IN VITRO STUDIES IN *Centella asiatica* (Linn.) Urban

Thesis submitted in part fulfillment of the requirements for the degree of ***Master of Science (Horticulture)*** *to the Tamil Nadu agricultural universityCoimbatore-641 003*

By

V.PANIMALAR

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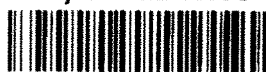

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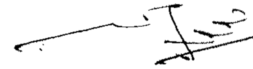

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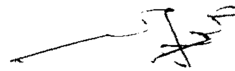
Chairman: 

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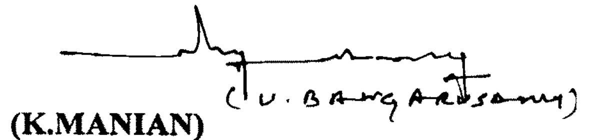

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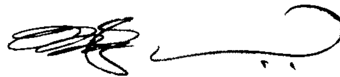
External examiner: 

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(V.PANIMALAR)

ABSTRACT

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In vitro studies in *Centella asiatica* (Linn.) Urban.

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Studies on standardization of micro propagation techniques for *Centella asiatica* were carried out at the Tissue culture Laboratory of the Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore.

Among the different explants used, nodal segments and leaf bits followed by petiole bits and stem bits gave good response. May–June was found to be the best season for explant collection, as maximum survival with minimum contamination was observed. Sterilization of nodal segments using $HgCl_2$ at 1 per cent concentration for 1 minute gave the maximum survival. Leaf bits, petiole bits and stem bits recorded maximum survival at 0.5 per cent concentration for 1–3 minutes.

Murashige and Skoog medium at full strength supplemented with BAP 2.0 mgl^{-1}, and BAP 1.0 mgl^{-1} with Kinetin 1.0 mgl^{-1} was found optimum for direct shoot regeneration from nodal segments, in 6–10 days. MS + BAP (3.0 mgl^{-1}) + NAA (0.3 mgl^{-1}) was optimum for inducing multiple shoots (5.16 shoots) from nodal segments. MS + BAP (2.0 mgl^{-1}) + GA_3 (0.5 mgl^{-1}) induced shoot elongation in nodal segments.

Callusing in nodal segments and leaf bits was achieved in 16–25 days on full strength MS medium supplemented with 3.0 mgl^{-1} NAA and 0.3 mgl^{-1} BAP. In petiole bits and stem bits MS + 4.0 mgl^{-1} NAA + 0.4 mgl^{-1} BAP was optimum in inducing callus in 22 days. Cent per cent response to callus proliferation in the initial 2 subcultures at 15 days interval was observed in all the four explants. Decrease in response and callus weight on subsequent sub culturing was noticed.

MS + BAP (4.0 mgl^{-1}) + NAA (1.0 mgl^{-1}) was found suitable for regeneration of shoots from callus of all explant sources, in 25 – 40 days. But a very low turnover in the number of shoots (a maximum of 4 shoots) regenerated per callus was observed. ½ MS + IBA (0.5 mgl^{-1}) and ½ MS + NAA (0.2 mgl^{-1}) induced rooting in regenerated shoots. A maximum of 15 roots per shoot was recorded.

Rooted plantlets showed maximum survival on transfer to a medium containing pot mixture (1 soil: 1 sand: 1 FYM) and Vermiculite in the ratio of 1: 1.

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ABBREVATIONS USED

BA	:	Benzyl adenine
BAP	:	6-benzyl amino purine
CD	:	Critical Difference
cm	:	Centimeter
2, 4-D	:	2, 4 – dichlorophenoxy acetic acid
EDTA	:	Ethylene Diamine Tetra Acetic acid
Fe	:	Iron
g	:	Gram
GA_3	:	Gibberellic 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
mgl^{-1} or mg / lit	:	Milligram per litre
min	:	Minute
ml	:	Milli litre
Mm	:	Milli Molar
MS	:	Murashige and Skoog's medium
NAA	:	α - naphthalene 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
$\mu E/m^2/s$	:	Micro Equivalent per minute square per second
µm	:	Micro molar
%	:	Percentage

INTRODUCTION

CHAPTER I

INTRODUCTION

Interest in medicinal plants as a re-emerging health aid has been fuelled by the rising costs of prescription drugs in the maintenance of personal health and well-being. India is one of the eight important Vavilovian centers of origin, immensely rich in medicinal and aromatic plants, occurring in diverse ecosystems. With increasing global demand for medicinal plants and a large base of local demand for plant based traditional medicines, the pressure on the existing population of medicinal plants has increased tremendously during the last few decades. Historically, most of these plants grow in the wild as a natural component of vegetation of a particular region and they are collected and sold by local people. Since there is no scientific system of collecting or regenerating these plants, several plants have either been completely lost or have become endangered.

It is recommended that more focused attention should be given to some 45 medicinal plants for the next 20 years, out of which 7 plants *Aloe vera, Bacopa monnieri, Centella asiatica, Rauwolfia serpentina, Catharanthus roseus, Taxus baccata* and *Artemesia annua* require concentrated attention. Thus *Centella asiatica* needs further investigations.

Commonly called as the Asiatic pennywort, it is also known as Brahama – manduki (Hindi), Mandukaparni (Sanskrit), Gotukola (Sinhala), and Vallarai (Tamil). A review on the cytology, culture, phytochemistry and nutritional uses was made by Peiris and Kays (1996).

Several botanical synonyms for *Centella asiatica* are listed in literature – *Hydrocotyle asiatica* L., *Hydrocotyle pallida* D.C., etc. The earlier classification as *Hydrocotyle asiatica* L. was subsequently corrected to *Centella asiatica* L. (Bagchi and Puri, 1988). *Centella* comprises of about 20 species found mainly in South Africa (Mabberley, 1987) and belongs to the family Apiaceae.

It is a perennial herb with long, slendar, horizontal stolons, characterized by long internodes, from which arise a cluster of ascending petiolate leaves at each node. Leaf size and margins vary from smooth to crenate or slightly lobed. Inflorescence of 1 – 4 simple umbels / node contain 1 – 4 flowers / umbel. The petals are white, often tinged rose, fruit ellipsoid, 3 – 4 mm long and 3 – 5 mm broad (Correll and Correll, 1982).

It is distributed form the Himalayas to Ceylon, up to an altitude of 2000 ft (Anonymous, 1988). It is also found in China, Sikkim, Malaysia and other tropical and subtropical regions of the world (Godfrey and Wooten, 1981). Plants grow along margins of lakes, ponds, drainage and irrigation canals, swampy areas and bunds of irrigated paddy fields, and prefer shade.

Cytological studies indicate the somatic chromosome number of 2n = 18 for *Centella* (Singha and Singha, 1977). Two cultivars Kayakirti and Majja Poshak have been registered for commercial cultivation by CIMAP (Shalini Mathur *et al.*, 1999).

The plants contain triterpene glycosides such as asiaticoside, madecassoside, asiatic acid and madecassic acid. It also contains essential oils such as Beta – caryophyllene, alpha humulene and Germacrene D. Health claims range from enhanced memory and longevity, to the treatment of leprosy (Kartnig, 1988). It is also used in the treatment of various skin diseases, ulceration, chronic rheumatism, malaria, fever, epilepsy and enlargement of glands.

Owing to its virtues, commercial cultivation of *Centella asiatica* is being attempted now. Seeds often fail to germinate and slips cannot be transported to far off places. An *in vitro* protocol is necessary for rapid multiplication. Hence it is important to standardize the suitable explants, medium and hormones, for tissue culture multiplication of *Centella asiatica*.

The present study has been contemplated on the micro propagation of *Centella asiatica* with the following objectives.

1. To evaluate ideal explant for maximum callusing and regeneration.
2. To standardize the sterilization procedure for explants.
3. To evaluate the callogenesis and organogenesis under different plant growth regulator fortification.
4. To evaluate microshooting and microrooting capability.
5. To standardize the hardening protocol for propagules.

REVIEW OF LITERATURE

CHAPTER II
REVIEW OF LITERATURE

Despite the increasing use of medicinal plants, their future seemingly is being threatened by complacency concerning their conservation. Reserves of herbs and stocks of medicinal plants in developing countries are diminishing and in danger of extinction as a result of growing trade demands for cheaper health care products and new plant – based therapeutic market in preference to more expensive target – specific drugs and biopharmaceuticals. The increasing demand for herbal medicines in recent years, due to their fewer side effects in comparison to synthetic drugs and antibiotics has highlighted the need for conservation and propagation of medicinal plants. Tissue culture provides efficient techniques for rapid and large-scale propagation of medicinal plants and their *in vitro* conservation of germplasm.

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. 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 auxin: cytokinin ratio and that organ differentiation could be regulated by changing the relative concentrations of these two substances in the medium. Murashige developed a standard method for *in vitro* propagation of several species ranging from ferns to foliage, flower and fruit crops. 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. 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.

Murashige (1978) gave the different stages for the production of plants via micro propagation. Murashige (1974), Thorpe (1978), Dodds and Roberts (1982) and Dixon (1985) have standardized and discussed many tissue culture techniques for mass multiplication of plants.

In nature, *Centella asiatica* is not over exploited. However factors such as endemicity, restricted distribution and small populations in accessible areas has caused its decline. The requirement is now met from the natural populations, leading to their gradual depletion. There is a wide scope for application of biotechnology for improvement of this important medicinal plant for which standardization of an efficient *in vitro* multiplication protocol is a crucial prerequisite. Therefore a review is made to comprehend the protocol for microprogagation of *Centella asiatica.*

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

2.1.1. 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) 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 over all quality of the plant from which the explants are obtained.

2.1.1.1.Type of explant

The morphogenetic potential of various plants varies depending on the explant used. For direct organogenesis without an intermediate callus, young shoot bases in Safed musli (Purohit *et al.*, 1994), meristem tips in *Dioscorea cayenensis* (Bernard Malaurie *et al.*, 1995) and leaf explants in *Withania somnifera* (Abhyankar and Chinchanikar, 1996) were found suitable. Manjula *et al.* (1997) reported good response via axillary and adventitious shoot regeneration in *Aristolochia indica.* Leaf segments devoid of petioles were more responsive than petioles in regenerating plantlets of *Centella asiatica* (Suchitra Banerjee *et al.*, 1999). Other type of explant reported as being suitable for direct regeneration of plants is nodal segments in *Vitex negundo* (Thiruvengadam and Jayabalan, 2001).

Indirect organogenesis via callus has been best achieved using stem explants in *Aegle marmelos* (Varghese *et al.*, 1993), young leaves and nodes in *Aristolochia bracteolate* (Remeshree *et al.*, 1994), seedling hypocotyls of *Papaver somniferum* (Wakhlu and Bajwa, 1986), and leaf, stem and petiole explants of *Centella asiatica* (Patra *et al.*, 1999).

2.1.1.2. Size of explant

Success in *in vitro* culture depends on the optimum size of explant, which varies from crop to crop. Differences in initial callus formation were attributed to variation in explant size in tomato (Zankowski and Rost, 1990). Nodal segments of about 10 cm length in *Agave* (Binh *et al.*, 1990) and nodal segments of 1.0 – 1.5 cm long in *Coleus forshkohlii* (Neelam Sharma *et al.*, 1991) gave multiple shoots, whereas nodal segments 3 – 5 cm long gave the maximum number of shoots in *Eclipta alba* (Franca *et al.*, 1995).

For callusing, 2.0 – 3.0 mm^2 leaf pieces in *Medicago suffruticosa* (Xiu – Qing Li and Yves Demarly, 1996), 1 cm long stem bits, 5.0 mm^2 leaf bits and 8 mm long leafstalk in *Artemisia absinthium* (Stefania Nin *et al.*, 1996).

2.1.1.3. Physiological age of explant

The morphogenic response in *Coleus forskohlii* varied as a function of the age of the explant, irrespective of hormones used. Shoot tips isolated from 30 d old seedlings yielded the maximum number of shoots (Jayanthi sen and Sharma, 1991a). Of the various explants used leaf explants of *Centella asiatica*, that were collected from five to six months old, glass house plants gave better callusing and regeneration response (Suchitra Banerjee *et al.*, 1999). Nodal segments taken from tender twigs, from five to six year old mature plants of *Gymnema sylvestre* gave good response for multiplication of shoots (Sairam Reddy *et al.*, 1999).

2.1.1.4 Season of explant collection

In *Beta vulgaris* it was reported that the best results were obtained in June and it was the favourable period for gynogenesis, which proved that physiological status of donor plant was important (Marylise Doctrinal *et al.*, 1989). But in contrast it was reported in *Sandersonia and Gloriosa* that there were no marked seasonal problems as often present in monocotyledons (Finnie and Van Staden, 1989). Sitakanta Pattnaick *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 to December. John (1996) reported in *Holostemma annulare* that the explants collected in the months of January to April gave lower contamination and higher survival percentages.

2.1.1.5. Position of 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 development between explants taken from the top, and the base of the shoot was 88.6 and 69.81 respectively. From the 'V' shaped tubers of *Gloriosa superba*, the tuber base and tips resulted to give shoots while the tuber center resulted in micro roots (Finnie and Van Staden, 1989).

2.1.1.6. Placement of the explant on the culture medium

The polarity and placement also influences the survival of explant on the medium. In petunia, the positional variation neither altered the pattern of development nor the capacity for bud formation (Rao *et al.*, 1973). 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). Irrespective of the vertical or horizontal positioning, all the shoot tips of *Rauwolfia micrantha* developed into single shoots either by longitudinal growth or by upward curvature and elongation (Sudha and Seeni, 1996).

