.782	0.621
CD (0.05)	13.240(8.598)**	0.516**	52.990**	1.383**

Values in parenthesis are arcsine-transformed values.

Fig. 5. Effect of BAP (3.0 mg/l) and kinetin (3.0 mg/l) on callus induction from shoot tip and nodal segment

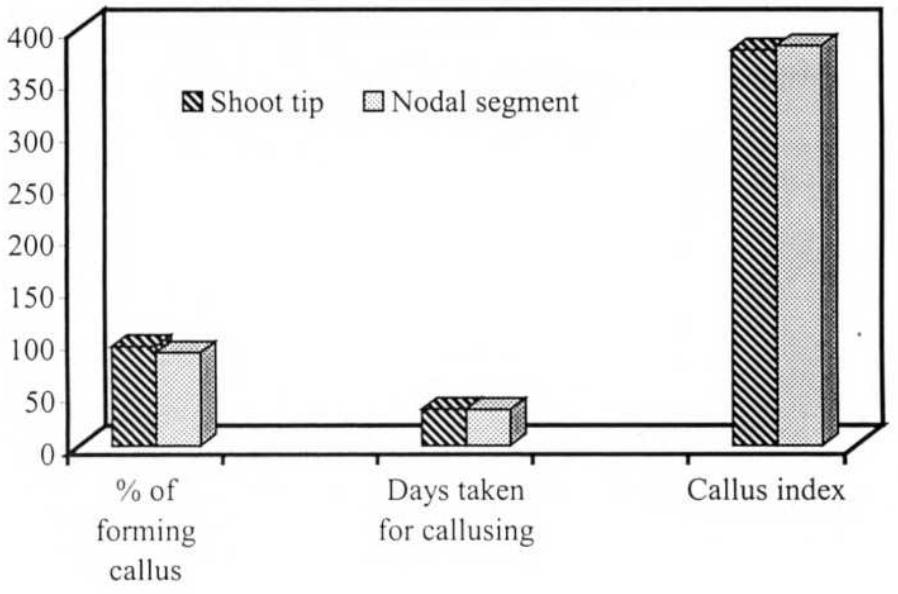


Table-12 Effect of BAP and GA_3 on weight of callus from shoot tip and nodal segment

Treatment (Concentration of BAP + GA_3) ($mg\ l^{-1}$)	Shoot tip (g/tube)	Nodal segment (g/tube)
T_0 (0.0 + 0.0)	--	--
T_1 (1.0 + 0.5)	0.69	0.42
T_2 (2.0 + 0.5)	1.48	1.04
T_3 (3.0 + 0.5)	1.03	0.85
T_4 (4.0 + 0.5)	0.68	0.51
T_5 (5.0 + 0.5)	0.62	0.41
Mean	**0.903**	**0.649**
SEd	0.138	0.044
CD (0.05)	0.309**	0.100**

using nodal segment as explant. The least weight of callus (0.41 g/tube) was obtained from nodal segment at BAP ($5.0mgl^{-1}$) and GA_3 ($0.5mgl^{-1}$). Increasing the concentration of BAP beyond $2.0mgl^{-1}$ reduced the callus weight (Table 12).

4.4.2.10. Combined effect of BAP and Kinetin on callus weight

The combination of two cytokinins were also tried for callus weight. The combination of BAP ($1.0\text{-}5.0mgl^{-1}$) and Kinetin ($1.0\text{-}5.0mgl^{-1}$) recorded good response for callus weight in case of shoot tip and nodal segments as explants. The highest callus weight 3.19 g/tube and 3.28 g/tube were recorded in shoot tip and nodal segment respectively cultured in MS medium supplemented with $3.0mgl^{-1}$ BAP and $3.0mgl^{-1}$ Kinetin. The least callus weight of 0.33 g/tube and 0.32 g/tube were observed in shoot tip and nodal segment respectively with $5.0mgl^{-1}$ BAP and $5.0mgl^{-1}$ Kinetin (Table 13, Fig.6).

4.4.2.11. Effect of continuous subculture on callus proliferation

White calli obtained from stem bit and leaf bit were subcultured four time in MS medium containing $1.0mgl^{-1}$ BAP + $50.0mgl^{-1}$ Glycine.

Green calli obtained from shoot tip and nodal segment were also sub-cultured four time in MS medium supplemented with $2.0mgl^{-1}$ BAP + $50.0mgl^{-1}$ Glycine.

It was found that there was considerable callus proliferation in the first and second subculture 3.61g and 3.73g were obtained from stem bit and shoot tip respectively. Beyond the second sub-culture, there was a decline in the proliferation rate (Table 14 & 15).

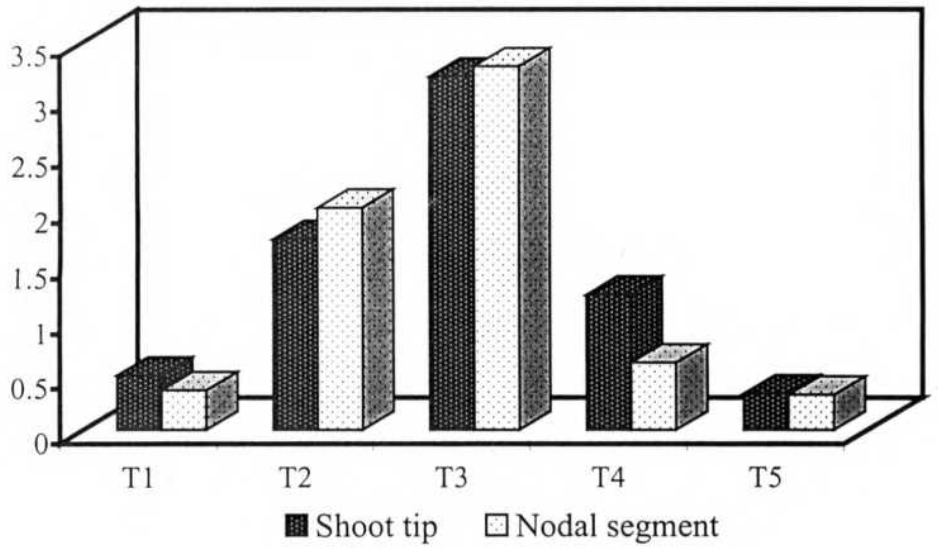
4.5.Direct organogenesis

When MS basal medium devoid of growth hormones were used, 50 per cent of the cultures survived, however, there was not much response to plant regeneration. The days for

Table-13 Effect of BAP and kinetin on weight of callus from shoot tip and nodal segment

Treatment (Concentration of BAP +kin) ($mg\ l^{-1}$)	Shoot tip (g/tube)	Nodal segment (g/tube)
T_0 (0.0 + 0.0)	--	--
T_1 (1.0 + 1.0)	0.50	0.36
T_2 (2.0 + 2.0)	1.73	2.01
T_3 (3.0 + 3.0)	3.19	3.28
T_4 (4.0 + 4.0)	1.23	0.61
T_5 (5.0 + 5.0)	0.33	0.32
Mean	**1.400**	**1.316**
SEd	0.215	0.115
CD (0.05)	0.479**	0.258**

Fig. 6. Effect of BAP and kinetin on weight of callus from shoot tip and nodal segment



T1-1.0 mg/l BAP + 1.0 mg/l kinetin

T2-2.0 mg/l BAP + 2.0 mg/l kinetin

T3-3.0 mg/l BAP + 3.0 mg/l kinetin

T4-4.0 mg/l BAP + 4.0 mg/l kinetin

T5-5.0 mg/l BAP + 5.0 mg/l kinetin

Table-14 Effect of subculture on callus proliferation from stem bit and leaf bit explants

S.No	Treatment ($mg\ l^{-1}$)	Number of subculture	Culture response (%)	Callus proliferation (15 days after subculture)	
				Weight of callus (g/tube)	Percentage increase
1.	1.0 BAP + Glycine 50	1	100.00	3.61	150.00
2.	1.0 BAP + Glycine 50	2	100.00	3.16	90.00
3.	1.0 BAP + Glycine 50	3	80.00	1.14	30.00
4.	1.0 BAP + Glycine 50	4	50.00	0.49	10.00

Table-15 Effect of subculture on callus proliferation from shoot tip and nodal segment

S.No	Treatment ($mg\ l^{-1}$)	Number of subculture	Culture response (%)	Callus proliferation (15 days after subculture)	
				Weight of callus (g/tube)	Percentage increase
1.	2.0 BAP + Glycine 50	1	100.00	3.73	150.00
2.	2.0 BAP + Glycine 50	2	100.00	3.66	95.00
3.	2.0 BAP + Glycine 50	3	70.00	2.07	40.00
4.	2.0 BAP + Glycine 50	4	30.00	1.00	20.00

regeneration of shoot was 14.99 days whereas when nodal segments used as explants about 29.55 days and no bud break was observed (Table 16 & 17).

4.5.1. Effect of various growth regulators on multiple shoot induction from different explants

The additions of growth regulators to the medium have positive effect on the shoot regeneration from nodal segments and shoot tip explants. Normally to induce shoot regeneration, higher concentration of cytokinin with lower concentration of auxin were employed to the MS basal medium.

Various growth regulators were tried and the results observed are presented here under.

4.5.1.1. Combined effect of BAP and GA3 on multiple shoot induction from shoot tip

The combination of BAP (1.0-5.0mgl^{-1}) and GA_3 (0.5mgl^{-1}) gave better response in shoot tip explants. In this combination multiple shoot regeneration was observed in shoot tip explants

BAP 2.0mgl^{-1} with GA_3 0.5mgl^{-1} gave survival percentage upto 83.91, which was significantly high, however, the least response was obtained (57.10%) at BAP 5.0mgl^{-1} + GA_3 0.5mgl^{-1} in shoot tip explant. The days taken for shooting in 14.99 days with 2.0mgl^{-1} BAP + GA_3 0.5mgl^{-1}. The shoot tip showed delayed (30.37 days) shooting with 4.0mgl^{-1} BAP + GA_3 0.5mgl^{-1}. About 5.15 shoots were formed with 2.0mgl^{-1} BAP + 0.5mgl^{-1} GA_3 in the case of shoot tip explant. However, a reduced number of shoots (3.38) was noticed at 3.0mgl^{-1} BAP and GA_3 0.5mgl^{-1} (Table 16, Fig.7).

4.5.1.2. Combined effect of BAP and GA_3 on multiple shoot induction from nodal segment

The combination of BAP (1.0-5.0mgl^{-1}) and GA_3 (0.5mgl^{-1}) were tried. Nodal segment showed high survival percentage (82.04) with BAP (2.0mgl^{-1}) and GA_3 (0.5mgl^{-1}). The least

Table-16 Effect of BAP and GA_3 on multiple shoot induction from shoot tip

Treatment (Concentration of BAP + GA_3) (mgl^{-1})	Survival percentage	Days taken for shooting	Number of shoots per explant	Length of shoot (cm)
T_0 (0.0 + 0.0)	--	--	--	--
T_1 (1.0 + 0.5)	77.57(61.74)	21.99	4.55	1.34
T_2 (2.0 + 0.5)	83.91(66.36)	14.49	5.15	1.83
T_3 (3.0 + 0.5)	74.84(59.89)	28.63	3.38	1.26
T_4 (4.0 + 0.5)	64.66(53.52)	30.37	3.75	1.61
T_5 (5.0 + 0.5)	57.10(49.08)	30.08	4.77	1.31
Mean	**71.619(58.123)**	**25.071**	**4.323**	**1.475**
SEd	0.735(0.463)	0.800	0.312	0.264
CD(0.05)	1.638(1.032)**	1.782**	0.694**	0.376**

Values in parenthesis are arcsine-transformed values

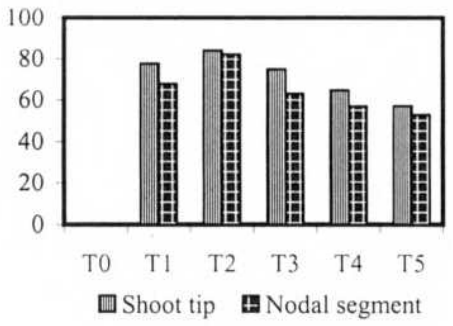
Table-17 Effect of BAP and GA_3 on multiple shoot induction from nodal segment

Treatment (Concentration of BAP + GA_3) (mgl^{-1})	Survival percentage	Days taken for shooting	Number of shoots per explant	Length of shoot (cm)
T_0 (0.0 + 0.0)	--	--	--	--
T_1 (1.0 + 0.5)	67.81(55.44)	24.29	4.66	1.41
T_2 (2.0 + 0.5)	82.04(64.94)	15.35	5.08	1.72
T_3 (3.0 + 0.5)	63.01(52.54)	28.64	3.98	1.23
T_4 (4.0 + 0.5)	56.90(48.97)	29.66	3.09	1.23
T_5 (5.0 + 0.5)	52.83(46.62)	28.99	3.60	1.07
Mean	**64.525(53.706)**	**25.391**	**4.086**	**1.335**
SEd	1.132(0.679)	0.310	0.318	0.089
CD(0.05)	2.522(1.514)**	0.690**	0.709**	0.200

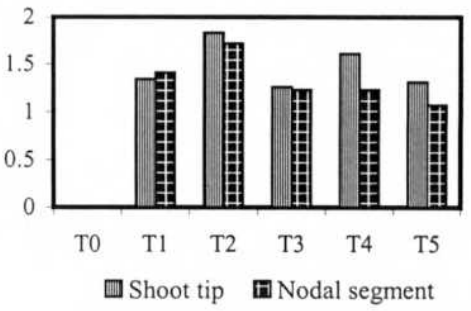
Values in parenthesis are arcsine-transformed values

Fig.7. Effect of BAP and GA_3 on multiple shoot induction from shoot tip and nodal segment

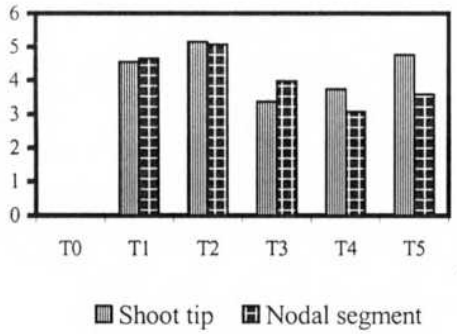
Survival percentage



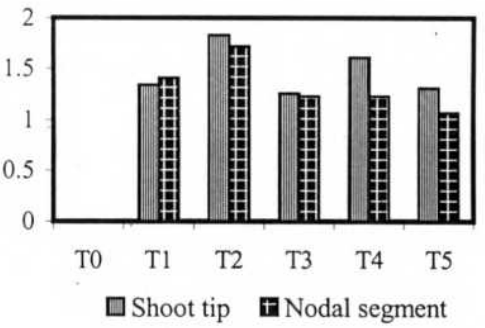
Days taken for shooting



Number of shoots per explant



Length of shoots (cm)



T0 – MS basal

T1 – 1.0 mg/l BAP + 0.5 mg/l GA_3

T2 - 2.0 mg/l BAP + 0.5 mg/l GA_3

T3 - 3.0 mg/l BAP + 0.5 mg/l GA_3

T4 - 4.0 mg/l BAP + 0.5 mg/l GA_3

T5 - 5.0 mg/l BAP + 0.5 mg/l GA_3

survival percentage (52.83) was obtained at BAP 5.0mgl^{-1} + GA_3 0.5mgl^{-1}. A gradual decline with increased concentration of BAP beyond 2.0mgl^{-1} was noticed.

The days taken for shooting in 15.35 days as in the case of nodal segments with 2.0mgl^{-1} BAP + GA_3 0.5mgl^{-1}. The nodal segments showed delayed (29.66 days) shooting with 4.0mgl^{-1} BAP + GA_3 0.5mgl^{-1}. The highest number of shoots (5.08) was observed with 2.0 mgl^{-1} BAP + 0.5 mgl^{-1} GA_3. The least number of shoots (3.09) was observed at 4.0mgl^{-1} BAP + GA_3 0.5mgl^{-1}. The increased shoot length (1.72 cm) was observed in nodal segment cultured in MS medium supplemented with 2.0mgl^{-1} BAP + 0.5mgl^{-1} GA_3.The least shoot length (1.07cm) was noticed at 5.0mgl^{-1} BAP + GA_3 0.5mgl^{-1} (Table 17, Fig.7).

4.5.1.3. Combined effect of BAP and Kinetin on multiple shoot induction from shoot tip

The combination of BAP (1.0-5.0mgl^{-1}) and kinetin (1.0-5.0mgl^{-1}) were tried for multiple shoot induction as in the case of shoot tip explants.The combination of BAP (3.0mgl^{-1}) and Kinetin (3.0mgl^{-1}) resulted in 95.00 per cent survival in shoot tip explants. The least survival percentage of shoot tips showed 23.33 with BAP (1.0mgl^{-1}) and Kinetin (1.0mgl^{-1}). The duration for multiple shoot induction was shorter (30.02 days) in shoot tip explants cultured in the medium containing BAP (3.0mgl^{-1}) and Kinetin (3.0mgl^{-1}). The longer duration (39.00 days) was observed at BAP (1.0mgl^{-1}) and Kinetin (1.0mgl^{-1}) The combination of BAP (3.0mgl^{-1}) and Kinetin (3.0mgl^{-1}) resulted in 6.05 shoots in case of shoot tip explants. The lowest number of shoots (2.77) was observed at BAP (1.0mgl^{-1}) and Kinetin (1.0mgl^{-1}) Shoot tips showed highest shoot length (1.73 cm) with BAP (3.0mgl^{-1}) and Kinetin (3.0mgl^{-1}) (Table 18, Fig.8).

4.5.1.4.Combined effect of BAP and Kinetin on multiple shoot induction from nodal segment

The combination of BAP (3.0mgl^{-1}) and Kinetin (3.0mgl^{-1}) resulted in 88.88 per cent survival in nodal segments. The least survival percentage of nodal segments showed 33.32 with BAP (1.0mgl^{-1})

Table-18 Effect of BAP and Kinetin on multiple shoot induction from shoot tip

Treatment (Concentration of BAP + Kin) (mgl^{-1})	Survival percentage	Days taken for shooting	Number of shoots per explant	Length of shoot (cm)
T_0 (0.0 + 0.0)	--	--	--	--
T_1 (1.0 + 1.0)	23.33(28.64)	39.00	2.77	1.15
T_2 (2.0 + 2.0)	75.00(60.07)	31.50	4.38	1.52
T_3 (3.0 + 3.0)	95.00(79.12)	30.02	6.05	1.73
T_4 (4.0 + 4.0)	70.55(57.15)	33.55	5.22	1.40
T_5 (5.0 + 5.0)	55.00(47.88)	37.88	3.83	1.26
Mean	**63.771(54.579)**	**34.383**	**4.452**	**1.414**
SEd	4.767(4.282)	0.818	0.520	0.186
CD(0.05)	10.621(9.542)**	1.822**	1.158**	0.414 NS

Values in parenthesis are arcsine-transformed values

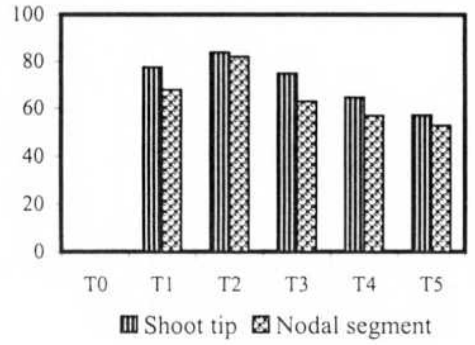
Table-19 Effect of BAP and Kinetin on multiple shoot induction from nodal segment

Treatment (Concentration of BAP + Kin) (mgl^{-1})	Survival percentage	Days taken for shooting	Number of shoots per explant	Length of shoot (cm)
T_0 (0.0 + 0.0)	--	--	--	--
T_1 (1.0 + 1.0)	33.32(35.21)	36.88	1.49	1.06
T_2 (2.0 + 2.0)	65.59(54.15)	31.80	2.94	1.28
T_3 (3.0 + 3.0)	88.88(70.93)	29.55	5.94	1.56
T_4 (4.0 + 4.0)	55.55(48.20)	33.11	5.05	1.33
T_5 (5.0 + 5.0)	38.59(38.26)	34.44	2.38	1.18
Mean	**56.389(49.345)**	**33.159**	**3.563**	**1.286**
SEd	5.942(3.856)	0.866	0.361	0.078
CD(0.05)	13.240(8.598)**	1.929**	0.804**	0.175**

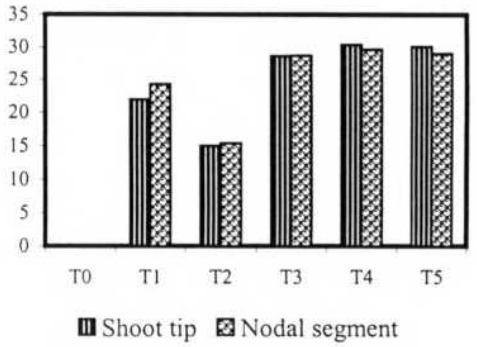
Values in parenthesis are arcsine-transformed values

Fig.8.Effect of BAP and kinetin on multiple shoot induction from shoot tip and nodal segment

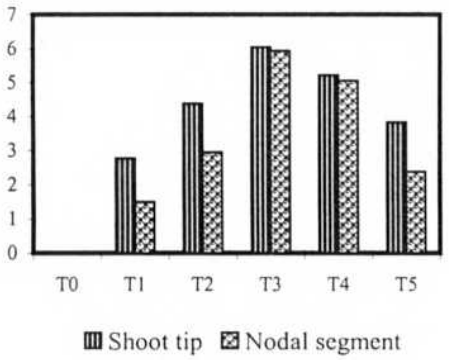
Survival percentage



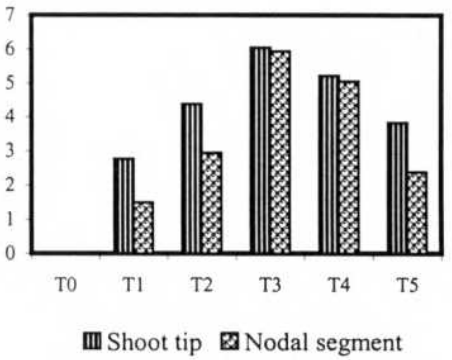
Days taken for shooting



Number of shoots per explant



Length of shoots (cm)



T0 – MS basal

T1 – 1.0 mg/l BAP + 1.0 mg/l kinetin

T2 - 2.0 mg/l BAP + 2.0 mg/l kinetin

T3 - 3.0 mg/l BAP + 3.0 mg/l kinetin

T4 - 4.0 mg/l BAP + 4.0 mg/l kinetin

T5 - 5.0 mg/l BAP + 5.0 mg/l kinetin

and Kinetin (1.0mgl^{-1}). The days taken for shooting in 29.55 days from nodal segments at BAP (3.0mgl^{-1}) and Kinetin (3.0mgl^{-1}). The duration taken for multiple shoot regeneration was longer (36.88 days) at BAP (1.0mgl^{-1}) and Kinetin (1.0mgl^{-1}). Combination of BAP (3.0mgl^{-1}) and Kinetin (3.0mgl^{-1}) resulted in 5.94 shoots in case of nodal segment explants. The lowest number of shoots (1.49) was observed at BAP (1.0mgl^{-1}) and Kinetin (1.0mgl^{-1}). Nodal segment showed highest shoot length (1.56cm) with BAP (3.0mgl^{-1}) and Kinetin (3.0mgl^{-1}). The lowest shoot length (1.06cm) was observed at BAP (1.0mgl^{-1}) and Kinetin (1.0mgl^{-1}) (Table 19, Fig 8).

Among various treatments, using various growth regulators tried, increasing the concentration of BAP and Kinetin, the height was decreased and sometimes drying up of shoots was observed. Micro shoots obtained from shoot tip and nodal segments did not elongate in response to addition of GA_3.

4.6. Culture response to explant position

Various explants were inoculated with both positive and negative polarity, in order to study the influence of explant position.

In case of shoot tip the positive polarity gave significantly more response (95.00%) that too at BAP (3.0) and Kin (3.0). The nodal segments in positive polarity with BAP (3.0) and Kin (3.0) combination gave 88.88% response. The stem bit and leaf bit in positive polarity with 2,4-D (4.0) and NAA (0.4) gave 86.45% and 64.57% respectively. Above the explants were futile with negative polarity (Table 20).