2.1.1.7. Surface sterilization

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).

The concentrations of surface sterilants used 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 the explants first with ethanol (45 %) for 3 minutes followed by a 10 minutes 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).

When tissue cultures were tested by microbiological indexing methods after initiation, most contaminants found during later stages of the micro propagation process were introduced accidentally 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 operators using inefficient aseptic techniques could rapidly spread contaminants during the handling of plant cultures. 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).

The use of plantlets developed from stolon segments in sand bed as the initial material had the advantage that only a low degree of contamination occurred in the initial cultures of Agave (Binh *et al.*, 1990). Unander (1991) suggested a sterilization procedure for explants of *Phyllanthus amarus*. Leifert *et al.* (1994) reported that initial culture of delphinium in a medium of pH 3.5 resulted in 65 % of shoots free of contamination, which shows the anti-bacterial preservative effect of medium acidification. Subjecting the shoot tips of *Gloriosa superba* to 0.5 % $HgCl_2$ for a period of 3 minutes reduced the contamination percent (15.00 %) (Munavarajan, 2000).

2.1.2. 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, amino acids and plant growth regulators improved the success

Research laboratories for instance, often employed coconut milk and banana homogenate was still a popular addition to media for orchid culture. Since 1962, most researchers have been using Murashige and Skoog (1962) medium. A variety of plant tissue and cell culture media now in practice include Lin and Staba (1961) medium, B5 medium developed by Gamborg *et al.* (1976), Huang and Murashige (1977) 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 micronutrients contained in it. The MS medium had the highest salt content and some workers found it beneficial to reduce its strength by half (Skirvin, 1980). He also 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 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 resulted in poor success, depicting the requirement for standardization of specified media for the crop species of varieties and plant part used as explant. Although attempts have been made to avoid the use of undefined compounds such as fruit juice, yeast extract and casein hydrolysate, improved results have sometimes been reported by their addition (George and Sherrington, 1984).

2.1.2.1. Type of medium

Jayanthi Sengupta *et al.* (1984) reported that both MS and modified Whites medium supported callusing as well as organogenesis in *Dioscorea floribunda* and similarly no significant difference was found between MS and B5 minerals for callus cultures of *Dysosma pleiantha* (Meng-jin Chauang and Wei-Chin Chang, 1987). Leaves

of *Rauwolfia* cultured on Whites' root culture medium (WCR) and Abou – Mandoues' medium (AM), showed that callus was induced on WCR, whereas growth of callus on AM media was almost negligible (Mahmood Akram and Riffat Nazir, 1994). The best callus growth was obtained from leaf explants on MS medium supplemented with 2mg NAA/l and 3mg BAP/l rather than the Phillips – Collins medium and modified MS medium in *Catharanthus roseus* (Akcam and Yurekli, 1995).

2.1.2.2. Mineral salts

Nitrate and ammonium salts are commonly used as nitrogen source in plant cell and tissue culture media. Addition of both ions results in a rapid increase of biomass. The ratio between the amounts of nitrate- N and ammonium – N is often a characteristic of a medium (Murashige and Skoog, 1962). Mac Carthy *et al.* (1980) studied the influence of the removal of Ca, Mg, K, Na and So_4^- from a modified Wood and Braun medium. None of these cations was completely removed during culture growth. Uptake of Na and K from the medium proceeded in parallel with culture growth Phosphate plays an important role in the regulation of growth and secondary metabolism. Rapid uptake of Po_4^- is a commonly observed phenomenon (Merillon *et al.*, 1983, Hoopen *et al.*, 1987). Ammonium nitrate at 0.30 % resulted in maximum shoot and root formation and alkaloid content of regenerated plant was increased with increase of NH_4No_3 in the culture of *Tylophora indica* (Soumitra Chattopadhyay *et al.*, 1992).

2.1.2.3. Carbon and energy source

Sucrose is probably the most widely used carbohydrate source in plant tissue culture media, but research has been carried out in identifying alternative sources of carbon. Kinnersley and Henderson (1988) reported that higher productivity was obtained with media containing corn syrups than with comparable media containing glucose or sucrose. It was reported that glucose was better for shoot proliferation, while sucrose for

rooting (Marino *et al.*, 1993; Mukherjee *et al.*, 1991). Biomass production was the maximum when the initial sucrose concentration was 1-3 % for callus growth in Ginseng (Choi *et al.*, 1994). The greatest biomass was obtained with 1% total carbohydrates (0.5 % fructose and 0.5 % sucrose) in *Taxus brevifolia* (Ketchus *et al.*, 1995) and an initial concentration of 4– 6%, which was 60 % greater than in 3 % sucrose medium (Yu Shao Xiong *et al.*, 1996). Glucose containing media was either superior or almost equal to sucrose containing media in inducing shoot organogenesis for 2 genotypes of Rose (Chi-ni Hsia and Korban, 1996).

2.1.2.4. Gelling agents

Agar is the widely used gelling agent in *in vitro* studies. Owen *et al.* (1991) reported that addition of gelling agents increased the post – autoclave pH of MS medium containing sucrose and could be alleviated by adjusting the pH after their addition but prior to autoclaving. Six cultivars of apple and two of raspberry produced equal or significantly better shoot proliferation on MS medium gelled with a mixture of cornstarch and Gelrite than on the same medium with agar (Zimmerman *et al.*, 1995). *Taxus medica* and *Agrostis stolonifer* calluses cultivated on liquid media with cotton fibres as medium support (25 ml medium / g cotton) grew better than calluses on agar (0.8 % W/V) and there was no significant difference between shoot organogenesis (Moraes – Cerdeira *et al.*, 1995).

2.1.2.5. Vitamins

The vitamins mostly used in plant tissue culture media are thiamine, nicotinic acid and pyridoxine. However, Murashige and Skoog (1962) vitamin mixture also contains Myo-inositol, Glycine and Ascorbic acid. Perik (1987) reported that vitamins used in most of the *in vitro* culture media were Myo-inositol, Thiamine, Calcium pentothenate, Folic acid, Riboflavin, Ascorbic acid, Nicotinic acid, Pyridoxine and Biotin.

2.1.2.6. Growth regulators

The success of an *in vitro* system was much dependant 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 cytokinins were lower than auxins, roots were induced, and at intermediate concentrations, the tissue grew as an unorganized callus.

Callus was induced in the presence of Kinetin in *Mimosa tenuiflora* (Villarreal *et al.*, 1993). Similarly high cytokinin and low auxin combination showed the best growth of callus in *Aristolochia bracteolate* (Remeshree *et al.*, 1994) and *Digitalis purpurea* (Palazon *et al.*, 1995). The shoot elongation was more associated with lower NAA/ BAP concentrations and callusing appeared to be linked to higher NAA / BAP concentration in cultures of *Dioscorea spps.* (Bernard Malaurie *et al.*, 1995). Abhyankar and Chinchanikar (1996) reported that a high cytokinin: low auxin ratio proved important for direct organogenesis in leaf discs of *Withania somnifera*. Bud break in nodal explants of *Centella asiatica* was dependent on 6– BAP, but the combination of 22.2 micro M BAP and 2.68 micro M NAA induced the optimum frequency (91 %) of shoot formation as well as shoot number (4 to 5 shoots / node) (Tiwari *et al.*, 2000).

2.1.2.7 Other additives

Some commonly used additives are coconut water, activated charcoal and fruit juices. A slight enhancement in sprouting was observed with addition of 30 % coconut water in *Gymnema sylvestre* (Anu *et al.*, 1994). MS medium supplemented with 0.2 % activated charcoal inhibited phenolic exudation and browning of medium in *Acorus calamus* (Harikrishnan *et al.*, 1997) and prevented hydrolysis of media on autoclaving (Wann *et al.*, 1997).

2.1.2.8 pH

pH of the culture medium effects nutrient availability and uptake, and has been shown to influence a number of plant developmental processes. Most tissue cultures are grown at pH 5.2 to 5.8, with pH adjustments being made prior to autoclaving. Doughall (1980) has reported the cause of pH changes in terms of ammonium (NH_4^+) and nitrate uptake (NO_3^-) from the medium. Skirvin *et al.* (1986) reported significant differences between initial pH levels and pH levels following autoclaving, particularly in the pH range of 5.7 to 8.5.

2.1.2.9 Aeration

Ducos and Pareilleux (1981), Pareilleux and Vinax (1983), and Hegarty *et al.* (1986) studied the effect of the aeration rate on culture growth. Too high and too low rates lead to a sub optimum level in dissolved CO_2 concentration, which inhibited growth. A cotton wool plug is highly permeable for oxygen but the reproducibility of this type of stopper is low. However silicon foam stoppers combine a good permeability with a good reproducibility (Meijer *et al.*, 1986).

2.1.3 Culture environment

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 30μ $E/m^2/s$ gave better results (Unander, 1991). In *Phyllanthus fraternus*, cultures maintained under 14h/10 h daily illumination with white, cool fluorescent light of photon flux 100 $\mu mol/m^2$ /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).

2.1.3.1 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°C.

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

2.1.3.2. 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. A relative humidity of 55 – 69 % was found optimum to induce good response in culture of *Centella asiatica* (Suchitra Banerjee *et al.*, 1999).

2.2. *In vitro* regeneration

2.2.1. Direct organogenesis

Axillary meristem proliferation from nodal segments was obtained in *Datura insignis* (Maria Cristina *et al.*, 1990) and *Adathoda beddomei* (Sudha and Sooriamuthu Seeni, 1994). Direct shoot multiplication was obtained from epidermal layers of

internodal segments without any intermediate callus formation in *Cephaelis ipecacuanha* (Pisipati Yamuna *et al.*, 1993). *In vitro* propagation of *Thevetia peruviana* via direct shoot morphogenesis, without any intermediate callus phase was established by culturing embryonic axis explants on MS medium supplemented with BAP and Kinetin (Kumar and Anjani Kumar, 1995). Nodes and tender leaves of *Plumbago rosea* on MS medium containing BAP (1.5 mg/l) + 1AA (0.5 mg/l) (Harikrishnan and Hariharan, 1996) and *Centella asiatica* (Suchitra Banerjee *et al.*, 1999) have been used for direct shoot multiplication.

2.2.1.1. Multiple shoots

Multiple shoot formation seems to be due to enhanced axillary branching (Hisejima, 1982). A combination of auxins and cytokinins strongly stimulated multiple shoots in *Withania somnifera* (Jayanthi Sen and Sharma, 1991 b), *Aegle marmelos* (Varghese *et al.*, 1993) and *Catharanthus roseus* (Yuan *et al.*, 1994). The maximum number of multiple shoots from nodal segments was achieved on MS medium supplemented with BAP 1 mg / 1 in *Achilles asplenifolia* (Wawrosch *et al.*, 1994) and *Centella asiatica* (Singh *et al.*, 1999).

2.2.2. 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.

2.2.2.1 Callus induction

Becker and Schrall (1980) reported that callusing was observed on all levels of NAA ranging from a moderate response at 1 – 2 mg/l to an extensive response at 3–4 mg/l. Wakhlu and Bajwa (1986) reported that IAA, Kinetin and 2, 4 – D induced callus in *Papaver somiferum.* Guimaraes *et al.* (1989) pointed out that lower concentration of auxin such as 2, 4 - D (0.5 mg /l) stimulated callus proliferation in *Cyphomandra*

betacea, whereas higher concentration of NAA (1 mg / l) favoured callus proliferation in *Trachyspermum ammi* (Sehgal and Syed Abbas, 1994). Young leaf explants produced a compact callus in MS medium supplemented with 0.5 mg 2, 4 - D and 0.5 mg Kinetin / l in *Centella asiatica* (Josekutty, 1998).

2.2.2.2. Regeneration from callus

Many scientists have reported the requirement of optimal concentration of auxins and cytokinins for better regeneration from explant tissues. Green nodular callus of *Hemidesmus indicus* turned morphogenetic upon subculturing on MS medium with 1.5 – 2.0 mgl^{-1} Kinetin along with 10 % coconut milk. Small shoots developed from the compact regions of the callus within 20 days (Sarason *et al.*, 1994). MS medium supplemented with 1.5 mgl^{-1} BAP and 0.25 mgl^{-1} 2, 4-D produced a single shoot along with 4-6 shoot primordial and numerous meristematic protuberances from callus initiated from young rhizomes of *Alpinia calcarata* (Martin and Molly Hariharan, 1997).

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

2.2.2.3.Rooting

Among the auxins, IBA and NAA have been most effective for root induction. High concentrations of auxin inhibited rooting in some species. No significant differences were observed in the rooting response of *Plantago ovata* cultures on MS medium supplemented with 0.5, 1.0 and 2.0 mg/l IBA. (Wakhlu and Barna, 1989). Low ionic strength medium like White's is frequently used for *in vitro* rooting. This may be due to the need for only a small amount of total nitrogen for rooting (George and Sherrington, 1984).

In *Aristolochia indica*, MS medium with IBA induced rhizogenesis, while White's medium containing 2.46 µm IBA induced healthy roots with profuse laterals (Manjula *et al.*, 1997). Excised shoots of *Rauwolfia serpentina* on half-strength MS media supplemented with 1.0 mg/l each of IBA and IAA, rooted within 3 weeks with 100 % success (Roy *et al.*, 1995), whereas in *Centella asiatica* half-strength MS basal medium supplemented with 0.5 mg IAA / l alone induced rooting (Patra *et al.*, 1998).