4.7. Rooting

Half strength MS media with various concentrations of IAA and IBA was involved for rooting. IBA (0.25 –1.25mgl^{-1}) with IAA (0.25-1.25mgl^{-1}) were tried. Among the treatments, a lower concentration of IBA (0.50mgl^{-1}) and IAA (0.50mgl^{-1}) showed higher rooting percentage

Table-20 Culture response to explant position

Treatments	Culture Response (%)	
	Positive polarity	Negative polarity
Shoot tip		
MS + BAP 3.0mg l^{-1} + Kin 3.0mg l^{-1}	95.00	-
MS + BAP 2.0mg l^{-1} + GA_3 0.5mg l^{-1}	73.87	-
Nodal segment		
MS + BAP 3.0mg l^{-1} + Kin 3.0mg l^{-1}	88.88	-
MS + BAP 2.0mg l^{-1} + GA_3 0.5mg l^{-1}	68.25	-
Stem bit		
MS + 2,4-D 4.0mg l^{-1}	62.94	-
MS + 2,4-D 4.0mg l^{-1} + NAA 0.4mg l^{-1}	86.45	-
Leaf bit		
MS + 2,4-D 4.0mg l^{-1}	58.63	-
MS + 2,4-D 4.0mg l^{-1} + NAA 0.4mg l^{-1}	64.57	-

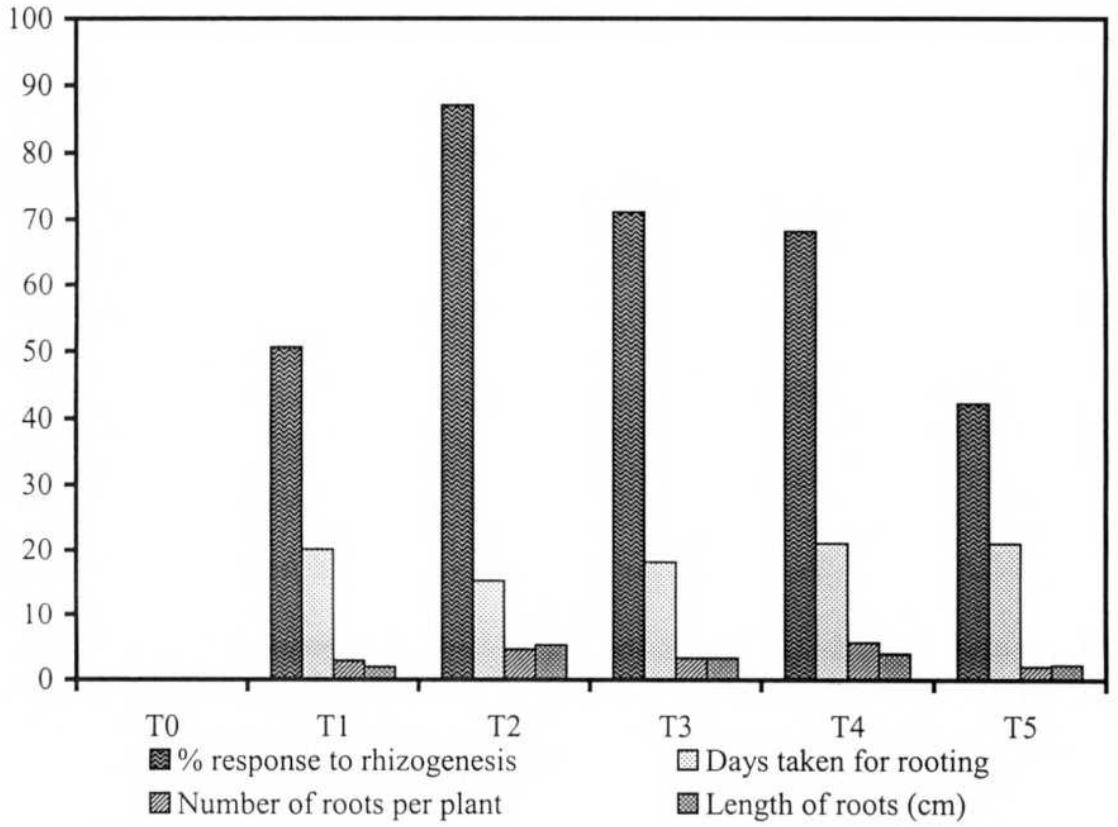
Statistically not analysed

Table-21 Effect of IAA and IBA on rhizogenesis

Treatment (Concentration of IAA + IBA) (mgl^{-1})	Percentage response to rhizogenesis	Days taken for rooting	Number of roots per plant	Length of root (cm)
T_0 (0.00 + 0.00)	--	--	--	--
T_1 (0.25 + 0.25)	50.63(45.36)	20.21	2.88	1.94
T_2 (0.50 + 0.50)	87.09(68.97)	15.31	4.69	5.34
T_3 (0.75 + 0.75)	71.18(57.53)	18.30	3.34	3.34
T_4 (1.00 + 1.00)	68.23(55.69)	21.20	5.77	4.04
T_5 (1.25 + 1.25)	42.30(40.57)	21.21	2.03	2.25
Mean	**63.890(53.629)**	**19.250**	**3.747**	**3.383**
SEd	0.896(0.656)	0.565	0.337	0.176
CD (0.05)	1.997(1.463)**	1.258**	0.751**	0.393**

Values in parenthesis are arcsine-transformed values

Fig.9.Effect of IAA and IBA on rhizogenesis



T0 – Half MS medium

T1 – Half MS + 0.25 mg/l IAA and 0.25 mg/l IBA

T2 - Half MS + 0.50 mg/l IAA and 0.50 mg/l IBA

T3 - Half MS + 0.75 mg/l IAA and 0.75 mg/l IBA

T4 - Half MS + 1.00 mg/l IAA and 1.00 mg/l IBA

T5 - Half MS + 1.25 mg/l IAA and 1.25 mg/l IBA

(87.09) followed by IAA (0.75mgl^{-1}) and IBA (0.75mgl^{-1}). The lowest rooting percentage was observed at IAA (1.25mgl^{-1}) and IBA (1.25mgl^{-1}). Earliest (15.31 days) rooting was observed at IAA (0.50mgl^{-1}) and IBA (0.50mgl^{-1}). The delayed (21.21 days) rooting was obtained at IAA (1.25mgl^{-1}) and IBA (1.25mgl^{-1}). The highest number of roots per explant recorded was 5.77 at IBA (1.00mgl^{-1}) and IAA (1.00mgl^{-1}). The lowest number of roots per explant was observed at IAA (1.25mgl^{-1}) and IBA (1.25mgl^{-1}). Combination of 0.5mgl^{-1} IAA and 0.5mgl^{-1} IBA showed the average root length (5.34cm) whereas lowest root length (1.94cm) was observed at IAA (0.25mgl^{-1}) and IBA (0.25mgl^{-1}) (Table 21, Fig. 9).

4.8. Hardening

When the plantlets attained a considerable size, they were transferred to hardening chamber. Two types of media *viz.*,

(1) Sand, soil and leaf mould (1:1:1)

(2) Sand and vermiculite (1:1) were tried.

Considerable extent of *in vivo* establishment was observed with sand, soil and leaf mould (16.66%) and sand and vermiculite (80.00%). But the growth was luxurious in vermiculite medium.to this 1/4th of MS medium was supplemented thrice a week and the plants were covered with polythene bags to maintain high humidity.Within 25 days, it was better established and transferred to pot with soil, sand, farmyard manure from where it can be taken for field planting (Table 22).

4.9. Biochemical studies

4.9.1. Estimation of phyllanthin and hypophyllanthin

An experiment was carried out to estimate the phyllanthin and hypophyllanthin from different types of cultures.

Table-22 Establishment of *Phyllanthus amarus* plantlets *in vivo*

Treatments	Number of plantlets transferred	Survival percentage		
		After 1 week	After 2 weeks	After 3 weeks
T_1	30	93.33	83.00	80.00
T_2	30	50.00	33.33	16.66

T_1 – Plantlets transferred to the medium containing sand and vermiculite (1:1)

T_2 – Plantlets transferred to the medium containing sand, soil and leaf mould (1:1:1)

Table-23 Estimation of phyllanthin and hypophyllanthin from different types of cultures

S.No.	Nature of culture & source of culture	Phyllanthin (%)	Hypophyllanthin (%)
1	Multiple shoot clumps with basal callus (Shoot tip)	0.345	0.198
2	Multiple shoot clumps with basal callus (Nodal segment)	0.323	0.189
3	Micro shoots without roots (Nodal segment)	0.492	0.285
4	Micro shoots with roots (Nodal segment)	0.521	0.296
5	Green callus (Shoot tip)	0.316	0.194
6	Green callus (Nodal segment)	0.331	0.209
7	White callus (Stem bit)	0.328	0.214
8	White callus (Leaf bit)	0.314	0.161

Among the various cultures, *in vitro* grown plants were recorded highest phyllanthin (0.521%) and hypophyllanthin (0.296%) cultured in ½ MS medium containing IBA (0.5mgl^{-1}) and IAA (0.5mgl^{-1}), followed by plants grown *in vitro* without roots. Lowest content of phyllanthin (0.314%) and hypophyllanthin (0.161%) were recorded in leaf bit derived white callus cultured in MS medium supplemented with 2,4-D (4.0mgl^{-1}) and NAA (0.4mgl^{-1}) (Table 23).

DISCUSSION

CHAPTER V

DISCUSSION

Phyllanthus amarus is one of the most important medicinal plants and is used against the treatment of jaundice. Conventionally it is being propagated through seeds, the germination percentage is only 50 per cent. Further the viability of seeds is also very low. The growing demand for commercial cultivation of this crop necessitates an alternative faster rate of multiplication. In recent times, tissue culture techniques are being widely used to produce uniform, quality and disease free plants at a faster rate within a limited space (Murashige, 1974, 1977; Thomas and Wernicke, 1978).

However, *in vitro* culture is a highly complex phenomenon and the success depends on a number of factors. The factors which significantly influence the *in vitro* cultures were documented by Murashige (1978). The nature of plant, genotype, culture media, stage and age of explant, method of inoculation, size of explant and hormonal influence are the most important areas of interaction which determines the success of *in vitro* culture. Thus the present study was formulated to optimize these elements of *in vitro* culture for *P. amarus*.

Surface sterilants and survival of explants

In the present study, when mercuric chloride was used as surface sterilant, the degree of contamination was found to reduce with increased concentration, with the optimal concentration being 1.0 per cent. The contamination level seemed to have been influenced by the incubation time also. Lesser the time of incubation greater was the contamination. However, increasing the period of exposure of the explants to sterilants caused an increase in the mortality of explants, conforming to the observation of Sommer and Caldas (1981); Karuppaiah and Rajasekaran (1993); Chandrasekaran and Narayanaswamy (1993). Concentration of mercuric cholride and

duration of exposure of explants at levels above the optimum value (1.0%) produced contaminant free cultures, however the percentage of death of explants was higher, which indicates the deleterious effect of sterilants when exposed to longer time. The softer tissues were more conducive in absorption of the $HgCl_2$, and when the limit exceeds it becomes toxic to cells.

Influence of season on survival percentage of explants

The season during which the explants were collected from the mother plant also have a profound influence on the survival percentage of explants *in vitro*. January – February proved to be the best period of explant collection which was on par with July – August and the response was poor during September – October. This might be due to the mild and humid environment during monsoon months, which encouraged the survival and growth of many microorganisms. This was in corroboration with the findings of Malik (1989), who expressed that microbes show varied growth response to different incubation temperatures at which organisms could grow. The months, July-August were the most appropriate season, which recorded the highest per cent survival of 70.25% with least per cent contamination of 30.25% in *Gloriosa superba* (Munavarjan, 2000).

The varying rate of growth of different parts of *P.amarus* during different seasons might be due to the hormonal changes, which influenced by the season. In July–August, the concentration of hormones needed for the growth might be more and the demand was met, ensuring accelerated growth than the other unfavourable seasons. This was in tune with earlier findings of Wodziki (1978), proved that seasonal variation of auxin in stem cambium of pines. John (1996) reported that in *Holostemma annulare*, the explants collected in the months of January to April gave lower contamination and higher survival percentages with the contamination rate lowest in the months of January and February.

Standardization of explants

The various explants like shoot tip, nodal segment, leaf bit, stem bit, root bit, fruit and seed were used. Among the various explants involved, shoot tip, nodal segment and stem bit performed well for induction of green and white/brown callus respectively, irrespective of growth regulators used, while leaf bits showed poor response. The reason for the success of shoot tip culture might be due to the high meristematic activity and less phenolic contents in the meristematic tissues (Surya Narmada, 2000).

Types of explant and callusability

Different types of explants of the same species have their own effect on callusability even though callusability of explants mainly depends on the absolute concentrations of phytohormones (Gresshoff and Doy, 1972; Padmanaban *et al.*, 1974). Such a variation in the explant performance has been obtained in the present study. Shoot tip, nodal segment and stem bit have recorded the best response. Haicour (1974) obtained callus cultures of leaf bit and stem bit of *Phyllanthus urinaria*. Unander (1991) found that the stem bit was ideal explants for callus induction in *P.amarus*.

However, their response varied significantly with variations in the growth regulators employed for the culture.Stem bit and leaf bit produced white callus with development of white hairy roots when 2,4-D and NAA were supplemented to the medium.This could be due to the increased level of endogenous auxin in stem and leaf bit. This type callus developed roots only and not the shoots. However, when the cytokinin was added, the leaf bit and stem bit did not respond while shoot tip and nodal segments produced green globular callus.

The variation in the performance of the explants could possibly be due to the variation in ratio of endogenous phytohormones present in the explant system, age of the explant, position

from where the explant was taken, types of cells present and their physiological and developmental stage (Baskarrajan, 1994).

Age of explant and callusability

In the present study, it was observed that the shoot tips (younger tissue) responded very well to callusing. The duration of callus induction also differed due to age of explant used, being shorter with younger tissue than that of older ones. Such a response would occur under two situation *viz.*, due to enhanced cell acitivity with increase DNA synthesis of protein incorporation in the younger ones or due to the presence of inhibitor compounds such as abscissic acid which is profound in the older tissue (Wright and Hiron, 1969) thus inhibiting or delaying callus induction.

Callus induction and proliferation

Callus is an undifferentiated mass of proliferating cells. Using tissue culture tecniques, callus can be induced with numerous plant organs and tissues.

Auxin and cytokinins, could be used to promote differential growth of embryogenic and non-embryogenic calli and accelerated callus growth. The duration of cell cycles is influenced by hormone concentration (Baylies,1977) and vary with cell types (King,1980).These hormones influence cell growth and division through different phases of cell cycles (King,1980).

Auxin alone can lead to cell enlargement but a combination of kinetin and auxin are essential for cell division. This has been shown by the studies with synthetic kinetin (Patau *et al.*, 1977). Callus growth hardly occurred in the absence of growth regulators. This is in agreement with the findings of Neelam Sharma *et al.* (1991) in *Coleus forkskohlii*, who reported that there was no callus growth in the basal medium without auxins or cytokinins. Surya Narmada (2000) found the same trend in *Gymnema sylvestre.*

In the present study, explants did not respond to callusing in the absence of growth regulators. The different growth regulators had their own effects on callus induction. When auxin alone (2,4-D or 2,4-D + NAA at higher concentration) was applied, the explant exhibited brown or white friable callus, but in contrast when cytokinin (BAP at lower concentration, $2.0mgl^{-1}$) was applied, the explants exhibited globular green callus production. Shoot tips and nodal segments recorded high callusability at a concentration of $3.0mgl^{-1}$ BAP + $3.0mgl^{-1}$ Kinetin. Green callus was observed in cytokinin rich medium. This was supported by the findings of Arockiasamy *et al.* (1999) in *Datura metel* where attempts of BAP ($0.5–3.0mgl^{-1}$) usage resulted in formation of green and compact callus from internodal explants.This might be due to increase in the chlorophyll content in cells when cytokinin was supplemented to the basal medium.

An increase in the concentration of all growth regulators except 2,4-D had a deleterious effect on culture induction.This inhibition could be attributed to the negative effect of supra-optimal levels of growth regulators on the process of callus induction. The callusability in treatment with 2,4-D might be due to the stimulatory effect of 2,4-D on other in enhancing cell division (George and Sherrington, 1984).

Callus regeneration

The possibility of callus regeneration that could be brought through the manipulation of hormone balance is a known factor (Skoog and Miller, 1957; Rossini,1969). The suitability of the hormones and their concentration required vary with plant types. For most of the plant species in *in vitro* system, certain ratio of auxin and cytokinin is widely used. The level of auxin and cytokinin alone or in combination decides the efficiency of callusability and organogenesis (Kohlenbach, 1977) and a high level of auxin in relation to cytokinin generally favoured root

formation, whereas increase in the level of cytokinin tilted in favour of shoot regeneration (Skoog, 1971; Duramd *et al.*, 1973).

This fact was reported to be well established in several species like pelargonium (Deberg and Maene, 1978); *Gloriosa peruviana* (Somani *et al.*, 1989), *Thevetia peruviana* (Kumar, 1992) and vettiver (Keshavachandran and Abdul khader, 1994).

In the present study, the callus clumps developed in an auxin rich medium were transferred to a cytokinin rich medium. Cytokinin (BAP) along with vitamin (Glycine) and auxin (IAA/IBA) were supplemented to the medium. Shoot tip and nodal segment derived callus showed better regeneration capacity of shoots at the effective concentration of cytokinin (BAP at 2.0mgl^{-1}) along with Glycine (50.0mgl^{-1}). Shoot tip and nodal segment derived callus showed better regeneration capacity of roots at the effective concentration of auxin (IAA at 0.5mgl^{-1}).

However, none of the treatments could induce shoots from stem bit and leaf bit derived calli.In the present study, the failure of calli to regenerate in the medium might be due to four possible reasons as pointed out by Halperin (1986). The first reason could be that the cells involved in the process might have been lacking totipotency. Secondly the cells might not have been able to react to the hormonal signals which initiate differentiation. However, this might be epigenetic and reversible under certain other condition. Thirdly, it might have been due to physiological factors ,i.e., the cells might be having genetic potential to show differentiation but they might not be expressive under such particular *in vitro* system. However, the leaf bit and stem bit derived calli started to regenerate roots during cultivation on MS medium with auxins (2,4-D and NAA). Using different auxin concentrations in MS culture medium observed typical rhizogenesis of stem bit and leaf bit at concentrations of 4.0mgl^{-1} 2,4-D and 0.4mgl^{-1} NAA. The lowest concentrations of 2,4-D at 1.0mgl^{-1} and NAA at 0.1mgl^{-1} did not induced any root

formation. The highest concentrations of auxin induced firstly callus formation, later roots of unorganized growth were regenerated from calli. Cellarova *et al.* (1983) found the same trend in *Matricaria chamomilla* and *Achillea collina*. Profuse rooting occurred from the hypocotyl callus of *Phyllanthus fraternus* on medium supplemented with 10.0μM BAP + 10.0μM NAA (Rajasubramaniam and Pardha Saradhi, 1994).

Direct organogenesis

Although organogenesis through various explants induced callus system is popular in many cases (Murashige 1974,1977 and 1978). There is always a possibility for variation in the genetic components and for shift in ploidy levels, leading to spontaneous mutation resulting in variability in the regenerants. Direct organogenesis differ from the above, with a possibility of producing a true to type. In this present study,direct organogenesis has been succesfully obtained from various explants *viz*., shoot tip and nodal segments.

Multiple shoot induction from shoot tip or nodal segment had been suggested as a potential tool for the mass multiplication of *in vitro* (Minochae, 1987).Though this method is less rapid than somatic embryogenesis or organogenesis via callus phase, it is a much reliable method of propagation for maintaining genetic stability.

Generally, to induce shoot regeneration,higher concentration of cytokinin and lower concentration of auxin are added to the MS basal media. In general, BAP had been frequently reported to induce better shoot multiplication than other cytokinin (Ahmad and Darus, 1989).

In the present study, BAP, GA_3 and kinetin were employed for multiple shoot induction from shoot tip and nodal segment explants. Of these, BAP in combination with both GA_3 and kinetin was the most effective in inducing multiple shoots from nodal segment and shoot tip

explants, while BAP in combination with kinetin produced the best response to multiple shoot regeneration from shoot tip and nodal segment explants.

The significant contribution of BAP was evident among the three growth regulators in inducing multiple shoots. The effective and efficient role played by the BAP in the shoot proliferation of various medicinal crops were emphasised by Harikrishnan *et al.*(1999) in *Acorus calamus*, Arockiasamy *et al.*(1999) in *Datura metel*, Al-Wasel (1999) in *Atropa belladona*, Smitha chetia and Handique (2000) in *Plumbago indica*, Selvakumar and Balakumar (2000) in *Acalypha fruiticosa* and Muthuram *et al.*(2000) in *Scoparia dulcis*.

GA_3 is known to have stimulatory effect on stem elongation in different plants. The same effect was seen in the present study when GA_3 was supplemented to the MS basal media at a lower concentration where shoot tip derived microshoots responded well to elongation (an average length of 1.83 cm). This was supported by the findings of Sitakanta Pattnaik and Pradeep (1996) in *Ocimum sanctum*. However, above the optimal level a negative correlation was found to exhibit between the concentration of GA_3 and the response to shoot elongation.

When kinetin was added to the culture medium along with BAP, remarkable effect was seen in the induction of multiple shoot. Axillary bud break was seen in shoot tip and nodal segments which inturn gave rise to multiple shoot induction. This was in agreement with the reports suggested by Vincent *et al.*(1992) who found that use of kinetin along with BAP in the culture medium enhanced multiple shoot induction in *Kaempferia galanga*. Similar effects have already been documented in *Gymnema elegans*, where kinetin in the culture medium enhanced bud break in explants (Komalavalli and Rao, 1997). Shoot tip explant showed high survival percentage (98.33) with 2.33 shoots per explant with average shoot length of 3.10cm in 26.67 days at BAP ($3.0mgl^{-1}$) and Kinetin ($0.4mgl^{-1}$) in *Gymnema sylvestre* (Surya Narmada, 2000).

Continuous subculturing for callus proliferation

The present study shows that continuous sub-culture resulted in the reduction in the rate of callus growth. A gradual decrease in the rate of callus growth has been observed at each sub-culture. The shoot and root regeneration capacity of the shoot tip and nodal segment callus were also drastically reduced by increasing the number of subcultures. This was supported by the findings of Munavarjan (2000) in *Gloriosa superba*.

The loss of morphogenetic potential in continuous sub-cultures might be due to the low levels of endogenous hormones (Wochok and Wetherell, 1972). Such failure of callus to regenerate was reported by Chen and Galston (1967) in pelargonium pits cells. They reported that shoot production from callus may be initiated from organised centres may gradually be lost through repeated subculturing and rapid unorganised cell division. Hill (1967) and Reinert and Backs (1968) observed a gradual loss of morphogenetic potential in terms of carrot cultures.In contrast some tissue and cell cultures retain morphogenetic potential over extended periods of subculturing. Similarly, Earle (1974) reported that Chrysanthemum propagated by callus containing meristematic areas retained morphogenetic potential over a 3.5 year period.

Polarity of explants

The placement of the explant on the medium various with the plant species and has been found to influence the survival of the culture. Generally, the type of movement of auxin is basipetal and the movement is similar in different organs of the plant such as coleoptile, stem, hypocotyl and petiole of the leaf. The polarity of transport of auxin was achieved by preferential activity of transport sites at the basal end of the cell (Pandey and Sinha, 1999).

However, a small quantity of auxin can also move in an acropetal direction and the quantity transported in to negligible that it is not of much significance in controlling physiological functions

of plants. In the present investigation also, a similar result was obtained, with the possitively polarised explants producing callus which there was the no response in the negatively polarised explants. In *Withania somnifera*, the leaf disc was cultured with its abaxial surface touching the medium, only then the shoot buds were initiated (Abhyankar and Chinchanikar, 1996). Similar results have been reported in Chrysanthemum (Chin-Yi Lu *et al.*,1990).

Rooting

Normally rooting is induced *in vitro* by inoculating the culture in half strength MS medium supplemented with any auxin mainly IAA/IBA alone or in combination. A small amount of total nitrogen enhanced the process of rooting (George and Sherrington, 1984). In the present study, half strength MS medium was used for rooting.

Axillary and adventitious shoots developed in culture in the presence of cytokinins generally lacked roots. To obtain full plants the shoots were transferred to a rooting medium, which differed, from shoot multiplication medium especially in its hormonal composition.

High auxin level could be undesirable in the phase after root initiation. The root elongation phase is very sensitive to auxin concentration and will be inhibited by high concentration as reported by Thimmann (1977). In the present study, IBA along with IAA was tried in half strength MS basal medium. IBA and IAA at lower concentration ($0.5mgl^{-1}$) gave good response (75% rooting). Further, addition of IAA ($1.25mgl^{-1}$) and IBA ($1.25mgl^{-1}$) also gave response to rooting, however the percentage of rooting, was only 15 per cent and yellowing of leaves was also observed. This might be due to overdose of auxin.

Often, where shoot multiplication was induced on full strength MS medium, the salt concentration was reduced to half (Zimmerman and Hordham, 1985) or quarter (Skirvin and Chu, 1979) for rooting. Defossard *et al.* (1978) have highlighted the low nitrate requirement for

rooting of *in vitro* shoots. In the present study, half strength MS medium gave better results for rooting.

Establishment of *Phyllanthus amarus* plantlets *in vivo*

The rooted plantlets were hardened prior to transfer to soil by immersing their roots in distilled water for 10minutes. During the initial place after transfer to mist chamber, the survival percentage was high. Later on, however, the survival percentage was declined.