2.3. Hardening

A potting mixture of soil, sand and pumice gave near 100 per cent survival in regenerated plantlets of *Aloe barbadensis* (Lucia Netali, 1990). Neelam Sharma *et al.* (1991) reported that 6 – 8 week old regenerated plantlets of *Coleus forshohlii* gave 100 per cent survival when they were washed free of agar, dipped in Bavistin and irrigated with ½ MS solution for 1 week.

Light, temperature and relative humidity are the 3 major factors to be controlled during acclimatization. Regenerated plantlets of *Clitoria ternatea* were grown in vermiculite for 15 days before transfer to a mixture of garden soil and sand (1: 3) (Kumar *et al.*, 1993). Plantlets of *Chlorophytum borivilianum* were washed thoroughly with water and maintained under high humidity in mini mist chambers in pots (Purohit *et al.*, 1994). Cultures of *Bacopa monnieri* were hardened on sterile distilled water and tap water sequentially (Tejavathi and Shailaja, 1999). Plantlets have also been successfully established in vermiculite soil in *Vitex negundo* (Thiruvengadam and Jayabalan, 2001).

2.4. Histological studies

Histological examination detected the presence of typical somatic embryos in the calli of *Medicago suffruticosa* (Xiu-Qing Li and Yves Demarly, 1996). Similar observations were made in the sections of calli of *Trachyspermum ammi* (Sehgal and Syed abbas, 1994).

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The present study of "*In vitro* studies in *Centella asiatica* L." was carried out at the Tissue culture laboratory of the Horticultural college and Research Institute, Tamil Nadu Agricultural University, Coimbatore.

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.

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 (IBA and NAA), cytokinins (Kinetin and BAP) and Gibberellins (GA_3) and the surface sterilant mercuric chloride were used in this study.

3.1.4. Plant materials

Plant materials were collected from the medicinal plants park at the Botanic Gardens, 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, and 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 Skoogs 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 200 ml distilled water. Na_2 EDTA solution was boiled added gently to $FeSO_4$ $7H_2O$ and then the volume made upto 500 ml.

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 made up using distilled water. 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 up to the final volume with distilled water.

3.2.1.2.1 Auxins

100 mg each of NAA, IBA were dissolved 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 0.1 N 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 0.1 N Hcl or 0.1N NaOH. The growth regulators at required concentrations were added, homogenized and the medium was then distributed to clean culture tubes of 25 x 150 mm 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 20 minutes (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, petiole bits, root bits, seeds and nodal segments.

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 inside the laminar air flow chamber in sterile conditions. The explant size taken for study were,

i.	Leaf bits (area)	:	0.5 – 1.0 cm^2
ii	Stem bits (length)	:	1 – 1.5 cm
iii.	Petiole bits (length)	:	1 – 1.5 cm
iv.	Root bits (length)	:	1 – 1.5 cm
v.	Nodal segments (length)	:	1 – 1.5 cm
vi.	Seed	:	Fresh

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 Mercuric chloride (0.1 – 1.0 %) solution for 1 – 5 minutes, 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 ultraviolet radiation (253.7°A). The instruments used for inoculation were autoclaved at 15 psi at 120°C for 20 minutes 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 on to 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°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 Completely Randomized Design (CRD), with 4 treatments and 5 replications. For each replication 20 test tubes were inoculated.

3.3.1. Seasonal effect

The inoculation of explant was carried out under different months (Jan – Feb, Mar – April, May – June, July – Aug, Sep – Oct and Nov – Dec) to record condusive 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.

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 5.0 minutes. 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., nodal segments were inoculated in MS basal medium supplemented with BAP (1.0 – 5.0 mgl^{-1}), BAP (1.0 – 5.0 mgl^{-1}) along with Kinetin (1.0 – 5.0 mgl^{-1}), BAP (1.0-5.0 mgl^{-1}) along with NAA (0.1-0.5 mgl^{-1}) and BAP (2.0 mgl^{-1}) with GA_3 (0.25-1.25 mgl^{-1}). Basal medium without any supplements served as the control.

Each treatment consisted of twenty tubes. The cultures were exposed to 1000 lux, and a light and darkness cycle of 16 hours and 8 hours respectively. The culture room temperature was maintained at 25 ± 2°C.

The following observations were recorded at regular intervals:

1. Percentage of responding cultures.
2. Days taken for shooting
3. Number of shoots regenerated per explant.

Visual counts were taken from the 1st week of inoculation.

4. 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, petiole bits and nodal segments. Sucrose (3.0 per cent), agar (0.8 per cent), cytokinin such as BAP (0.1-0.5 mgl^{-1}) along with an auxin NAA (1.0-5.0 mgl^{-1}) were added to the medium. The culture was kept in darkness by covering them with a black cloth.

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 (CI)

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 free of agar and the mean weight in grams was recorded.

Sub culturing was done at 15 days interval for callus proliferation on the medium which induced callusing. Percent response and callus weight were recorded in each subculture.

3.3.5.2. Callus regeneration

Three –four weeks after callus initiation, the callus was transferred to a regeneration medium. The regeneration medium consisted of MS basal medium supplemented with BAP (1.0 – 5.0 mg l^{-1}) and NAA (0.25-1.25 mg l^{-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°C. Sub culturing was done at an interval of 4 weeks.

Observations on percentage response to regeneration, days taken, number of shoots regenerated and length of shoots were recorded.

3.3.6 Rooting

After separating the multiple shoots, each individual shoot was sub cultured onto half MS medium containing various concentrations of IBA (0.25 – 1.25 mgl^{-1}) and NAA (0.25 – 1.25 mgl^{-1}) for the development of roots.

The cultures were exposed to the same physical environment as those of 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).

Rooted plantlets were carefully removed from the culture tubes and placed over a sterile petridish. Root length was measured in centimeters 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 transfer to small perforated plastic containers containing sterilized pot mixture, coir compost, vermiculite and their combinations (1: 1). The transferred plantlets were kept in the culture room for 15 – 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. The plantlets were initially covered with perforated polythene bags for 10 days to prevent. After hardening, the plantlets were transferred to bigger pots containing red earth, sand and farm yard manure (1: 1: 1) and maintained in the green house.

3.3.8. Anatomical studies

With a view to elicit information on the callus and regeneration of shoots, anatomical studies were carried out by adopting the method suggested by Johansen (1960). The callus was collected at different days after inoculation.

3.3.8.1. Killing and fixing

The calluses were killed and fixed in FAA for minimum 12 hours (FAA: 10:50:5:35 proportion of formalin, alcohol, acetic acid and water).

3.3.8.2. Dehydration

After killing and fixing the materials in FAA solution, the buds were washed with 50 per cent ethanol and then transferred to tertiary butyl alcohol series of 60, 70, 80, 99 and 100 per cent for one hour followed by 12 hours in 100 per cent TBA.

3.3.8.3. Infiltration with wax

Dehydrated buds were transferred to TBA + wax series of 2/3 +1/3,1/2+1/2,1/3+2/3 and absolute wax two times for 30 to 45 minutes in each series.

3.3.8.4. Embedding

After infiltration, the material was embedded in wax with melting point of 52 to 54°C . The molten wax was poured into a paper boat, with inner side smeared with glycerine. The infiltrated callus pieces were placed in molten wax in the proper orientation. The blocks were cut in such a way that each block contained one specimen.

3.3.8.5. Sectioning

The blocks were mounted to a microtome holder and sections were taken of 12μ thicknesses, using Spencer's rotary microtome.

3.3.8.6. Dewaxing and staining

The Dewaxing was done using xylene alcohol mixture. The slides containing sections were kept for ½ hour in pure xylene (2 times), ethnol+xylene (1:1), 90, 70, 50 per cent ethanol.

The slides were kept in saffranine solution for 12 hours, then subsequently transferred to 50,70,90 per cent ethanol for 10 minutes in each series. Then the slides were dipped in alcohol: ammonia and alcohol: picric acid mixture. Then the slides were transferred to pure alcohol, 70 per cent alcohol for 2-3 minutes. Fast green solution was added over the sections and stain was drained with clove oil and washed with distilled water. Slides were transferred to alcohol: xylene mixture for 5 minutes and in pure xylene (2 times) for 10 minutes.

3.3.8.7. Mounting

The sections were mounted in neutral synthetic mounting medium (DPX mountant) and air-dried.

3.3.8.8. Photomicroscopy

The sections were observed for different stages.

3.4. Statistical analysis

The observations were tabulated and statistical analysis was carried out in a Completely randomized design, as per Sukhatme and Amble (1985) and the results were interpreted.

EXPERIMENTAL RESULTS

CHAPTER IV

EXPERIMENTAL RESULTS

The results of the present study on standardization of protocol for mass multiplication of *Centella asiati*ca L. are presented in this chapter.

4.1. Explant standardization

4.1.1. Culture response

The explants like nodal segments, leaf bits, petiole bits, stem bits, root bits and seeds were sourced from the stock plant, that were maintained for the purpose of micro propagation. Among the various explants used, nodal segments gave the highest significant response (90.18 %), followed by leaf bits (81.45 %), petiole bits (77.22 %), stem bits (62.56 %) and root bits (20.20 %). Seeds did not respond (Table 1, Figure 1).

4.1.2. Contamination

Leaf bits were significantly superior in combating contamination, with the least rate of contamination (20.00 %), followed by nodal segments (24.00 %), petiole bits (29.20 %) and stem bits (33.80 %). The highest contamination was recorded in seeds (51.00 %) followed by root bits (50.80) (Table 1, Figure 1).

4.1.3. Culture survival

Of the various explants observed for survival under *in vitro* conditions, leaf bits showed the highest survival (61.00 %), followed by nodal segments (53.00 %), petiole bits (43.80 %), stem bits (39.20 %) and root bits (24.20 %). Seeds did not survive under *in vitro* conditions (Table 1, Figure 1).

4.1.4. Mortality

Leaf bits recorded the least mortality (19.00 %), closely followed by nodal segments (23.00 %). Not much of a difference was recorded in the mortality of root bits

Table 1. Standardisation of explants for micro propagation of *Centella asiatica* L.

Explants	Culture response (%)	Contamination (%)	Culture survival (%)	Mortality (%)
Nodal segments	90.18 (71.74) a	24.00 (29.33) d	53.00 (46.72) b	23.00 (28.65) e
Leaf bits	81.45 (64.49) b	20.00 (26.56) e	61.00 (51.35) a	19.00 (25.84) f
Petiole bits	77.22 (61.49) c	29.20 (32.71) c	43.80 (41.44) c	26.20 (30.79) c
Stem bits	62.56 (52.27) d	33.80 (35.54) b	39.20 (38.76) d	27.00 (31.31) b
Root bits	20.20 (26.71) e	50.80 (45.46) a	24.20 (29.47) e	25.00 (30.00) d
Seeds	00.00 (00.18) f	51.00 (45.57) a	00.00 (00.18) f	49.00 (44.42) a
Mean	**55.27 (46.15)**	**34.80 (35.86)**	**36.86 (34.65)**	**28.20 (31.84)**
SEd	0.413 (0.294)	0.200 (0.121)	0.200 (0.122)	0.115 (0.075)
CD (0.05)	0.852 (0.608)**	0.412 (0.249)**	0.412 (0.252)**	0.238 (0.154)**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

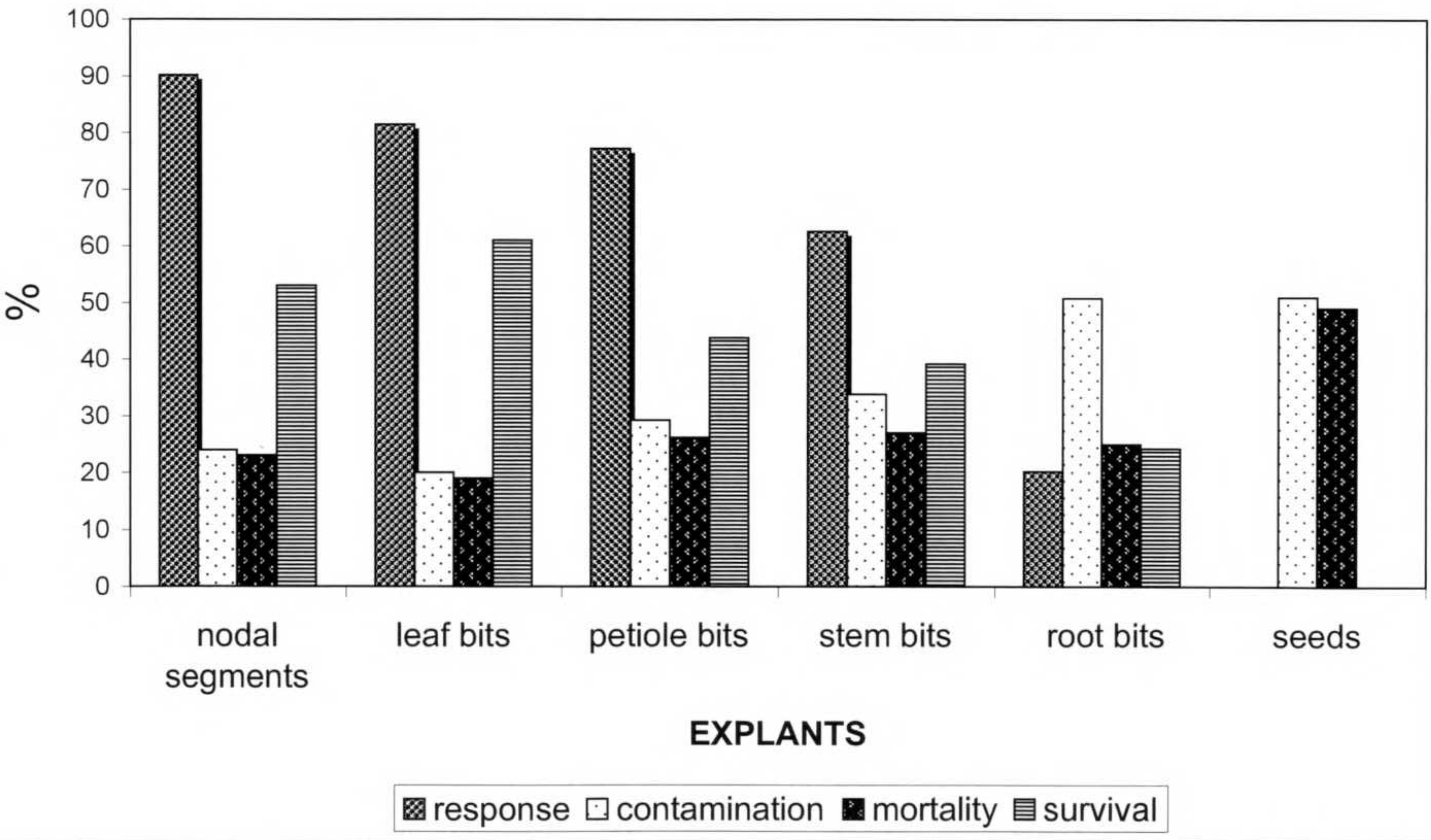


Figure 1.Standardisation of explants for micro propagation of *Centella asiatica* L.