Lloyd *et al.* (1988) attributed that the poor survival might be due to the fact that the plantlets often develop a single long and unbranched root which breaks easily on transfer to soil or may even degenerate after transfer.

Estimation of phyllanthin and hypophyllanthin

In *P. amarus*, isolation of antiviral compounds (phyllanthin and hypophyllanthin) and elucidation of their action on the hepatitis virus have been achieved. Various factors, such as biological, chemical and physical factors, play a vital role in improving product synthesis had been illustrated, along with differentiation at the cellular and morphological level.

Plant growth regulators are effective inducers of secondary metabolism (Bohm, 1980). Similarly both quality and quantity of auxins initially present in the tissue or administered during the course of culture development have a marked effect on secondary metabolite production. In the present study, auxins were employed for production of phyllanthin and hypophyllanthin from *in vitro* grown plants. This was supported by the findings of Shiio and Ohta (1973) along with Takahashi and Yamada (1973). They reported that lower concentration of auxins viz., IAA, NAA and 2,4-D promote nicotine synthesis in tobacco cell cultures and higher concentrations of auxins inhibit nicotine synthesis in tobacco cell suspension cultures. Lowest amounts were investigated in white callus maintained in the medium containing 2,4-D ($4.0mgl^{-1}$) and NAA

($0.4mgl^{-1}$). A persual of literature revealed that 2,4-D was completely inhibited the anthraquinone synthesis from callus of *Morinda citrifolia* (Zenk *et al.*, 1975).

In the present study, the quantity of phyllanthin and hypophyllanthin was more in *in vitro* grown plants than in callus. Thus, it seems that in many cases morphological differentiation may be necessary to obtain higher yields of secondary metabolites. Dhar and Pal (1988) have demonstrated that pyrethrin was being synthesized more in *Chrysanthemum cinerariaefolium* with shoots than the roots and its content was even more lower in undifferentiated callus culture.

It is known that essential oils and flavour compounds in plant accumulate in morphologically specialised structures, such as glands in mint, oil ducts in celery, swollen leaf bases in onion, secretory ducts, latex components found in laticifers. In *C. cinerariaefolium*, pyrethrins are localized in the oil glands and ducts present on the achenes (Chandler, 1951) and on leaves (Zito *et al.*, 1983). These oil glands were the site of pyrethrin biosynthesis.

The establishment of protocols for the successful multiplication of *P.amarus* through different *in vitro* routes is useful for conservation as well as biotechnological improvement of this pharmacologically important plant species.

Further studies are needed for isolation and purification of other compounds apart from phyllanthin and hypophyllanthin which is present in different parts of *P.*amarus.

SUMMARY

CHAPTER VI

SUMMARY

Investigations were undertaken to standardize the micropropagation techniques in *Phyllanthus amarus* at the Tissue Culture Laboratory of the Horticulture College and Research Institute, Coimbatore. The salient findings of the study are summarized hereunder.

1. Amongst the various explants investigated, shoot tip, nodal segment, stem bit and leaf bit showed better culture response. But the other explants did not respond to the *in vitro* culturing of *P. amarus*.
2. Surface sterilant mercuric chloride was tried for sterilization of different explants at different concentration for different period of exposure. The contamination level was almost controlled when mercuric chloride used at among concentration of 1.0 % for 3 minutes.
3. Whereas in case of leaf bit, the contamination was reduced by treating the explants with 1.0 % $HgCl_2$ for 1 minute exposure time.
4. Explants taken during January-February gave good response for survival of all the explants followed by July-August.
5. Among the different explants used, stem bit and nodal segment responded well for induction of white callus (325.62) and green callus (383.95) respectively and shoot tip and nodal segments responded well for multiple shoot induction.
6. Murashige and Skoog' medium at full strength proves to best for callus induction and multiple shoot formation.
7. The best treatment for obtaining green callus (383.95) from nodal segment was MS + BAP ($3.0mgl^{-1}$) + Kin ($3.0mgl^{-1}$).

8. For induction of white callus, stem bit and leaf bit explant treated with MS + 2,4-D ($4.0mgl^{-1}$) + NAA ($0.4mgl^{-1}$) gave good response.
9. When the explants were subcultured in full strength MS media fortified with BAP $3.0mgl^{-1}$ + kin $3.0mgl^{-1}$, showed more callus weight (3.28 g/tube) in nodal segment explant.
10. The best treatment for obtaining multiple shoot from shoot tip (5.22 shoots/explant) and nodal segments (5.94 shoots/explant) was MS + BAP ($4.0mgl^{-1}$) + kin ($4.0mgl^{-1}$).
11. The survival percentage of cultures was recorded more in shoot tip explants (95.00%) than nodal segments (88.00%).
12. Addition of GA_3 to the medium, enhance the length of multiple shoot.
13. On subculturing the multiple shoots in MS medium supplemented with BAP ($2.0mgl^{-1}$) and GA_3 ($0.5mgl^{-1}$) resulted in 7.0 multiple shoots formation.
14. When multiple shoots regenerated from callus in MS + BAP ($2.0mgl^{-1}$) + Glycine ($50.0mgl^{-1}$).
15. Rooting (87.09%) of the shoots was best achieved in ½ MS + IBA ($0.5mgl^{-1}$) + IAA ($0.5mgl^{-1}$).
16. The positively polarised explants resulted in higher response.But the negatively polarised explants failed to show any response.
17. Rapid subculture of explants gave poor response than the explants under went 1 or 2 cycles of subculture.
18. The culture per cent was very low with higher frequency of subculture.
19. For the estimation of phyllanthin and hypophyllanthin, *in vitro* grown plants recorded higher amount of phyllanthin (0.521%) and hypophyllanthin (0.296%) which was cultured in ½ MS medium supplemented with IBA ($0.50mgl^{-1}$) + IAA ($0.50mgl^{-1}$).

REFERENCES

REFERENCES

Abhyankar,G.A. and G.S.Chinchanikar. 1996. Response of *Withania somnifera* Dunal leaf explants *in vitro*. **Phytomorophology, 46**(3): 249-252.

Adolfina.R.Koroch, Jubiani,H.R. and V.S.Trippi. 1997. Micropropagation and acclimatization of *Hedeome maltiflorum*. **Plant Cell Tiss. Org. Cult., 48**(3): 213-217.

Ahmad,H. and Daurus. 1989. Micropropagation of *Acacia mangium* from aseptically germinated seedlings. **Journal of Tropical Forest Science. 3**(3) : 204-208.

Al-Wasel,A.S. 1999. *In vitro* multiplication of *Atropa belladonna* L. using nodal segments. **Alexandria J. Agrl. Res., 44**(3): 263-274.

Aminuddin, Johri,J.K., Mohal Aris and V.R.Balasubrmanyam. 1993. Regeneration of *Piper betel* from callus tissue. **Curr. Sci., 65**(10): 793-96.

Anjanevulu,A.S.R., Rao,k.J., Row,L.R. and C.Subramanyam. 1973. Crystalline constituents of Euphorbiaceae. XII. Isolation and structural elucidation of three new lignans from the leaves of *Phyllanthus niruri* Linn. **Tetrahedon, 29**: 1291-1298.

Anu,K.I. 1993. Standardisation of *in vitro* propagation techniques in *Gymnema sylvetre* R.Br. M.Sc. (Hort) Thesis. Kerala Agricultural University, Vellanikkara.

Anu,K.I., Nazeem,P.A., Lisamma Joseph and N.K.Vijayakumar. 1994. Response of 'Gurmar' (*Gymnema sylvestre*) for *in vitro* propagation. **Sou.Ind. Hort.,42(6)**: 365-368.

Arditti,J. 1982. Orchid seed germination and seedling culture – a manual. In orchid biology. **Reviews and Perspectives-II** J. Arditti (Ed.). 244-370.

Arockiasamy,D.I.; Muthukumar,B.and S.J.Britto. 1999. In vitro plant regeneration from internodal segments of *Datura metel* L. **Adv. in Plant. Sci. 12**(1): 227-231.

Banerjee,N. and E.De Langhe. 1986. *In vitro* propagation of *Crocus sativus*. **Plant Cell Rep., 4**: 351-354.

Baskarrajan,G.1994. Studies on in vitro multiplication of Patchouli (*Pogoestemon patchouli*, Hook). M.Sc. (Hort). Thesis submitted to Annamalai University, India.

Basu,A., Sethi,U. and S.Gunamukherjee. 1988. Factors affecting *in vitro* clonal propagation. **J.Exp. Bot., 39**: 1735-1742.

Baylies, M.M. 1977. The effect of 2,4-D on growth and mitosis in suspension cultures. **Plant Sci. Lett., 8:** 99 – 103.

Behagel,H.A. 1971. In: Effects of sterilization on components in nutrient media. Van Bragt,J., Mossel,D.A.A., Pieric,R.L.M. and H.Veldstra (Eds.). Miscellaneous papers hand bou whoe school, Wageningen, The Netherlands: 117-120.

Berlin,J. 1984. Plant cell cultures-a future source of natural products.**Endeavour 8**:5-8.

Bhat,S.R.; Kackar,A. and K.P.S.Chandel.1992. Plant regeneration from callus cultures of *Piper longum* L. by organogenesis. **Plant Cell Rep., 11**:525-528.

Bhojwani,S.S. and M.K.Rajdan. 1983. In: Plant tissue culture. Theory and Practice. Elselvier Amsterdam, Oxford.

Biswajit Ghosh and Sumitra Sen. 1992. Stable regeneration in *Asparagus cooperi* Baker as controlled by different factors. **Plant Sci., 182**:119-124.

Bohm,H. 1980. The formation of secondary metabolism in plant tissue and cell cultures. **Intl. Rev. Cytol. Suppl. 11 B**: 183-208.

Bonga,J.M. 1982. Tissue culture techniques. In: Tissue culture in Forestry. Bonga,J.M. and D.J.Durzan (Eds.). Martinus Nijhoff, Dr.W.Junk Publishers, London. p.4-35.

Boxus,P.H. and J.M.Terzi. 1987. Control of accidental contamation during mass propagation. **Acta Hort., 225**: 189-191.

Cellarova, E., Repeakova, K., Repeak, M. and R. Honeariv. 1983. Morphogenesis in tissue culture of some medicinal plants. **Acta Hort., 132**: 249-254.

Chandler,S.E., 1951. Botanical aspects of pyrethrum I. **Pyrethrum** post, **2**: 1-19.

Chandrasekar,C.P.S. and Narayanaswami. 1993. *In vitro* studies on tomato with special reference to Fusarial wilt disease. M.Sc. (Ag.) Thesis, Faculty of Agriculture. Annamalai University, India.

*Charaka (1st century A.D.). Charaka Samhita Vol.I. Chap.23, Verses, 1-5, cited in Ancient Hindi medicine by Chandra Chakrabarthy, Pub. by Ramachandracharabarti, Calcutta (1923).

Chen, H.R. and A.W. Galston. 1967. Growth and development of *Pelargonium* pith cells *in vitro*. II initiation of organised development. **Physiol. Plant, 20**: 539.

Chin-Yi Lu, Greg Nugent and Terese Wardely. 1990. Efficient, direct plant regeneration from stem segments of chrysanthemum. **Plant Cell Rep., 8**: 733-736.

Chua,B.Y., Kunisaki,J.T. and Y.Sagawa. 1981. *In vitro* propagation of *Dracena margenata*. **Hort. Sci., 16**: 494.

Cousson,A., Toubart,P. and Tran Thank Vank. 1989. *In vitro* propagation of chrysanthemum. **Can. J. Bot., 67**: 650-654.

Custers,J.B.N. and J.H.W.Bergervoet. 1980. *In vitro* adventitious bud formation on seedling and embryo explants of *Cucumis sativus*. *Cucubit*. **Genetics Coop., 3**: 2-4.

Danby,S., Berger,f., Howitt,D.J., Wilson,A.R., Dawson,S. and Leifert. 1993. Fungal contaminants of Primula, Coffee, Musa and Iris tissue cultures. **Plant Growth Regulators.**

David,A. 1982. *In vitro* propagation of Gymnosperm. In: Tissue culture in Forestry. Bonga,J.H. and D.J.Durzan (Eds.), Martinus Nijhoff, Dr.W.Junk publishers, London. 72-101.

Debergh, P. and L. Maene. 1978. Rapid clonal propagation of pathogen-free Pelargonium plants starting from shoot tips and apical meristems. **Acta Hort., 78**: 449-454.

Defossard,R.A. 1976. Tissue culture for plant propagators, University of New England, Armidale.

Dhar,K. and Pal,A. 1988. Pyrtherin content in unorganised and organised callus cultures of *Chrysanthemum cinerariaefolium* Vis. **Fitoterapia, LXIV**: 336-340.

Dodds,J.H. and L.W.Roberts. 1982. Experiments in plant tissue culture.p.231.Cambridge University Press, Cambridge.

Duell-Pfaff, N. and E. Wellmann. 1982. Involvement of phytochrome and a blue light photoreceptor in UV-B induced flavonoid synthesis in Parsely (*Petroselinum hortense* Hoffm.) **Planta, 156**: 213-217.

Dunwell,J.M. 1985. Embryogenesis from pollen *in vitro*. In: Biotechnology in Plant Science. Raitlin,M., Day, Pand Holande (Eds.). Relevance to Agriculture in the Eighties, Academic press. 49-76.

Durand, J., Potryken, I. and G. Donn. 1973. Plants tissues de protoplasts de penteroia. 2 pflomzen physiol 69: 26-34. In: Plant tissue culture and its biotechnological applications (eds.) Berg, W., Reinfard, E., zenk, M.H. Springler verlag, Berlin.

Earanna, N. and C.K.Suresh. 2001. *Phyllanthus amarus*-A weed in need. **Kissan world,** (1): 58.

Earle, A.B., Varge, A. and G. Staritsky. 1974. Callus growth and rooting of cassava stem segments cultured *in vitro*. **Acta Bot. Neerl., 23**: 315-320.

Evans, D.A., Sharp,W.R. and C.E. Flinck. 1981. Growth and behaviour of cell cultures.Embryogenesis and organogenesis. In: Plant tissue culture, methods and application in Agriculture Thorpe,T.A. (Ed.).Academic press. New York, pp.45-114.

Gamborg,O.L., Murashige,T. and Thorpe,T.A. and I.K.Vasil. 1976. Plant tissue culture media. ***In vitro*, 12:** 473-478.

George,E.F. and P.D.Sherrington. 1984. Plant propagation by tissue culture. Exogetics Limited, England: 1-690.

Gresshoff,P.M. and C.N.Doy. 1972. Development and differentiation of haploid *Lycopersicon esculentum*. **Plant** (Berlin), **107** : 161-170.

Grout,B.W. and M.J.Aston. 1978. Transplanting cauliflower plants regenerated from meristem culture. Water loss and water transfer related to changes in leaf wax and to xylem regeneration. **Hort. Res., 17**: 1-7.

Guney,A.L. and P.S.Rao. 1980. *In vitro* propagation of hybrid tomato plants using hypocotyl and cotyledon explants. **Ann. Bot., 45**: 205-207.

Gupta,P.K., Mascarenhas,A.F. and V.Jagannathan. 1981. Tissue culture of forest trees – clonal propagation of mature trees of *Eucalyptus citriodera*. **Plant. Sci. Lett., 20**: 195-201.

*Haberlandt,G. 1902. Cultureversuche mit isolierten pflanzenllen. kl. sitzungsber. Math. Naturwiss. Akad. Wiss. Wien., 11: 6992.

*Haicour, R. 1974. Comparaison chez *Phyllanthus urinaria* L. de l'activite antibact erienne desd'ecoctions de diverses portions de la plante et al., de cultures de tissue qui en proviennent. CR Seances Acad Sci Paris S'er D 278:3323-3325.

Halperin,W. 1986. Attainment and retention of morphogenetic capacity *in vitro*. In : Cell culture and somatic cell genetics of plants. Vol.3, I.K. Vasil (Ed.), pp. 3-47 Academic Press, New York.

*Hanning,E. 1904. Zur physiologic pflanzenlicher. Embryonen 1. Uber Die kultur von crucifercen embryonen austern halb der embryo sackes. **Bot., 62**: 45-80.

Harikrishnan,K.N. and Molly Hariharan. 1997. *In vitro* clonal propagation of sweet flag (*Acorus calamus*)-a medicinal plant In:Plant tissue culture and biotechnology:emerging trends Jan: 29-31.

Hasegava,P.M. 1980. Factors affecting shoot and root initiation from cultured rose shoot tips. **J. Ame. Soc. Hort. Sci., 105**: 216-220.

Hill, G.P. 1967. Morphogenesis in stem-callus cultures of *Convolvulus arvensis*. **Ann. Bot., (London) 31**: 437.

Holm- Nielson, L. B. 1979. Comments on the distribution and evolution of the genus *Phyllanthus* (Euphorbiaceae). In: Tropical botany.Larsen,K.and L.B.Holm-Nielson, (eds). Academic Press, New York, pp. 277-290.

Hu, C.Y. and P.J.Wang. 1983. Meristem, shoot tip and bud cultures.In:Evans,D.A., Sharp, W.R., Ammirato Huber,J., Constabel,F., Gamborg,O.L, (eds) Handbook of plant cell culture, Vol 1. Macmillan, NewYork, pp. 177-227.

Huang,L.C. and T.Murashige. 1977. Plant tissue culture media. Major constituents their preparation and some application. **Tiss. Cult. Assoc. Manual, 3**: 539-548.

Hughes,K.W. 1981. Ornamental species. In: Cloning Agricultural plants via *in vitro* technique. Conger,B.V. (Ed.). CRC Press. INL, Florida. 633.

*Hunault,G. 1985. Interaction acides organiques, pH ammonium nitrate surla croissance des tissus dasperge cultivars *in vitro*. **Ann. Sci. Naturelles, Botanique, Paris, 13**: 63-75.

Jin Yuan, Tsung.- Ting. Hu and Yu.-Min. Yang. 1994. Effects of auxins and cytokinins on formation of *Catharanthus roseus* G.Don multiple shoots. **Plant Cell Tiss. Org. Cult., 37**:193-196.

Johansson,L. 1983. Effects of activated charcoal in anther culture. **Physiol. Plant, 59**: 397-403.

John,S.A. 1996. Standardisation of *in vitro* techniques for rapid multiplication of *Holostemma annulare* K.Schum. M.Sc. (Hort) Thesis. Kerala Agricultural University, Vellanikkara.

Josekutty, P.C., Ndima, T.B. and E.Cloete. 1998. Direct organogenesis from leaf explants of *Scilla natalensis* planch. **Phyton., 63** (1/2):109-112.

Kaiser,W.M. and W.Hartung. 1981. Influence of activated charcoal in *in vitro*. **Plant Physiology, 68**: 202-208.

Kar,D.K. and S.Sen. 1985. Propagation of *Asparagus racemosus* through tissue culture.**Plant Cell Tiss. Org. Cult., 5**:89-95.

Kartha,K.M., Chanpouse,S., Gamborg,O.L. and Pahl. 1977. *In vitro* propagation of tomato by shoot apical meristem culture. **J. Ame. Soc. Hort. Sci., 102**: 346-349.

Karuppaiah,P. and L.R. Rajasekaran. 1993. Standardization of tissue culture methods and *in vitro* manipulation to evolve cell lines tolerant to NaCl stress in tomato. M.Sc. (Hort.) Thesis, Faculty of Agriculture, Annamalai University, India.

Kavikishor, P.B. and A.R. Mehta. 1987. Ergot alkaloid production in suspension cultures of *Ipomoea batatas* Poir. **Curr. Sci., 56**(15):781-783.

Keshava chandran,R. and A.D.Abdul khader.1994. In vitro regeneration of *Vetiveria zizanioides* (L) Nash. through somatic organogenesis. **Sou. Ind. Hort., 42** (3): 180-188.

King,P.J. 1980. Plant tissue culture and the cell cycle. In : Advances in biochemical engineering. Ficchten,A. (Ed.) Vol.18, **Plant Cell Cult., 11** : 11-38.

Kohlenbach,H.W. 1977. Basic aspects of differentiation and plant regeneration from cell and tissue cultures. In : Plant Tissue Culture and its Biotechnological Application. W.Berg, E.Reinland and M.H.Zenk (Eds.) Springler Verleg, Berlin.

Komalavalli,N. and M.V.Rao. 1997. *In vitro* micropropagation of *Gymnema elegans* W&A.– A rare medicinal plant. **Ind. J. Exp. Biol., 35**: 1088-1092.

Krikorian,A.D. 1982. Cloning higher plants from aseptically cultured tissues and cells. **Biol. Res., 57**: 151-218.

Kumar, A.1992. Morphogenesis in foliar callus of apocynaceous tree, *Thevetia peruviana* L. **Ind. J. Exp. Biol., 30**: 749-750.

Lakso,A.N., Reich,R.I., Mortensen,J. and M.N.Roberts. 1986. Carbondioxide enrichment for stimulation of growth of *in vitro* propagated grape vines after transfer from culture. **J. Ame. Soc. Hort. Sci., 111**: 636-638.

LangFord,P.H. and H.Wainwright. 1987. Effects of sucrose concentration on photosynthetic.

Leifert,C., Camotta,H. and W.M.Waites. 1992. Effect of combinations of antibiotics on micropropagated climates, Dalphinium, Hosta, Iris and Photiria. **Plant Cell Tiss. Org. Cult., 29**: 153-160.

Leifert,C., Ritchie,J. and W.M.Waite. 1991. Contaminants of plant tissue and cell culture. **World J. Microbiol. Biotechnol., 7**: 452-469.

Leifert,C., Nicholas,J.R. and W.M.Waites. 1990. Yeast contaminants of micropropagated plant culture. **J. Appl. Bacterial., 69**: 471-476.

Letham,D.S. 1974. Regulators of cell division in plant tissue xx. The cytokinins of coconut milk. **Physiol. Plant, 32**: 347.

Lin,M.L. and E.J.Staba. 1961. Peppermint and Spearmint Tissue cultures/callus formation and submerged culture. **Lloyelia, 24**: 139-145.

Linsmaier,E.M. and F.Skoog. 1965. Organic growth factors requirements for tobacco tissue culture. **Physiol. Plant, 18**: 100-127.

Lloyd,D., Roberts, A.V. and K.C.Short. 1988. The induction of in vitro of adventitious shoots in *Rosa*. **Euphytica, 37**: 31-36.

Lloyd,G. and B.McCown. 1980. Commercially feasible micropropagation of mountain *Laurel almia latifolia*, by use of shoot-tip culture. **Pros. Int. Plant Prop. Soc., 30**: 421-427.

Malathy,S. and J.S.Pal. 1998. *In vitro* propagation of *Hemidesmus indicus*. **Filoterapia, 69(6):** 533-536.

Malik, V.S. 1989. Biotechnology-The golden age. **Adv. in. Appl. Micrbiol., 34**:263-305.

Manjula,S., Anita Thomas, Benny Daniel and G.M.Nair. 1997.*In vitro* plant regeneration of *Aristolochia indica* through axillary shoot multiplication and organogenesis. **Plant Cell Tiss. Org. Cult., 51**:145-148.

Maria Cristina,F.dos Santos.; Maria A.Esquibel and Antonio,V.P.dos Santos. 1990. In vitro propagation of the alkaloid producing plant *Datura insignis* Barb. Rodr. **Plant Cell Tiss. Org. Cult., 21**:75-78.

Maroti,M. and E.Levi. 1977. Hormonal regulation of the organization from meristem cultures. In: Use of tissue culture in plant breeding. Novak,F.J. (Ed.). Czechoslovak Academy of Sciences, Prague. 337-352.

Martin, K.P.; Molly Hariharan. 1997. Rapid multiplication of *Alpinia calcarata* Rosc.-a medicinal plant through organogenesis. In: Plant tissue culture and biotechnology:emerging trends.(Jan) 29-31.

Martin,S.M. 1980. In: Plant tissue culture as a source of bio chemicals. Staba,E.J. (Ed.). CRC press. Boca Raton. 143-148.

Martin,S.M. and D.Rose. 1975. Growth of plant cell suspension cultures at controlled pH levels. **Can. J. Bot., 54**: 1264-1270.

Mathur,J., Ahiya,P.S., Lal,N. and A.K.Mathur. 1989. Propagation of *Valeriana officinalis* using encapsulated apical and axial shoot buds. **Plant Sci., 60**: 111-116.

*Mengal,K. 1984. Ernahrung and Stoffwcchset der pflanze cpp 134-173. Gustav fischer verlag, Stuttgart.

Millman,I., Venkateswaran,P.S. and B.S. Blumberg. 1987. Effect of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: *In vivo* and *in vitro* studies. **Proc. Natl. Acad. Sci. USA. 84**:274-278.

Minochae,S.C. 1987. Cell and tissue culture in propagation of forest trees. In : Plant cell cultures : Results and perspectives. Sale,F., Parisi,B. and Cella (Eds.). pp. 295-300 Elselvier, Amsterdam.