(25.00 %), petiole bits (26.20 %) and stem bits (27.00 %). Seeds showed the highest mortality (49.00 %) (Table 1, Figure 1).

4.2. Effect of season on explant growth

4.2.1. Culture survival

Explants inoculated during the months of May – June gave significantly higher survival (74.00 %), followed by January – February (66.00 %). The survival during January – February (66.00 %) was on par with March – April (66.00 %). The least survival per cent (29.00 %) was observed during the months of September – October (Table 2, Figure 2)

4.2.2. Contamination

The least contamination was observed during May – June (26.00 %), followed by January – February (34.00 %) and March – April (34.00 %). Highest contamination rate was observed during September – October (71.00 %) (Table 2, Figure 2).

4.3. Sterilization of explants

4.3.1. Contamination

All the four types of explants used showed the least contamination (5.0 %) at 1.0 % $HgCl_2$ for a duration of 5 minutes. Maximum contamination was observed in nodal segments (88.00 %) at 0.1 % $HgCl_2$ treated for 1 minute. Similarly contamination was more in all the other explants like leaf bits (86.00 %), stem bits (85.00 %) and petiole bits (82.00 %) at 0.1 % $HgCl_2$ for 1 minute (Table 3).

4.3.2. Survival

Petiole bits recorded maximum survival (88.00 %) at 0.5 % $HgCl_2$ for 1 minute, followed by stem bits (85.00 %) at 0.5 % $HgCl_2$ for 1 minute, nodal segments (83.00 %) at 1.0 % $HgCl_2$ for 1 minute and leaf bits (72.00 %) at 0.5 % $HgCl_2$ for 3 minutes. The survival percent decreased as the concentration and duration of $HgCl_2$ treatment increased, in all the explants (Table 3).

Table 2. Effect of season on explant growth in *Centella asiatica* L.

Season	Culture Survival (%)	Contamination (%)
January – February	66.00 (54.34) b	34.00 (35.66) d
March – April	66.00 (54.34) b	34.00 (35.66) d
May – June	74.00 (59.36) a	26.00 (30.64) e
July – August	51.00 (45.57) c	49.00 (44.42) c
September – October	29.00 (32.57) e	71.00 (57.43) a
November – December	41.00 (39.81) d	59.00 (50.19) b
Mean	**54.50 (47.67)**	**45.50 (42.33)**
SEd	1.414 (0.864)	1.414 (0.864)
CD (0.05)	2.918 (1.783)**	2.918 (1.783)**

Values in parentheses are arcsine-transformed
Column figures followed by different letters are significantly different from each other at 5% level

Figure 2.Effect of season on explant growth of *Centella asiatica* L.

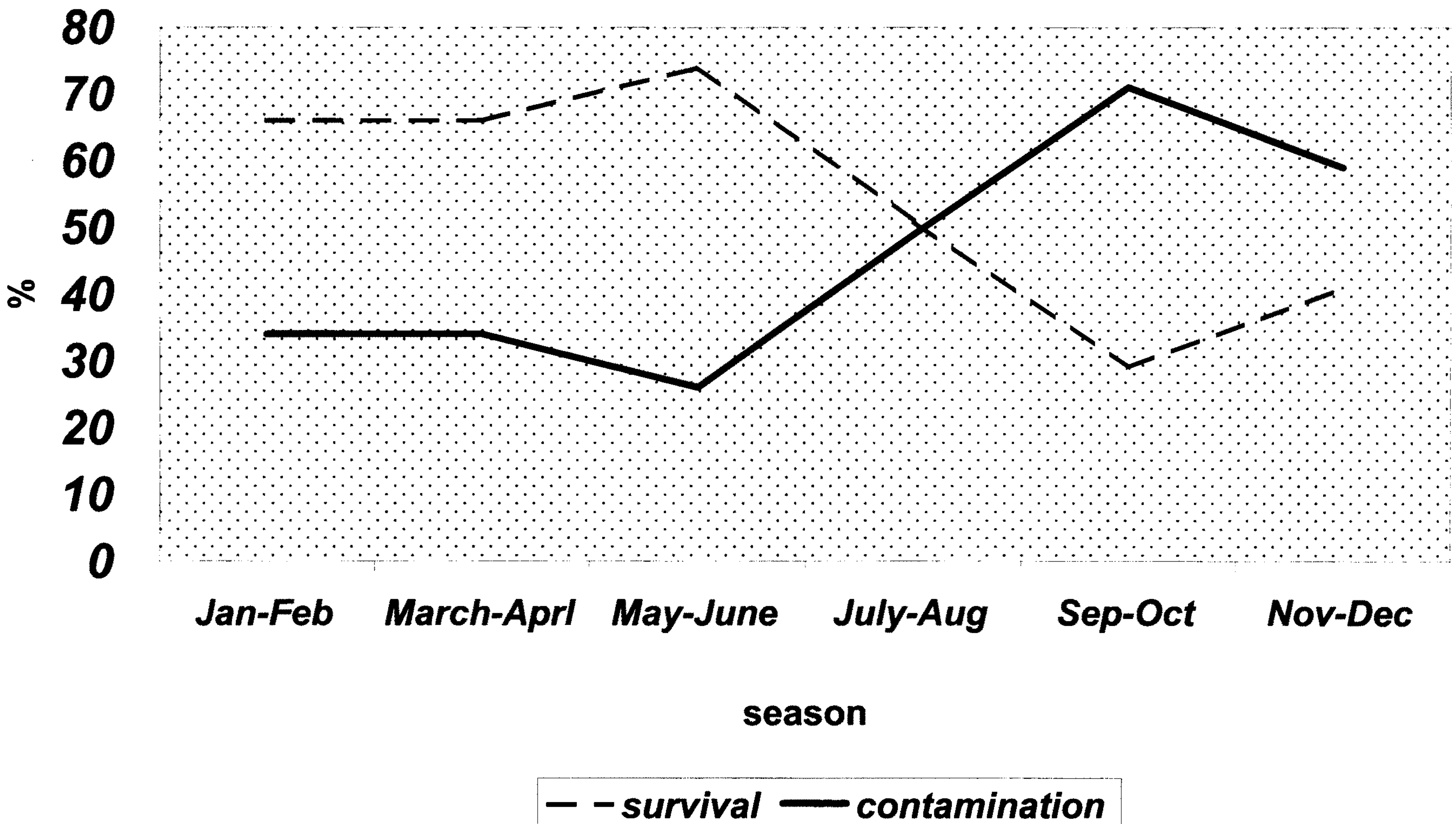


Table 3. Sterilization fixation of various explants of *Centella asiatica* L.

Explants	Duration of Exposure (min)	Contamination (%)			Survival (%)			Mortality (%)		
		$HgCl_2$ concentration (%)								
		0.10	0.50	1.00	0.10	0.50	1.00	0.10	0.50	1.00
Nodal segment	1	88.00	52.00	11.00	6.00	30.00	83.00	6.00	18.00	6.00
	3	78.00	43.00	7.00	13.00	37.00	52.00	9.00	20.00	41.00
	5	68.00	34.00	5.00	23.00	33.00	35.00	9.00	33.00	60.00
Leaf bits	1	86.00	21.00	8.00	9.00	64.00	42.00	5.00	15.00	50.00
	3	80.00	8.00	7.00	11.00	72.00	40.00	9.00	20.00	53.00
	5	46.00	7.00	5.00	39.00	65.00	36.00	15.00	28.00	59.00
Petiole bits	1	82.00	5.00	5.00	13.00	88.00	52.00	5.00	7.00	43.00
	3	65.00	5.00	5.00	28.00	75.00	30.00	7.00	20.00	65.00
	5	32.00	5.00	5.00	57.00	65.00	10.00	11.00	30.00	85.00
Stem bits	1	85.00	5.00	8.00	10.00	85.00	26.00	5.00	15.00	66.00
	3	32.00	7.00	5.00	60.00	53.00	17.00	8.00	40.00	78.00
	5	7.00	6.00	5.00	84.00	42.00	8.00	9.00	52.00	87.00

Statistically not analysed

4.3.3. Mortality

All the four explants recorded minimum mortality at lower concentration of $HgCl_2$, treated for a lesser duration. The mortality of leaf bits (5.0 %), petiole bits (5.0 %), stem bits (5.0 %) and nodal segments (6.0 %) was the lowest at 0.1 % $HgCl_2$ for 1 minute. The maximum mortality in stem bits (87.00 %), followed by petiole bits (85.00 %), nodal segments (60.00 %) and leaf bits (59.00 %) was recorded at 1.0 % $HgCl_2$ for 5 minutes (Table 3).

4.4. DIRECT ORGANOGENESIS – MICROSHOOTING

4.4.1. Effect of BAP on shooting in nodal segments

4.4.1.1. Culture response

No response was recorded in control T_0 (0.0 mgl^{-1}) where BAP was not supplemented with MS medium. Significantly maximum response (89.00 %) was recorded at an optimum BAP concentration of 2.0 mgl^{-1} (T_2). BAP concentrations of 3.0 mgl^{-1}(T_3) and 1.0 mgl^{-1} (T_1) also recorded good response of 69.00 per cent and 64.00 per cent, respectively. The response was the lowest (39.00 %) at the highest concentration of 5.0 mgl^{-1} (T_5) among the treatments (Table 4, Figure 3, Plates 3-4).

4.4.1.2. Days taken for shooting

Earliest response for shooting (6.85 days) was observed at a BAP concentration of 2.0 mgl^{-1} (T_2). Time taken for shooting at 1.0 mgl^{-1} (9.16 days) and 3.0 mgl^{-1} (9.28 days) were on par. Cultures at the highest concentration of 5.0 mgl^{-1} (T_5) took the maximum number of days (16.32 days) (Table 4, Fig. 3).

4.4.1.3. Number of leaves

A maximum of 3.04 leaves per explant was recorded at 2.0 mgl^{-1} BAP (T_2). The number of leaves (2.16) at 3.0 mgl^{-1} (T_3) and 1.0 mgl^{-1} (T_1) were on par. A minimum of 1.0 leaf per explant was recorded at 5.0 mgl^{-1} (T_5) BAP (Table 4, Figure 3).

Table 4. Effect of BAP on shooting in nodal segments of *Centella asiatica* L.