Misson,J.P., Boxus,P.H., Coumaus,M., Wright,P.P. and P.Gaspar. 1983. **Rijksuniv Gent., 116**: 1151-1157.

Morel,G. 1960. Producing virus free cymbidium. **Ame. Orchid Soc. Bull., 29**: 495-497.

Munavarjan, S. 2000. Micropropagation in *Gloriosa superba* Linn. M.Sc. (Hort). Thesis submitted to Horticultural College and Research Institute, TNAU, Coimbatore.

Murashige,T. 1974. Plant propagation through tissue culture.**Ann. Rev. Plant Physiol., 25**: 136-166.

Murashige,T. 1977. Manipulation of organ initiation in plant tissue cultures. **Bot. Bull. Acad. Sci., 18**: 1-24.

Murashige,T. 1978. Principle of rapid propagation. In : Propagation of higher plants through tissue cultures. A bridge between research and application. Hughas,K.W. Henke,R. and N.Constantin (Eds.). Tech. Inf. Centre, SUDE, Univ. Ternesse, USA. 14-24.

Murashige,T. and F.Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiol. Plant, 15**: 437-497.

Muthuram,G., Brinda,P. and T.S.Lokeswari. 2000. *In vitro* propagation of *Scoparia dulcis* Linn., a medicinal herb. In: Recent Trends in Spices and Medicinal Plants Research. Amit Krishna De (Eds.). Associated Publishing Co., New Delhi. pp. 100-105.

Narayanaswamy,S. 1977. Regeneration of plants from tissue cultures. In : Plant cell tissue and Orgen. culture. Reihert,J., Bajaj,Y.P.S. (Eds.). Springer Verlag, New York: 119-248.

Nataraj,K. 1975. Morphogenesis in embryonal cells of *Euphoiba* spp. *in vitro*. **Curr. Sci., 44**: 136-137.

Neelam Sharma, Chandel,K.P.S. and Srivastava,V.K. 1991. *In vitro* propagation of *Coleus forshkohlii* a threatened medicinal plant. **Plant Cell Rep., 10**: 67-70.

Nekrosova,T.S. 1964. The culture of isolated buds of fruit trees. **Soc. Plant. Physiol., 11**: 107.

Padmanabhan,V.; Paddock,E.J. and W.R.Sharp.1974. Plantlet formation from *Lycopersicon esculantum* leaf callus. **Can. J. Bot., 52**:1429-1432.

Pandey,S.N. and B.K.Sinha. 1999. In: **Plant physiology** (Third revised edition), (Pandey,S.N. and B.K.Sinha, Eds.). UBS Publishers, New Delhi. pp. 282.

Panse,V.G. and P.V.Sukhatme. 1978. Statistical methods for agricultural workers. ICAR, New Delhi.

*Patau, K., Das, N.k. and F. Skoog. 1977. Induction of auxin and cytokinin an morphogenesis of 3 varieties of *Episcia*. MS Thesis, The university of Tennessee khoxville.

Perik,R.L.C. 1987. *In vitro* culture of higher plants. Martinus Nijhoff Publishers, Dordrect.

Petra Bohmer, Beate Meyer and Hans-Jorg Jacobsen. 1995. Thidiazuron – induced high frequency of shoot induction and plant regeneration in protoplast derived pea callus. **Plant Cell Rep., 15**: 26-29.

Pillai,S.K. and A.C.Hildebrandt. 1969. Induced differentiates of Gerenium plants from undifferentiated callus *in vitro*. **Ame. J. Bot., 56:** 52-58.

Plas-van-der,L.H.W.; Eijkelboom,C. and Hamjmendoorn,M.Jr. 1995. Relation between primary and secondary metabolism in plant cell suspesions.**Plant Cell Tiss. Org. Cult., 43**:111-116.

Raghavan,T.S.V. 1977. Dicts and culture media for plant embryos. In : Hand Book series in Nutrition and Food. Vol.IV. Rechigal M.J. (Ed.) CRC press, Boca Raton, Florida: 369-372.

Rajasubramaniam,S. and P. Pardha Saradhi. 1994. Organic nitrogen stimulates caulogenesis from hypocotyl callus of *Phyllanthus fraternus*. **Plant Cell Rep., 13**:619-622.

Rajmohan,K., Mohankumaran and N.Mohan Babu. 1989. Application of tissue culture for the improvement of tree crops. Paper presented during the seminar on Molecular biology and biotechnology held at CMS College, Kottayam, 1-4, Jan. 1989: 1-8.

Rastogi,R. and V.K.Sawhney. 1986. The rate of plant growth regulators. Sucrose and pH in the development of floral buds of tomato cultured *in vitro*. **J. Plant Physiol., 128**: 285-295.

Reeves,D.W., Horton,B.D. and G.A.Couvillion. 1983. Effect of activated charcoal in tissue culture media. **Sci. Hort., 21**: 353-357.

Reinert, J. and D.Backs. 1968. Control of totipotency in plant cells growing *in vitro*. **Nature,** (London) **31**: 437.

Remashree, A.B., Molly Hariharan and K.Unnikrishnan. 1994. Micropropagation and callus induction of *Aristolochia bracteolata* Lam-A medicinal plant. **Phytomorphology, 44** (3&4):247-252.

Remashree,A.B., Hariharan,M. and K.Unnikrishnan. 1997. *In vitro* organogenesis in *Aristolachia indica*. **Phytomorphology, 47**(2): 161-165.

Roest,S.B. and G.S.Bokelmann. 1989. Vegetative propagation of carnation *in vitro* through multiple shoot development. **Sci. Hort., 14**: 357-366.

*Rossini. 1969. One nogevelle method de culture *in vitro* de cellules parenchymatessus separess de fevilles de *Calystegia sepium* L. CR. Acad. Sci., Paris, In : Plant Tissue Culture and its biotechnological application Berg,W, Rainard,E., Zenk,M.H.(Eds.). Springer Venlarg, Berlin.

Rout,G.R.; Saxena,C.;Samantaray,S.; Das,P. 1999. Rapid plant regeneration from callus cultures of *Plumbago zeylanica*. **Plant Cell Tiss. Org. Cult., 56**(1): 47-51.

Row, L.C.; Srinivasulu,C.;Smith,M. and G.S.R.S.Rao. 1996. Crystalline constituents of Euphorbiaceae.V.New lignans from *Phyllanthus niruri* Linn: The constitution of phyllanthin.**Tetrahedron, 22**:2899-2908.

Sahoo,Y.E. and P.K.Chand. 1998. *In vitro* multiplication of a medicinal herb, *Tridex procumbens*: Influence of explanting season, growth regulator synerges, culture passage and planting substrate. **Phytomorphology, 48**(2): 195-205.

Sairam Reddy,P., Ramagopal,G. and G.Lakshmi Sita. 1999. *In vitro* multiplication of *gymnema sylvestre* – An important medicinal plant. **http:/www.iise.ernet.in/**

Sarsan,V.; Soniya,E.V. and G.M.Nair.1994. Regeneration of Indian Sarsaparilla, *Hemidesmus indicus* Roxb. Through organogenesis and somatic embryogenesis. **Ind.J.Exp. Biol., 32**:284-287.

*Scheffer,F. and P.Schachtschabel. 1979. Lebrbuch der Boden kunde: 143-187. (Ferdinand Enke verlag. Stuffigart).

Schenk,N., Hsiao,K.C. and C.H.Bomman. 1991. Clonal multiplication of azaleas through culture. **Plant Cell Rep., 10**: 115-119.

Schenk,R.V. and A.C.Hildebrandt. 1972. Medium and techniques induction and growth of mono cotyledons and dicotyledons plant cell culture. **Can. J. Bot., 50**: 199-204.

*Schleiden,M.J. 1838. Beitrage Zur Phytogenesis. Muller Arch. Anat. Physiology Wiss. Med. pp.137-176.

*Schwann,T. 1839. Mikroskopische untersuch ungen uber die ubereinstimmung in der strukker and derm waschstume der. Tiere and Pfanzen (Ostwalds Klassiker der Exaketch Wissen schafften, No.176 Engelmann, Leipzin (1910).

Scott,J.N. and G.S.Ellen. 1990. Stability of IAA and IBA in nutrient medium to several Tissue culture procedures. **Hort. Sci., 25**(7):800-802.

Seema,B.J. 1997. Standardisation of in vitro techniques for rapid multiplication of *Trichopus zeylanicus*. M.Sc. (Hort) Thesis. Kerala Agricultural University, Vellanikkara.

Sehrawat,A.R.; Uppal,S.; Sehrawat,K.D. 1999. Effect of phytohormones on callus induction from different explants of *Catharanthus roseus* (L.) G.Don. **Ann. Agr. Bio. Res., 4**(2):183-187.

Selvakumar,V. and T.Balakumar. 2000. *In vitro* induction of axillary branching in the medicinal plant *Acalypha fruiticosa* Forsk. In: Recent Trends in Spices and Medicinal Plants Research. Amit Krishna De (Eds.). Associated Publishing Co., New Delhi. pp.96-99.

Shantz,E.M. and F.C.Steward. 1952. Coconut milk factors. The growth promoting substances in coconut milk. **J. Ame. Chem. Soc., 74**: 393.

Sharma,K.S., Maesatio,K., Hara,T. and Y.Sohoda. 1990. *In vitro* production of stigma like structures form stigma explants of *Crocus sativus*. **J.Exp. Bot., 41**(227): 745-748.

Shiio,I. And S.Ohta. 1973. Nicotine production by tobacco callus tissues and effect of plant growth regulators. **Agr. Bio. Chem., 37**: 1857-1864.

Singh,S., Bissonettle,G.K. and M.L.Double. 1987. Methods for sterilizing instruments contaminated with *Bacillus* sp. from plant tissue cultures. **Hort. Sci., 22**: 659.

Sitakanta Pattnaik and Pradeep K.Chand. 1996. *In vitro* propagation of the medicinal herbs *Ocimum americanum* L.syn. *Ocimum canum* Sims. and *Ocimum sanctum* L. **Plant Cell Rep., 15**:846-850.

Skirvin,R.M. 1980. Fruit crops. In : Cloning Agriculture Plants via *In vitro* Techniques. Corger.B.V. (Eds.). CRC Press. BocaRaton, Florida: 51-139.

Skirvin,R.M. and M.C.Chu. 1979. *In vitro* propagation of "Forever yours" rose. **Hort.Sci., 14** : 608-610.

Skoog,F. and C.O.Miller. 1957. Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. **Soc. Exp. Biol. Symp., 11**: 118-131.

Skoog. F. 1971. Aspects of growth factor interaction in morphogenesis of tobacco tissue cultures. In: Plant tissue culture and its biotechnological applications (eds.) Berg, W., Reinfard, E., Zenk, M.H. Springler verlag, Berlin.

Smita Chetia and P.J. Handique. 2000. High frequency *in vitro* shoot multiplication of *Plumbago indica*, a rare medicinal plant. **Curr. Sci. 78** (10): 1187-1188.

Smith,D.L. and A.D.Krikorian. 1990. pH control of carrot somatic embryogenesis. In: Progress in plant cellular and molecular biology: 449-454. Nijkanp,H.J., Van der plas,L.H.W. and Van Aartrijk,J. (Eds.). Kluwer Academic Pubishers, Dordrecht.

Sommer,H.E. and L.S.Caldas. 1981. *In vitro* methods applied to forest trees. In: Plant Tissue culture: Methods and applications in agriculture. Thorpe,T.A. (Ed.). Academic Press, New York: 149-358.

Srivastava,N. and M.A.Padhya. 1995. 'Punarnavine' profile in the regenerated roots of *Boerhaavia diffusa* L. from leaf segments.**Curr. Sci.,68**:653-656.

Straus,J. and R.E.Rodney, 1960. Response of *Cupressus funebris* tissue culture to Gibberlins, **Sci., 131** : 1806-1807.

Strickland, S.G., Nichol, J.W., Mc call, C.M. and D.A. Stuart. 1987. Effect of carbohydrate source on alfalfa somatic embryogenesis. **Plant Sci., 48**: 113-121.

Suchitra Banerjee, Mehar Zehra and Sushil Kumar. 1999. *In vitro* multiplication of *Centella asiatica*. A medicinal herb from leaf explants. **Curr. Sci., 76** (2):147-148.

Sudha,C.G. and S.Seeni, 1996. In vitro propagation of *Rauwolfia micrantha*, a rare medicinal plant. **Plant Cell Tiss. Org. Cult., 44**: 243-248.

Sukumar,S. and S.R.Sree Rangaswamy. 1988. Response of *in vitro* leaf culture for regeneration and evaluation of the regeneration in tomato. **Curr. Sci., 57**(16): 89-92.

Surya Narmada, T. 2000. *In vitro* studies in *Gymnema sylvestre*. M.Sc.(Hort). Thesis submitted to Horticultural College and Research Institute, TNAU, Coimbatore.

*Sushruta (6th Century B.C.). Sushruta Samhita Vol.II Under chikitsa sthanam, Chapter XII on Prambhar Pidaka verse 9, referred from an English translation of the Sushruta Samhita by K.K.Zhishgrabna. pp.379-392. Published in 1911.

Syamasunder,K.V.; Singh,B.; Thakur, R.S.; Husain,A.; Kiso,Y. and Hikino,H. 1985. Antihepatotoxic principles of *Phyllanthus niruri* herbs. **J.Ethnopharmacol., 14**:41-44.

Tadahiko Sato, Oh Chang Kwon, Hirsohi Miyakc, Takeshi Taniguchi and Eizo Maeda. 1995. Regeneration of plantlets from petiole callus of wild viola (*Viola patrinii*). **Plant Cell Rep., 14**: 768-772.

Takahsi,M. and Y. Yamada. 1973. Regulation of nicotine production by auxins in tobacco cultured cells *in vitro*. **Agr. Bio. Chem., 37**:1755-1757.

Takayama,S. and M.Misawa. 1979. Differentiation in Lilium bulb scales grown *in vitro* effect of various cultural conditions. **Physiol. Plant, 46**: 184-190.

Teli, N.P., Patil, N.N., Pathak, H.M., Bhalsingh, S.R.and V.L.Maheshwari.1999. *Withania somnifera* (Ashwagandha): regeneration through meristem culture. **J. Plant. Biochem. Biotech. 8**(2):109-111.

Thimmann,K.V. 1977. Hormone action in the whole life of plant, University of Massachusetts Press, Amherst.

Thomas,E. and W.Wernicke, 1978. Morphogenesis in habaceous crop plants. In: Frontiers of plant tissue culture. Thorpe T.A. (Ed.). Univ. of Calgary Press. Calgay. 403-410.

Thorpe,T.A. 1978. Physiological and biochemical aspects of organogenesis *in vitro*. In: Frontiers of plant tissue culture. Thorpe,T.A. (Ed.). University of Calgary press, Canada. 49-58.

Toivonen,L.; Leakso,S. and H. Rosenqvisit. 1992. The effect of temperature on hairy root cultures of *Catharanthus roseus*:Growth, indole alkaloid accumulation and membrane lipid composition. **Plant Cell Rep.,11**:395-399.

Unander, D.W., Venkateswaran, P.S., Millman, I., Bryan, H.H. and B.S.Blumberg. 1990. *Phyllanthus amarus*:a possible source of new antiviral compounds.In: Advances in new crops (eds), Janick, J. and J.E.Simon, Timber Press, Portland, Oregon, pp.518-521.

Unander,W.1991. Callus induction in *Phyllanthus* species and inhibition of viral DNA polymerase and reverse transcriptase by callus extracts.**Plant Cell Rep., 10**:461-466.

Van Overbreak,J., Conklin,M.E. and Blakeske. 1941. Factors in coconut milk essential for growth and development of very young *Datura* embryos. **Sci., 94**: 350-351.

Van Staden,J. and S.E.Drewes. 1974. Identification of cell division including compounds from coconut milk. **Physiol. Plant, 32**: 347.

Vasil,I.K. and V.Vasil. 1980. Clonal propagation. **Int. Rev. Cytol. Sup. Plant., 114**: 145-193.

Verma,D.C. and D.K.Dougall. 1977. Influence of carbohydrates on quantitative aspects of growth and embryo formation in wild carrot suspension cultures. **Plant Physiol., 59**: 81-85.

Vincent, K.A., Bejoy, M., Hariharan, M. and M.K. Mathew. 1992. Plantlet regeneration from callus cultures of *Kaempferia galanga* Linn. A medicinal plant. **Indian J. Plant Physiol., 34**(4): 396-400.

Vuylsteke.D. and E.C. De Langhe. 1985. Feasibility of *in vitro* propagation of tomato and plantains. **Trop Agric., 62** : 323-328.

Wainwright,H. 1988. Overcoming problems in establishing micropropagules guidelines for growers. **Prof. Hort., 2**: 67-72.

Weatherhead,M.A., Burdon,J. and C.G.Henshaw. 1978. Some effects of activated charcoal as an additive to plant tissue culture media. **Z. Pflenzen Physiol., 89**: 141-144.

Webster, G.L. 1970. A revision of *Phyllanthus* (Euphorbiaceae) in the continental United States. **Brittonica, 22**:44-76.

White,P.R. 1934. Potentially unlimited growth of excised tomato root tip in a liquid medium. **Plant. Physiol., 9**: 585-600.

Wickson,M. and K.V.Thimmann. 1958. The antagonism of auxin and kinetin in apical dominance. **Physiol. Plant, 11**: 62-74.

Wochok, Z.S. and D.F. Wetherell. 1972. Restoration of declining morphogenetic capacity in long term cultures of *Daucus carota*. **Ame. J. Bot., 41**: 321-326.

Wodziki, T.J. 1978. Seasonal variation of auxin in stem cambial region of *Pinus sylvestris*. **Acta Soc. Bot. Polon., 47**:225-231.

Wright,S.T.C. and R.W.P.Hiron. 1969. Abscissic acid, the growth inhibitor induced in detached wheat leaves by a period of wilting. **Nature, 224** : 719-720.

Yanagi,M., Unoura,M., Kobayashi,K., Hattori,N. and S.Murakami. 1989.Inhibitory effect of an extract from *Phyllanthus niruri* on reaction of endogenous HBV-DNA polymerase and other DNA synthetases. In: Abstracts of papers presented at the 1989 meeting on hepatitis B viruses, september 25-28, 1989.Cold Spring Harbor Laboratory. Cold Spring Harbor, Newyork. (Abstr.), pp. 77.

Yemon and Mc Cold. 1977. The present development and future of plant cell and tissue culture in agriculture, forestry and horticulture. In: Plant tissue culture and its Agricultural Applications. (Eds.) Withers, L.A. and A.G Alderson, Butter worths, London, pp. 485-500.

Yemon,M.M.(Ed.).1986.Plant Cell Culture technology. Blackweel scientific publications. Melbourne, pp. 33.

Zapato, N.; Ramakrishnan,T. and C.S.Vaidyanathan. 1981. Regeneration of hybrid tomato plants from leaf callus. **Curr. Sci., 47**(13):458-460.

Zenk,M.H., El-Shagi,H. and Schulte,U. 1975. Anthraquinone production by cell suspension cultures of *Morinda citrifolia*. **Planta Med.,** (Suppl.):79-101.

Zhang,B. and L.P.Stoltz. 1989. Effect of nutrient media pH in *in vitro* culture. **Hort. Sci., 24 :** 503-504.

Zimmerman,R.H. and I.Hordham. 1985. Simplified method for rooting apple cultivars *in vitro*. **J. Ame. Soc. Hort. Sci., 110**: 34-38.

Zito,S.W., Zieg,R.G. and E.J.Staba. 1983. Distribution of pyrethrin in oil glands and leaf tissues of *Chrysanthemum cinerariafolium*. **Planta Med., 47**: 205-207.

Ziv.,M. 1986. *In vitro* hardening and acclimatization of tissue culture plants. In: Plant tissue culture and its agricultural application. Withers,L.A. and P.G.Alderson (Eds.). Butter Worths, London: 187-196.

* *Originals not seen*

ANNEXURE

ANNEXURE I

Composition of Murashige and Skoog's medium (Murashige and Skoog, 1962)

S. No.	Constituents	Molecular weight	Amount (mg/lit)
I.	**Macronutrients**		
	NH_4NO_3	80.04	1650
	KNO_3	101.11	1900
	$CaCl_2.2H_2O$	147.02	0440
	$MgSO_4.7H_2O$	246.47	0370
	KH_2PO_4	136.09	0170
II.	**Micronutrients**		
	KI	166.01	00.83
	K_3BO_3	61.83	06.20
	$MnSO_4.4H_2O$	223.01	22.30
	$ZnSO_4.7H_2O$	287.54	08.60
	$Na_2MoO_4.2H_20$	241.95	00.25
	$CuSO_4.5H_2O$	249.68	00.025
	$CoCl_2.6H_2O$	237.93	00.025
III.	**Iron EDTA**		
	$FeSO_4.7H_2O$	278.30	27.80
	Na_2–EDTA.$2H_2O$	372.25	37.30
IV	**Organic nutrients**		
	Myo-inositol	180.16	100.00
	Nicotinic acid	123.11	0.50
	Pyridoxine HCl	205.64	0.50
	Thiamine HCl	337.29	0.10
	Glycine	075.07	2.00

ANNEXURE II

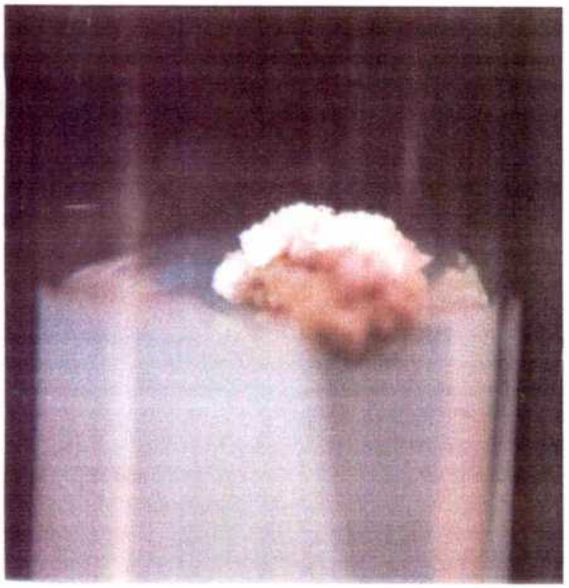
Stock solutions for Murashige and Skoog's medium

S. No.	Constituents	Amount (mg/lit))	Volume of stock/L of medium (ml)
I.	**Stock-A (X20)**		
	NH_4NO_3	33000	
	KNO_3	38000	
	$CaCl_2$ (Fused)*	6643.363	50
	$MgSO_4.7H_2O$	7400	
	KH_2PO_4	3400	
II.	**Stock-B (X200)**		
	Kl	166	
	H_3BO_3	1240	
	$MnSO_4.4H_2O$	4460	
	$ZnSO_4.7H_2O$	1720	5
	$Na_2MoO_4.2H_2O$	50	
	$CuSO_4.5H_2O$	5	
	$CoCl_2.6H_2O$	5	
III.	**Stock-C (X200)**		
	$FeSO_4.7H_2O$	5560	
	Na_2–EDTA.$2H_2O$	7460	5
IV.	**Stock-D (X200)**		
	Meyo inositol	20000	
	Nicotinic acid	100	
	Pyridoxine HCl	100	
	Thiamine HCl	20	
	Glycine	400	

ANNEXURE III

S. No.	Compound	Common abbreviations	Molecular weight	Quantity (mg) per 50 ml stock	Solvent	Dilutant	Storage
I.	**Auxins**						
1.	Indole-3-acetic acid	IAA	175.18	50	1 N NaOH	Distilled water	0°C
2.	3-indolebutyric acid	IBA	203.23	50	1 N NaOH	Distilled water	0°C
3.	α-napthalene acetic acid	NAA	186.20	50	1 N NaOH	Distilled water	0-5°C
4.	2,4-dichlorophenoxy acetic acid	2,4-D	221.0	50	1 N NaOH	Distilled water	0-5°C
II.	**Cytokinins**						
5.	6-benzylamino purine	BAP	22.520	10	1 N NaOH	Distilled water	0-5°C
6.	6-furfurylamino purine	KN	215.21	50	1 N NaOH	Distilled water	0°C

PLATES



Callus induction from stem bit in MS + 2,4 – D (4 mg/l)

Callus induction from stem bit in MS + 2,4 – D (4 mg/l) + NAA(0.4 mg/l)

Callus induction from nodal segment in MS + BAP 3 mg/l + kinetin 3 mg/l

Callus induction from nodal segment in MS + BAP 2 mg/l + GA_3 0.5 mg/l

Multiple shoot regeneration from shoot tip in MS + BAP 2 mg/l + GA_3 0.5 mg/l

Multiple shoot regeneration from shoot tip in MS + BAP 3 mg/l + Kinetin 3 mg/l

Profuse growth of multiple shoots after subculture onto MS + BAP 2 mg/l + GA_3 1mg/l

In vitro rooting in ½ MS + IAA 0.5 mg/l + IBA 0.5 mg/l

Hardening of plantlet in humid condition

Plantlet transferred to pot mixture