Treatment (Concentration of BAP) (mgl^{-1})	Culture response (%)	Days taken for shooting	No. of leaves	Length of shoot (cm)
T_0 (0.0)	00.00 (00.18) f	0.00 e	0.00 e	0.00 e
T_1 (1.0)	64.00 (53.13) c	9.16 c	2.16 b	1.43 d
T_2 (2.0)	89.00 (70.69) a	6.85 d	3.04 a	2.36 a
T_3 (3.0)	69.00 (56.18) b	9.28 c	2.16 b	2.04 b
T_4 (4.0)	49.00 (44.42) d	14.48 b	1.12 c	1.52 c
T_5 (5.0)	39.00 (38.64) e	16.32 a	1.00 d	1.50 c
Mean	**51.67 (43.87)**	**9.35**	**1.58**	**1.48**
SEd	1.291 (0.849)	0.068	0.045	0.033
CD (0.05)	2.664 (1.752)**	0.141**	0.090**	0.068**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

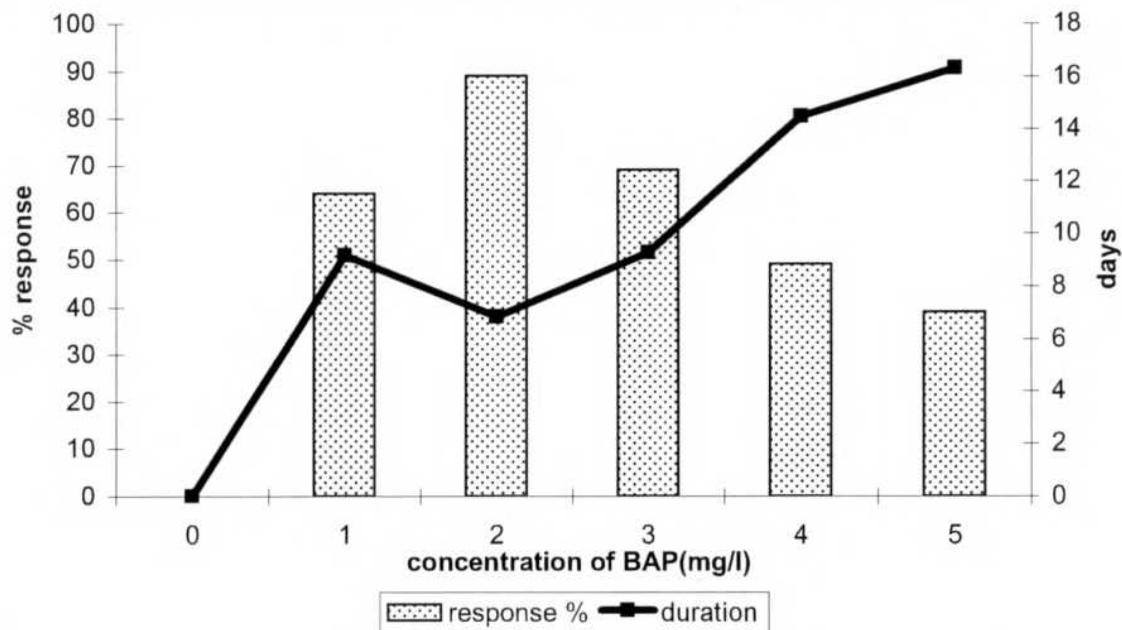


Figure 3(a). Effect of BAP on shooting in nodal segments of Centella asiatica L.
% response
days
100
90
80
70
60
50
40
30
20
10
0
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
concentration of BAP(mg/l)
response %
duration

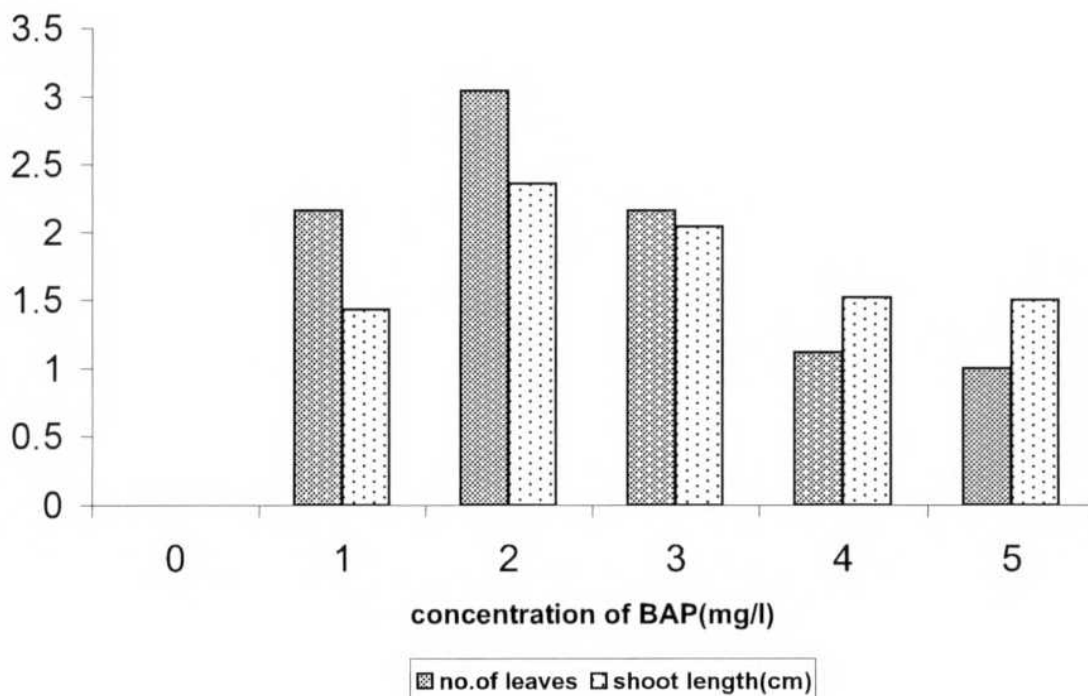


figure 3b
3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
concentration of BAP(mg/l)
no.of leaves
shoot length(cm)

4.4.1.4. Length of shoot

A concentration of 2.0 mgl^{-1} BAP (T_2) was found optimum for maximum shoot length of 2.36 cm. The shoot length gradually decreased towards extreme concentrations of 1.0 mgl^{-1} (T_1) and 5.0 mgl^{-1} (T_5) BAP, to 1.43 and 1.50 cm respectively (Table 4, Figure 3).

4.4.2. Effect of BAP and Kinetin on shooting in nodal segments

4.4.2.1. Culture response

A significantly maximum response (91.00 %) was recorded when the lowest concentrations of BAP and Kinetin were used in treatment T_1 (1.0 + 1.0 mgl^{-1}). It was followed by a much lesser response (65.00 %) at 2.0 mgl^{-1} each of BAP and Kinetin (T_2). The lowest response (11.00 %) was observed at 5.0 mgl^{-1} BAP and Kinetin (T_5). No response was observed in the absence of growth regulators (T_0) (Table 5,Plate 5).

4.4.2.2. Days taken for shooting

The treatment where a minimum concentration (1.0 mgl^{-1}) of BAP and kinetin were used (T_1) recorded the least number of days (6.68) for shooting from nodal segments. It took a maximum of 19.48 days for shooting where high concentrations (5.0 mgl^{-1}) of BAP and Kinetin were used (T_5) (Table 5).

4.4.2.3. Number of leaves

A maximum of 2.56 leaves per explant was recorded when 1.0 mgl^{-1} each of BAP and Kinetin were used (T_1). Treatments with 4.0 mgl^{-1} (T_4) and 5.0 mgl^{-1} (T_5) each of BAP and Kinetin were on par producing 1.0 leaf per explant (Table 5).

4.4.2.4. Length of shoot

A maximum shoot length (2.42 cm) was observed in T_1 (1.0 + 1.0 mgl^{-1} BAP and Kinetin) and minimum shoot length (1.21 cm) was observed in T_5 (5.0 + 5.0 mgl^{-1}) (Table 5).

Table 5. Effect of BAP and Kinetin on shooting in nodal segments of *Centella asiatica* L.

Treatment (Concentration of BAP + Kinetin) (mgl^{-1})	Culture response (%)	Days taken for shooting	No. of leaves	Length of shoot (cm)
T_0 (0.0 + 0.0)	00.00 (00.18) f	0.00 f	0.00 d	0.00 f
T_1 (1.0 + 1.0)	91.00 (72.67) a	6.68 e	2.56 a	2.42 a
T_2 (2.0 + 2.0)	65.00 (53.73) b	9.12 d	1.29 b	2.04 b
T_3 (3.0 + 3.0)	40.00 (39.23) c	13.52 c	1.28 b	1.67 c
T_4 (4.0 + 4.0)	19.00 (25.81) d	16.52 b	1.00 c	1.42 d
T5 (5.0 + 5.0)	11.00 (19.31) e	19.48 a	1.00 c	1.21 e
Mean	**37.67 (35.15)**	**10.88**	**1.18**	**1.46**
SEd	1.00 (0.921)	0.0631	0.003	0.026
CD (0.05)	2.63 (1.901)**	0.130**	0.006**	0.053**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

4.4.3. Effect of BAP and NAA on multiple shoot induction from nodal segments

4.4.3.1. Culture response

A significantly high response (91.00 %) was observed when 3.0 mgl^{-1} BAP and 0.3 mgl^{-1} NAA (T_3) were used. It was closely followed by the treatment using 4.0 mgl^{-1} BAP and 0.4 mgl^{-1} NAA (T_4), where it recorded a response of 81.00 per cent. The least response (31.00 %) was recorded in T_1 (1.0 mgl^{-1} BAP and 0.1 mgl^{-1} NAA). No response was observed where growth regulators were not used (T_0) (Table 6,Plate 6).

4.4.3.2. Days taken for multiple shoot induction

The treatment that showed the highest response (T_3) with 3.0 mgl^{-1} BAP and 0.3 mgl^{-1} NAA, also recorded a significantly minimum number of days (15.64) for multiple shoot induction. The number of days gradually increased with decreasing concentration of growth regulators and the treatment with 1.0 mgl^{-1} BAP and 0.1 mgl^{-1} NAA (T_1) recorded 29.28 days (Table 6).

4.4.3.3. Number of shoots

A maximum of 5.16 shoots was recorded in the treatment T_3 (3.0 mgl^{-1} BAP and 0.3 mgl^{-1} NAA) and the least number of shoots (2.0) in treatment T_5 (5.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA) (Table 6).

4.4.3.4. Length of shoot

Not much variation in shoot length was observed between treatments, but a maximum of 2.45 cm was recorded in the treatment T_3 (3.0 mgl^{-1} BAP and 0.3 mgl^{-1} NAA). A minimum shoot length (2.04 cm) was recorded in the treatment using 1.0 mgl^{-1} BAP and 0.1 mgl^{-1} NAA (T_1). The shoot length (2.35 cm) was on par between treatments T_2 (2.0 mgl^{-1} BAP and 0.2 mgl^{-1} NAA) and T_4 (4.0 mgl^{-1} BAP and 0.4 mgl^{-1} NAA) (Table 6).

Table 6. Effect of BAP and NAA on multiple shoot induction from nodal segments of *Centella asiatica* L.

Treatment (Concentration of BAP + NAA) (mgl^{-1})	Culture response (%)	Days taken for multiple shoot induction	No. of shoots	Length of shoot (cm)
T_0 (0.0 + 0.0)	00.00 (00.18) f	0.00 f	0.00 f	0.00 e
T_1 (1.0 + 0.1)	31.00 (33.82) e	29.28 a	2.24 d	2.04 d
T_2 (2.0 + 0.2)	51.00 (45.57) d	28.52 b	2.68 c	2.37 b
T_3 (3.0 + 0.3)	91.00 (72.67) a	15.64 e	5.16 a	2.45 a
T_4 (4.0 + 0.4)	81.00 (64.19) b	20.12 d	4.08 b	2.35 b
T_5 (5.0 + 0.5)	61.00 (51.36) c	25.44 c	2.00 e	2.20 c
Mean	**52.50 (44.63)**	**19.83**	**2.69**	**1.90**
SEd	1.291 (0.973)	0.078	0.063	0.025
CD (0.05)	2.665 (2.008)**	0.161**	0.130**	0.053**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

4.4.4. Effect of BAP and GA_3 on shoot elongation in nodal segment

4.4.4.1. Culture response

Of the various GA_3 concentrations used, 0.5 mgl^{-1} of GA_3 in combination with 2.0 mgl^{-1} BAP (T_2) gave a significantly good response (79.00 %). The lowest response (6.00 %) was recorded with the highest concentration of GA_3 used (1.25 mgl^{-1}) along with 2.0 mgl^{-1} BAP (T_5). There was no response in control (T_0), in the absence of growth regulators (Table 7,Plate 7).

4.4.4.2. Days taken for shoot elongation

Shoot elongation occurred in a significantly minimum number of days (8.16), when 0.5 mgl^{-1} GA_3 was used along with 2.0 mgl^{-1} BAP (T_2). It was closely followed by treatment T_1 (2.0 mgl^{-1} BAP + 0.25 mgl^{-1} GA_3), where shoot elongation took place in 9.32 days. The maximum number of days for shoot elongation (17.48) was recorded in the treatment T_5, where the highest concentration of GA_3 (1.25 mgl^{-1}) was used (Table 7).

4.4.4.3. Increased shoot length

A visible difference with increased shoot length (3.72 cm) was observed in the treatment where 0.5 mgl^{-1} GA_3 was used along with 2.0 mgl^{-1} BAP (T_2). The lowest increase in shoot length (1.51 cm) was observed in the treatment where 1.25 mgl^{-1} GA_3 with 2.0 mgl^{-1} BAP (T_5) was used (Table 7).

4.4.4.4. Number of leaves

An optimum concentration of GA_3 (0.5 mgl^{-1}) used along with 2.0 mgl^{-1} BAP (T_2) significantly increased the number of leaves (3.96). The number of leaves decreased with increasing concentration of GA_3, and a minimum of 1.0 leaf was recorded with the highest concentration of GA_3 used (1.25 mgl^{-1}) among the treatments (Table 7).

Table 7. Effect of BAP and GA_3 on shoot elongation in nodal segments of *Centella asiatica* L.

Treatment (Concentration of BAP + GA_3) (mgl^{-1})	Culture response (%)	Days taken for shoot elongation	Increased shoot length (cm)	No. of leaves
T_0 (0.0 + 0.0)	00.00 (00.18) f	0.00 f	0.00 f	0.00 f
T_1 (2.0 + 0.25)	69.00 (56.18) b	9.32 d	3.05 c	2.09 b
T_2 (2.0 + 0.50)	79.00 (62.75) a	8.16 e	3.72 a	3.96 a
T_3 (2.0 + 0.75)	51.00 (45.57) c	10.32 c	3.12 b	2.04 c
T_4 (2.0 + 1.00)	24.00 (29.31) d	14.08 b	2.34 d	1.24 d
T_5 (2.0 + 1.25)	6.00 (14.02) e	17.48 a	1.51 e	1.00 e
Mean	**38.17 (34.67)**	**9.89**	**2.29**	**1.72**
SEd	1.291 (0.977)	0.080	0.015	0.007
CD (0.05)	2.664 (2.017)**	0.165**	0.031**	0.014**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

4.5. INDIRECT ORGANOGENESIS

4.5.1. Effect of NAA and BAP on callus induction from nodal segment

4.5.1.1. Cultures forming callus

Nodal segments responded well to callusing with 70.80 per cent of cultures producing callus when 3.0 mgl^{-1} NAA was used along with 0.3 mgl^{-1} BAP (T_3). It was closely followed by T_4 (4.0 mgl^{-1} NAA and 0.4 mgl^{-1} BAP), where 50.20 per cent responded to callusing. The least response (15.20 %) was observed in the treatment with the highest concentration of growth regulators, 5.0 mgl^{-1} NAA and 0.5 mgl^{-1} BAP (T_5). No callusing was observed in the absence of growth regulators (T_0) (Table 8).

4.5.1.2. Relative growth

Profuse callusing (4.12) was observed in cultures inoculated on a medium supplemented with 3.0 mgl^{-1} NAA and 0.3 mgl^{-1} BAP (T_3). Moderate callusing (3.16) was observed in the treatment T_4 (4.0 mgl^{-1} NAA and 0.4 mgl^{-1} BAP). Slight callusing was observed in two treatments, T_2 (2.0 mgl^{-1} NAA and 0.2 mgl^{-1} BAP) and T_5 (5.0 mgl^{-1} NAA and 0.5 mgl^{-1} BAP), where the relative growths were 2.24 and 2.16 respectively. Both the treatments were on par. Poor callusing (1.36) was observed in T_1 where the concentration of NAA and BAP were the lowest and were 1.0 mgl^{-1} and 0.1 mgl^{-1} respectively (Table 8).

4.5.1.3. Callus index

Maximum callusability (292.20) was recorded in the treatment with 3.0 mgl^{-1} NAA and 0.3 mgl^{-1} BAP (T_3). The callusability decreased in both extremes of concentrations of NAA and BAP. It was 28.40 in T_1 (1.0 mgl^{-1} NAA and 0.1 mgl^{-1} BAP) and 32.40 in T_5 (5.0 mgl^{-1} NAA and 0.5 mgl^{-1} BAP (Table 8).

Table 8. Effect of NAA and BAP on callus induction from nodal segments of *Centella asiatica* L.

Treatment (Concentration of NAA + BAP) (mgl^{-1})	Culture response (%)	Relative growth	Callus index	Days taken for callusing
T_0 (0.0 + 0.0)	00.00 (00.18) f	0.00 e	0.00 f	0.00 f
T_1 (1.0 + 0.1)	20.20 (26.70) d	1.36 d	28.40 e	40.42 a
T_2 (2.0 + 0.2)	24.80 (29.86) c	2.24 c	53.60 c	37.16 c
T_3 (3.0 + 0.3)	70.80 (57.29) a	4.12 a	292.20 a	22.05 e
T_4 (4.0 + 0.4)	50.20 (45.11) b	3.16 b	161.00 b	33.80 d
T_5 (5.0 + 0.5)	15.20 (22.94) e	2.16 c	32.40 d	39.86 b
Mean	**30.20 (30.35)**	**2.17**	**94.60**	**28.88**
SEd	0.258 (0.175)	0.065	1.496	0.160
CD (0.05)	0.532 (0.361)**	0.135**	3.089**	0.330**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

4.5.1.4. Days taken for callusing

Callusing was observed in 22.05 days from the date of inoculation when 3.0 mgl^{-1} NAA and 0.3 mgl^{-1} BAP were used (T_3). The longest duration for callusing (40.42 days) was recorded in treatment T_1 (1.0 mgl^{-1} NAA and 0.1 mgl^{-1} BAP) (Table 8).

4.5.1.5. Appearance of callus

The callus from nodal segments was dull white in colour and it was compact.

4.5.2. Effect of NAA and BAP on callus induction from leaf bits

4.5.2.1. Cultures forming callus

Of the four explants used, leaf bits gave the highest significant response (91.00 %), when 3.0 mgl^{-1} NAA and 0.3 mgl^{-1} BAP were used (T_3). The lowest response to callusing (16.00 %) was observed in treatment T_1 (1.0 mgl^{-1} NAA and 0.1 mgl^{-1} BAP). No response to callusing was observed in control (T_0) (Table 9, Figure 4).

4.5.2.2. Relative growth

Leaf bits produced profuse callus (3.96) when moderate concentration of both NAA and BAP were used in T_3 (3.0 mgl^{-1} and 0.3 mgl^{-1}), followed by treatment T_4 (4.0 mgl^{-1} NAA and 0.4 mgl^{-1} BAP) where a relative growth of 3.13 was recorded. Poor callusing (1.24) was observed when the least concentration of NAA and BAP of all treatments were used in T_1 (1.0 mgl^{-1} and 0.1 mgl^{-1}) (Table 9, Figure 4).

4.5.2.3. Callus index

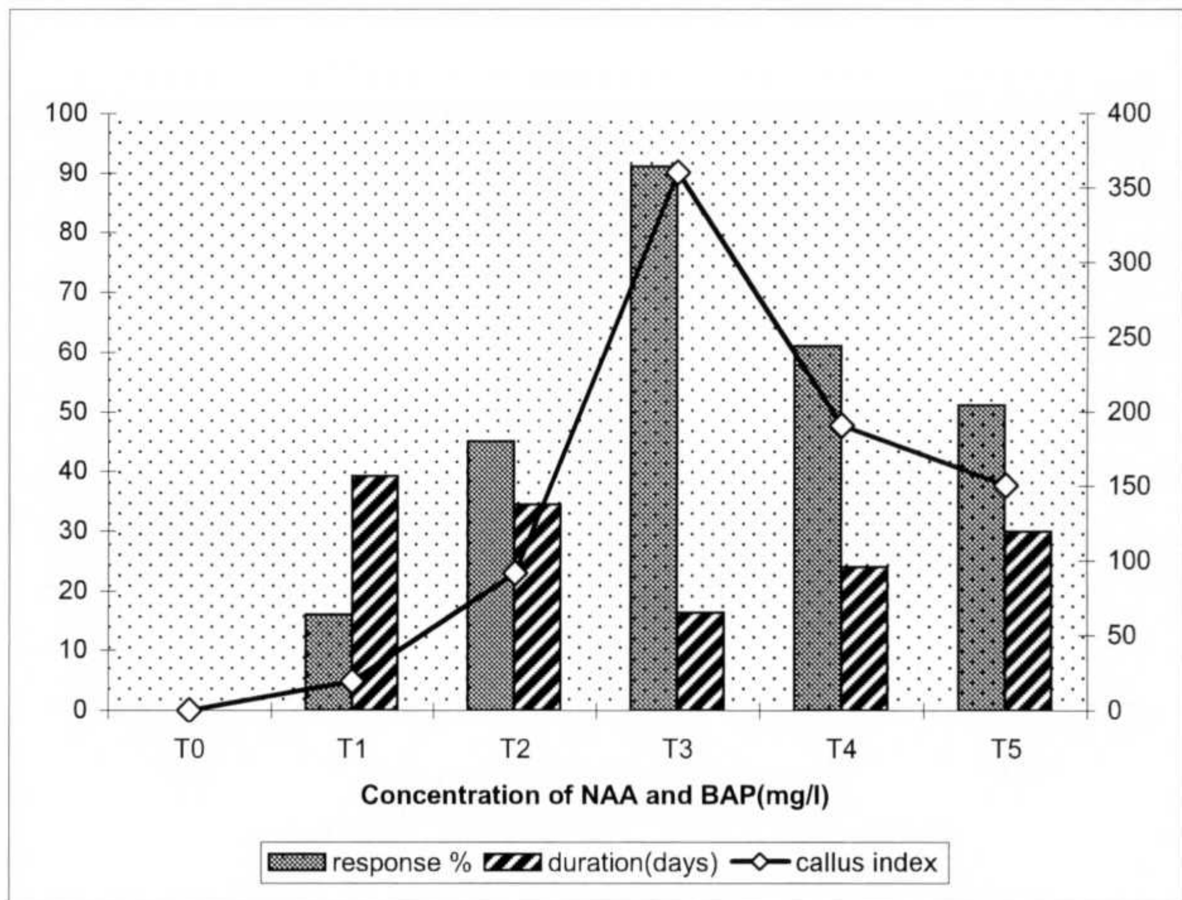
The medium supplemented with 3.0 mgl^{-1} NAA and 0.3mgl^{-1} BAP (T_3) recorded the highest callusability (360.20). A minimum callusability (19.60) was observed at very low concentration of NAA and BAP (1.0 mgl^{-1} and 0.1 mgl^{-1}) (T_1) (Table 9, Figure 4).

Table 9. Effect of NAA and BAP on callus induction from leaf bits of *Centella asiatica* L.

Treatment (Concentration of NAA + BAP) (mgl^{-1})	Culture response (%)	Relative growth	Callus index	Days taken for callusing
T_0 (0.0 + 0.0)	00.00 (00.18) f	0.00 f	0.00 f	0.00 f
T_1 (1.0 + 0.1)	16.00 (23.54) e	1.24 e	19.60 e	39.18 a
T_2 (2.0 + 0.2)	45.00 (42.13) d	2.04 d	91.80 d	34.40 b
T_3 (3.0 + 0.3)	91.00 (72.67) a	3.96 a	360.20 a	16.30 e
T_4 (4.0 + 0.4)	61.00 (51.36) b	3.13 b	190.68 b	23.90 d
T_5 (5.0 + 0.5)	51.00 (45.58) c	2.96 c	150.80 c	29.92 c
Mean	**44.00 (39.24)**	**2.22**	**135.51**	**23.95**
SEd	1.155 (0.907)	0.053	1.011	0.119
CD (0.05)	2.383 (1.872)**	0.109**	2.088**	0.246**

Values in parantheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

Figure 4.Effect of NAA and BAP on callus induction from leaf bits of *Centella asiatica* L.



4.5.2.4. Days taken for callusing

Leaf bits responded to callusing in 16.30 days on a medium supplemented with 3.0 mgl^{-1} NAA and 0.3 mgl^{-1} BAP (T_3). The duration for callusing increased with decreasing concentrations of growth regulators, and it was highest (39.18 days) at 1.0 mgl^{-1} NAA and 0.1 mgl^{-1} BAP (T_1) (Table 9, Figure 4).

4.5.2.5. Appearance of callus

Greenish white callus, which was hard and compact, developed from leaf bits. The callus appeared to be rhizogenic (Plate 8-9).

4.5.3. Effect of NAA and BAP on callus induction from petiole bits.

4.5.3.1. Culture response

The best response to callusing by petiole bits (75.20 %) was observed in the media supplemented with 4.0 mgl^{-1} NAA and 0.4 mgl^{-1} BAP (T_4), followed by 50.00 per cent response with 5.0 mgl^{-1} NAA and 0.5 mgl^{-1} BAP (T_5). The lowest response (10.20 %) was observed with the lowest concentration of NAA and BAP (1.0 mgl^{-1} and 0.1 mgl^{-1}) (T_1). There was no response to callusing in the absence of growth regulators (T_0) (Table 10).

4.5.3.2. Relative growth

Profuse callusing (3.97) was observed with 4.0 mgl^{-1} NAA and 0.4 mgl^{-1} BAP (T_4), followed by 3.17 with 5.0 mgl^{-1} NAA and 0.5 mgl^{-1} BAP. Poor callusing (1.33) was observed with 2.0 mgl^{-1} NAA and 0.2 mgl^{-1} BAP (T_2) (Table 10).

4.5.3.3. Callus index

A significantly high callusability (298.39) was observed with 4.0 mgl^{-1} NAA and 0.4 mgl^{-1} BAP (T_4), followed by 158.30 in a medium supplemented with 5.0 mgl^{-1} NAA and 0.5 mgl^{-1} BAP (T_5). The lowest callusability (14.28) was observed with the lowest concentration of NAA and BAP (T_1)(1.0 mgl^{-1} and 0.1 mgl^{-1}).

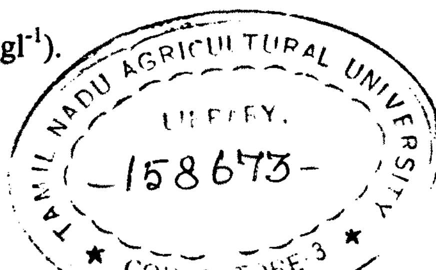

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Table 10. Effect of NAA and BAP on callus induction from petiole bits of *Centella asiatica* L.

Treatment (Concentration of NAA + BAP) (mgl^{-1})	Culture response (%)	Relative growth	Callus index	Days taken for callusing
T_0 (0.0 + 0.0)	00.00 (00.18) f	0.00 f	0.00 f	0.00 f
T_1 (1.0 + 0.1)	10.20 (18.62) e	1.40 d	14.28 e	39.52 a
T_2 (2.0 + 0.2)	19.80 (26.42) d	1.33 e	26.37 d	37.20 b
T_3 (3.0 + 0.3)	39.80 (39.11) c	2.05 c	81.59 c	29.62 c
T_4 (4.0 + 0.4)	75.20 (60.13) a	3.97 a	298.39 a	22.90 e
T_5 (5.0 + 0.5)	50.00 (45.00) b	3.17 b	158.30 b	26.00 d
Mean	**32.50 (31.57)**	**1.98**	**96.49**	**25.87**
SEd	0.230 (0.171)	0.003	0.600	0.178
CD (0.05)	0.477 (0.352)**	0.006**	1.239**	0.368**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

4.5.3.4. Days taken for callusing

Petiole bits responded to callusing in 22.90 days in a medium supplemented with 4.0 mgl^{-1} NAA and 0.4 mgl^{-1} BAP (T_4). The duration for callusing (39.52) increased with decreasing concentrations of NAA and BAP (1.0 mgl^{-1} and 0.1 mgl^{-1})(T_1) (Table 10).

4.5.3.5. Appearance of callus

Petiole bits produced greenish white compact callus (Plate 10).

4.5.4. Effect of NAA and BAP on callus induction from stem bits

4.5.4.1. Culture response

The best response (70.00 %) to callusing was obtained with 4.0 mgl^{-1} NAA and 0.4 mgl^{-1} BAP (T_4). The lowest response (10.00 %) to callusing was observed at a lower concentration of NAA and BAP (1.0 mgl^{-1} and 0.1 mgl^{-1})(T_1). The medium without growth regulators (T_0) did not initiate callusing (Table 11).

4.5.4.2. Relative growth

Profuse callusing (3.97) was observed with 4.0 mgl^{-1} NAA and 0.4 mgl^{-1} BAP (T_4), followed by 3.40 with 5.0 mgl^{-1} NAA and 0.5 mgl^{-1} BAP (T_5). Poor callusing (1.25) was observed with 2.0 mgl^{-1} NAA and 0.2 mgl^{-1} BAP (T_2) (Table 11).

4.5.4.3. Callus index

A significantly high callusability (277.76) was recorded in the medium supplemented with 4.0 mgl^{-1} NAA and 0.4 mgl^{-1} BAP (T_4). The lowest callusability (14.20) was observed in treatment T_1 (1.0 mgl^{-1} NAA and 0.1 mgl^{-1} BAP) (Table 11).

4.5.4.4. Days taken for callusing

Stem bits inoculated on a medium supplemented with 4.0 mgl^{-1} NAA and 0.4 mgl^{-1} BAP, produced callus in 22.90 days. The number of days increased with decreasing concentration of NAA and BAP. 44.20 days were recorded for callusing with 1.0 mgl^{-1} NAA and 0.1 mgl^{-1} BAP (T_1) (Table 11).

Table 11. Effect of NAA and BAP on callus induction from stem bits of *Centella asiatica* L.

Treatment (Concentration of NAA + BAP) (mgl^{-1})	Culture response (%)	Relative growth	Callus index	Days taken for callusing
T_0 (0.0 + 0.0)	00.00 (00.18) f	0.00 f	0.00 f	0.00 f
T_1 (1.0 + 0.1)	10.00 (18.43) e	1.42 d	14.20 e	44.20 a
T_2 (2.0 + 0.2)	19.00 (25.81) d	1.25 e	23.75 d	42.10 b
T_3 (3.0 + 0.3)	40.00 (39.23) c	2.37 c	94.72 c	35.90 c
T_4 (4.0 + 0.4)	70.00 (56.79) a	3.97 a	277.76 a	22.90 e
T_5 (5.0 + 0.5)	45.00 (42.13) b	3.40 b	153.00 b	26.90 d
Mean	**30.67 (30.43)**	**2.07**	**93.91**	**28.67**
SEd	0.577 (0.436)	0.013	0.769	0.163
CD (0.05)	1.192 (0.900)**	0.026**	1.587**	0.337**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

4.5.4.5. Appearance of callus

Stem bits produced dirty white compact callus (Plate 11).

4.5.5. Effect of sub culture on callus proliferation from nodal segments and leaf bits

4.5.5.1. Culture response

Callus obtained from nodal segments and leaf bits was sub cultured five times at 15 days interval on a media supplemented with 3.0 mgl^{-1} NAA and 0.3 mgl^{-1} BAP. A significant 100.00 per cent response to callus proliferation was observed during the first two subcultures in both nodal segment and leaf bits. This response gradually declined on subsequent subcultures in both the explants. The response was reduced to 50.20 per cent and 41.20 per cent in nodal segments and leaf bits respectively at the fifth subculture (Table 12).

4.5.5.2. Weight of callus

The maximum callus weight in both the explants was recorded at the first subculture, which were 3.17 g and 3.50 g for nodal segments and leaf bits respectively. The callus weight gradually reduced to 0.58 g and 0.66 g for nodal segments and leaf bits respectively at the fifth subculture (Table 12).

4.5.6. Effect of subculture on callus proliferation from petiole bits and stem bits.

4.5.6.1. Culture response

Callus from petiole bits and stem bits was sub cultured five times at 15 days interval on a media supplemented with 4.0 mgl^{-1} NAA and 0.4 mgl^{-1} BAP. The response to callus proliferation was cent percent in both the explants, till the second subculture. This response gradually decreased with increasing number of subcultures. The response recorded at the fifth subculture was 50.80 per cent and 50.00 per cent for petiole and stem bits respectively (Table 13, Figure 5).

Table 12. Effect of subculture on callus proliferation from nodal segments and leaf bits of *Centella asiatica* L.

Treatment (Concentration of NAA + BAP) (mgl^{-1})	Number of subcultures (15 days interval)	Nodal segments		Leaf bits	
		Culture response (%)	Weight of callus (g / tube)	Culture response (%)	Weight of callus (g / tube)
3.0 + 0.3	1	100.00 (89.36) a	3.17 a	100.00 (89.36) a	3.50 a
3.0 + 0.3	2	100.00 (89.36) a	2.82 b	100.00 (89.36) a	3.16 b
3.0 + 0.3	3	91.60 (73.16) b	1.32 c	80.60 (63.87) b	2.28 c
3.0 + 0.3	4	68.20 (55.67) c	0.91 d	54.80 (47.75) c	0.78 d
3.0 + 0.3	5	50.20 (45.12) d	0.58 e	41.20 (39.93) d	0.66 e
Mean		**82.00 (70.53)**	**1.76**	**75.32 (66.06)**	**1.73**
SEd		0.236 (0.191)	0.013	0.236 (0.152)	0.002
CD (0.05)		0.493 (0.399)**	0.028**	0.493 (0.318)**	0.005**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

Table 13. Effect of subculture on callus proliferation from petiole bits and stem bits of *Centella asiatica* L.

Treatment (Concentration of NAA + BAP) (mgl^{-1})	Number of subcultures (15 days interval)	Petiole bits		Stem bits	
		Culture response (%)	Weight of callus (g / tube)	Culture response (%)	Weight of callus (g / tube)
4.0 + 0.4	1	100.00 (89.36) a	2.46 a	100.00 (89.36) a	2.18 a
4.0 + 0.4	2	100.00 (89.36) a	2.25 b	100.00 (89.36) a	2.02 b
4.0 + 0.4	3	70.20 (56.91) b	1.90 c	70.20 (56.91) b	1.52 c
4.0 + 0.4	4	62.20 (52.06) c	1.42 d	60.20 (50.88) c	0.92 d
4.0 + 0.4	5	50.80 (45.46) d	0.90 e	50.00 (45.00) d	0.62 e
Mean		**76.64 (66.63)**	**1.78**	**76.08 (66.30)**	**1.45**
SEd		0.358 (0.208)	0.013	0.179 (0.108)	0.028
CD (0.05)		0.746 (0.434)**	0.027**	0.373 (0.226)**	0.059**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

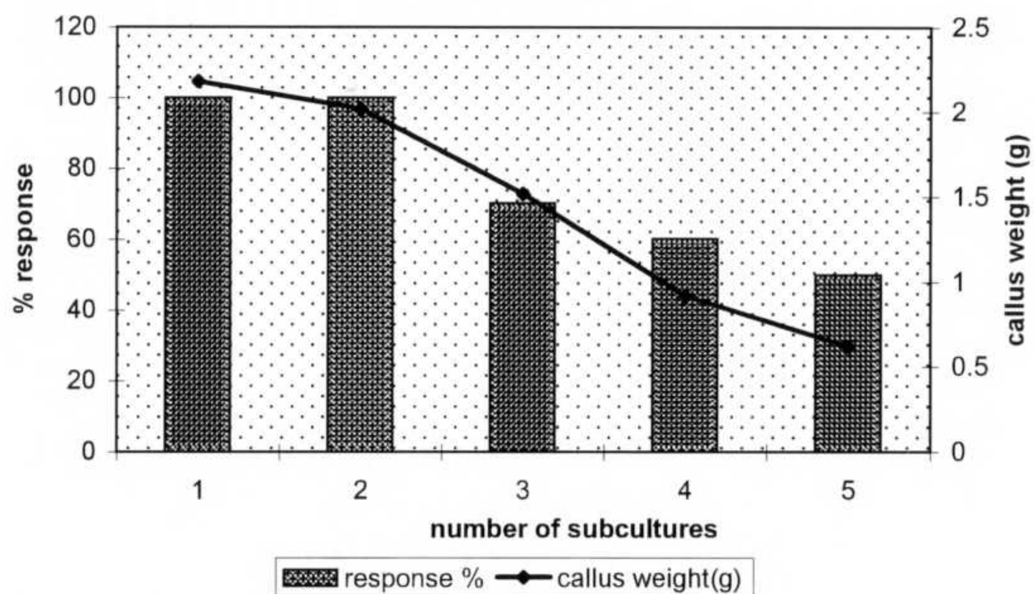
Figure 5(a).Effect of subculture on callus proliferation in stem bits of *Centella asiatica* L.
120
100
80
60
40
20
0
% response
2.5
2
1.5
1
0.5
0
callus weight (g)
1
2
3
4
5
number of subcultures
response %
callus weight(g)

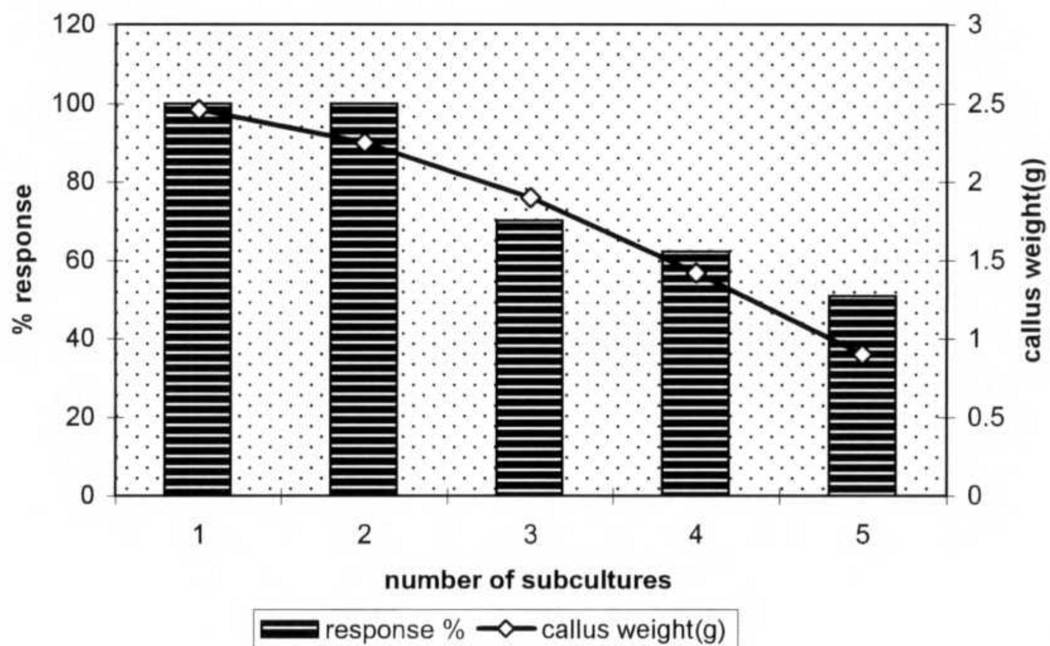
Figure 5(b).Effect of subculture on callus proliferation from petiole bits of *Centella asiatica* L.
120
100
80
60
40
20
0
% response
3
2.5
2
1.5
1
0.5
0
callus weight(g)
1
2
3
4
5
number of subcultures
response %
callus weight(g)

4.5.6.2. Weight of callus

Maximum callus weight of 2.46 g in petiole bits and 2.18 g in stem bits was recorded at the first subculture. This gradually declined to 0.90 g in petiole bits and 0.62g in stem bits at the fifth subculture (Table 13, Figure 5).

4.5.7. Effect of BAP and NAA on callus regeneration from nodal segments

4.5.7.1. Culture response to shoot differentiation

At BAP 4.0 mgl^{-1} with NAA 1.0 mgl^{-1} (T_4), 65.20 per cent of callus inoculated regenerated into shoots. It was closely followed by a 55.20 per cent response to regeneration on a media with 5.0 mgl^{-1} BAP and 1.25 mgl^{-1} NAA (T_5). A very low response of 10.20 per cent was recorded at a very low concentration of BAP and NAA (1.0 and 0.25 mgl^{-1})(T_1). No regeneration from callus was observed on a media without growth regulators (T_0) (Table 14, Figure 6,Plate 16-17).

4.5.7.2. Days taken for shoot differentiation

Shoots regenerated from callus in 25.38 days with 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA (T_4). The duration for regeneration increased with decreasing concentration of growth regulators and a maximum of 47.70 days was recorded for regeneration with 1.0 mgl^{-1} BAP and 0.25 mgl^{-1} NAA (T_1) (Table 14, Figure 6).

4.5.7.3. Number of regenerated shoots

A maximum of 3.96 shoots regenerated from callus on media supplemented with 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA (T_4). A minimum of 1 shoot / callus regenerated on a media with 1.0 mgl^{-1} BAP and 0.25 mgl^{-1} NAA (T_1) (Table 14, Figure 6).

4.5.7.4. Length of shoots

The maximum shoot length (3.16 cm) was recorded on a media with 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA (T_4). The lowest shoot length (1.90 cm) was recorded on a media with 1.0 mgl^{-1} BAP and 0.25 mgl^{-1} NAA (T_1) (Table 14, Figure 6).

Table 14. Effect of BAP and NAA on callus regeneration from nodal segments of *Centella asiatica* L.

Treatment (Concentration of BAP and NAA) (mgl^{-1})	Culture response to shoot differentiation (%)	Days taken for shoot differentiation	Number of shoots regenerated	Length of shoots (cm)
T_0 (0.0 + 0.0)	00.00 (00.18) f	0.00 f	0.00 f	0.00 f
T_1 (1.0 + 0.25)	10.20 (18.62) e	47.70 a	1.00 e	1.90 e
T_2 (2.0 + 0.50)	15.00 (22.78) d	44.59 b	2.00 d	2.02 d
T_3 (3.0 + 0.75)	39.80 (39.11) c	35.62 c	2.21 c	2.52 c
T_4 (4.0 + 1.00)	65.20 (53.85) a	25.38 e	3.96 a	3.16 a
T_5 (5.0 + 1.25)	55.20 (47.98) b	28.08 d	2.96 b	2.98 b
Mean	**30.90 (30.42)**	**30.23**	**2.02**	**2.09**
SEd	0.231 (0.159)	0.093	0.036	0.030
CD (0.05)	0.476 (0.329)**	0.192**	0.074**	0.063**

Values in parentheses are arcsine – transformed

Column figures followed by different letters are significantly different from each other at 5% level

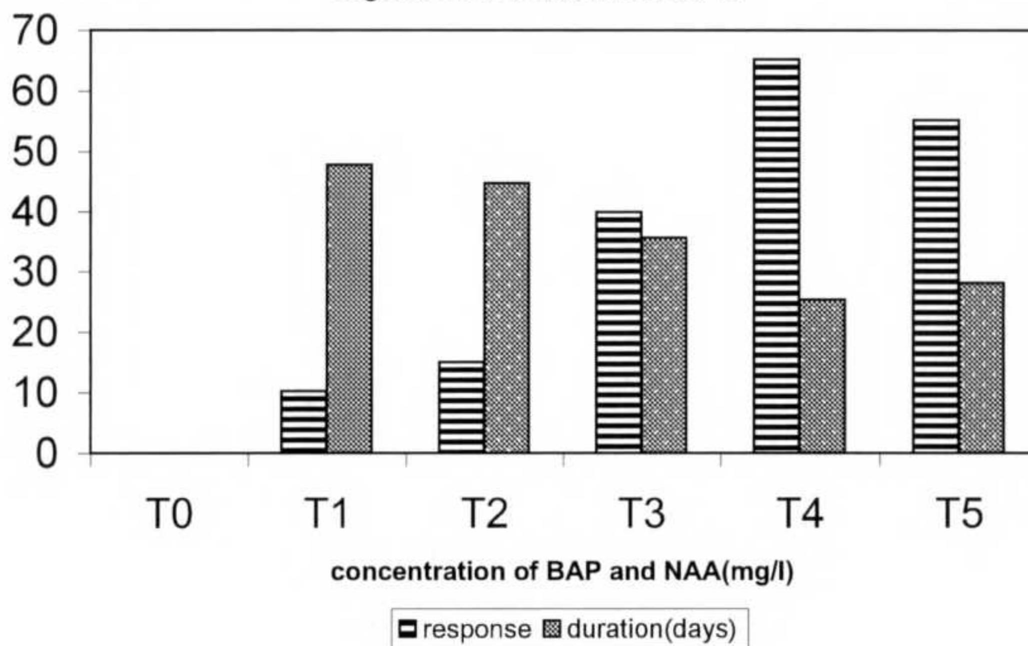
Figure 6(a).Effect of BAP and NAA on callus regeneration from nodal segments of *Centella asiatica* L.
70
60
50
40
30
20
10
0
T0
T1
T2
T3
T4
T5
concentration of BAP and NAA(mg/l)
response
duration(days)

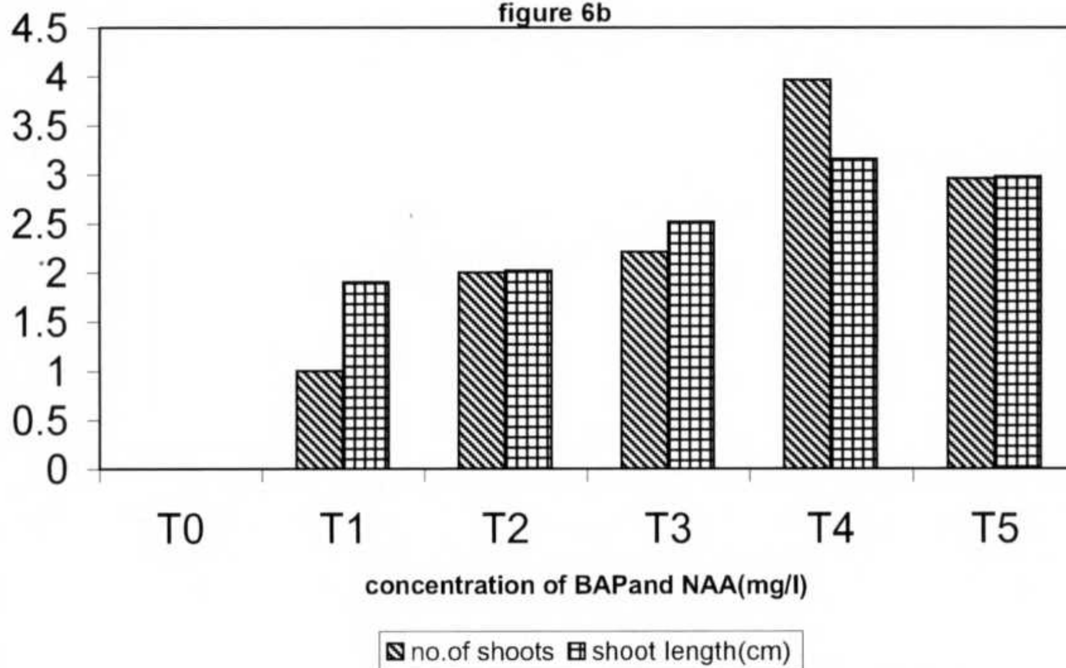
figure 6b
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
T0
T1
T2
T3
T4
T5
concentration of BAPand NAA(mg/l)
no.of shoots
shoot length(cm)

4.5.8. Effect of BAP and NAA on callus regeneration from leaf bits

4.5.8.1. Culture response

Callus from leaf bits did not regenerate into shoots both in control (T_0) and when the lowest concentration of growth regulators (1.0 mgl^{-1} BAP and 0.25 mgl^{-1} NAA) among treatments was used (T_1). A significantly good response (70.20 %) was recorded on a media supplemented with 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA (T_4). The lowest response (15.20 %) to regeneration was observed with 2.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA (T_2) (Table 15).

4.5.8.2. Days taken for shoot regeneration

The least number of days (33.20) for regeneration from callus was observed in two-growth regulator combinations viz., 4.0 mgl^{-1} BAP with 1.0 mgl^{-1} (T_4) and 5.0 mgl^{-1} BAP and 1.25 mgl^{-1} NAA (T_5). Callus regeneration took a maximum of 48.80 days with 2.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA (T_2) (Table 15).

4.5.8.3. Number of regenerated shoots

A maximum of 4.0 shoots / callus was recorded on a media with 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA (T_4). The lowest number of shoots (1.04) regenerated at low concentration of BAP and NAA (2.0 mgl^{-1} and 0.5 mgl^{-1}) (T_2) (Table 15).

4.5.8.4. Length of shoots

The length of shoots ranged from 1.79 cm at 2.0 mgl^{-1} BAP with 0.5 mgl^{-1} NAA (T_2), to a maximum of 3.18 cm at 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA (T_4) (Table 15).

4.5.9. Effect of BAP and NAA on callus regeneration from petiole bits

4.5.9.1. Culture response

Of the different combinations of BAP and NAA the treatment with 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA (T_4) gave the best response (54.80 %) in regenerating shoots from callus.

Table 15. Effect of BAP and NAA on callus regeneration from leaf bits of *Centella asiatica* L.

Treatment (Concentration of BAP and NAA) (mgl^{-1})	Culture response to shoot differentiation (%)	Days taken for shoot differentiation	Number of shoots regenerated	Length of shoots (cm)
T_0 (0.0 + 0.0)	00.00 (00.18) e	0.00 d	0.00 e	0.00 e
T_1 (1.0 + 0.25)	00.00 (00.18) e	0.00 d	0.00 e	0.00 e
T_2 (2.0 + 0.50)	15.20 (22.94) d	48.80 a	1.04 d	1.79 d
T_3 (3.0 + 0.75)	35.20 (36.39) c	40.20 b	2.04 c	2.22 c
T_4 (4.0 + 1.00)	70.20 (56.91) a	33.20 c	4.00 a	3.18 a
T_5 (5.0 + 1.25)	55.20 (47.98) b	33.20 c	3.04 b	2.92 b
Mean	**29.30 (27.43)**	**25.90**	**1.68**	**1.68**
SEd	0.230 (0.151)	0.230	0.040	0.017
CD (0.05)	0.476 (0.312)**	0.476**	0.082**	0.036**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

Table 16. Effect of BAP and NAA on callus regeneration from petiole bits of *Centella asiatica* L.

Treatment (Concentration of BAP and NAA) (mgl^{-1})	Culture response to shoot differentiation (%)	Days taken for shoot differentiation	Number of shoots regenerated	Length of shoots (cm)
T_0 (0.0 + 0.0)	00.00 (00.18) e	0.00 d	0.00 e	0.00 e
T_1 (1.0 + 0.25)	00.00 (00.18) e	0.00 d	0.00 e	0.00 e
T_2 (2.0 + 0.50)	9.80 (18.24) d	52.20 a	1.00 d	1.59 d
T_3 (3.0 + 0.75)	24.80 (29.86) c	46.20 b	1.96 c	2.36 c
T_4 (4.0 + 1.00)	54.80 (47.75) a	42.80 c	3.96 a	3.19 a
T_5 (5.0 + 1.25)	45.00 (42.13) b	42.80 c	3.00 b	2.98 b
Mean	**22.40 (23.06)**	**30.66**	**1.65**	**1.69**
SEd	0.200 (0.152)	0.230	0.032	0.013
CD (0.05)	0.413 (0.313)**	0.476**	0.067**	0.028**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

The least response (9.80 %) was observed with 2.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA (T_2). No response was observed in treatments T_0 and T_1, where no growth regulators and 1.0 mgl^{-1} BAP with 0.25 mgl^{-1} NAA were added respectively (Table 16).

4.5.9.2. Days taken for regeneration for shoots

The number of days for regeneration (42.80) was similar in two treatments T_4 and T_5, where the media was supplemented with 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA, and 5.0 mgl^{-1} and 1.25 mgl^{-1} NAA respectively. The maximum number of days (52.20) was recorded with 2.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA (T_2) (Table 16).

4.5.9.3. Number of regenerated shoots

The maximum number of shoots (3.96) was recorded on a media with 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA (T_4), closely followed by 3.0 shoots with 5.0 mgl^{-1} BAP and 1.25 mgl^{-1} NAA (T_5). The lowest number of shoots (1.0) was observed with 2.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA (T_2) (Table 16).

4.5.9.4. Length of shoots

The maximum shoot length (3.19 cm) was recorded with 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA (T_4), and the lowest shoot length (1.59 cm) was observed on a media supplemented with 2.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA (T_2) (Table 16).

4.5.10. Effect of BAP and NAA on callus regeneration from stem bit

4.5.10.1. Culture response

The media supplemented with 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA (T_4), recorded the maximum response (40.20 %). The least response (9.80 %) was observed with 2.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA (T_2). No response to regeneration was observed on media without growth regulators (T_0) and with 1.0 mgl^{-1} BAP and 0.25 mgl^{-1} NAA (T_1) (Table 17).

Table 17. Effect of BAP and NAA on callus regeneration from stem bits of *Centella asiatica* L.

Treatment (Concentration of BAP and NAA) (mgl^{-1})	Culture response to shoot differentiation (%)	Days taken for shoot differentiation	Number of shoots regenerated	Length of shoots (cm)
T_0 (0.0 + 0.0)	00.00 (00.18) e	0.00 e	0.00 c	0.00 e
T_1 (1.0 + 0.25)	00.00 (00.18) e	0.00 e	0.00 c	0.00 e
T_2 (2.0 + 0.50)	9.80 (18.24) d	45.80 a	1.00 b	1.68 d
T_3 (3.0 + 0.75)	19.80 (26.42) c	42.20 b	1.04 b	2.08 c
T_4 (4.0 + 1.00)	40.20 (39.35) a	38.20 d	3.04 a	2.69 a
T_5 (5.0 + 1.25)	35.20 (36.39) b	40.20 c	3.00 a	2.59 b
Mean	**17.50 (20.13)**	**27.73**	**1.34**	**1.51**
SEd	0.231 (0.170)	0.231	0.033	0.017
CD (0.05)	0.476 (0.352)**	0.476**	0.067**	0.034**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

4.5.10.2. Days taken for shoot regeneration

The minimum number of days (38.20) for regeneration was recorded with 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA (T_4). The maximum duration (45.80 days) was recorded with 2.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA (T_2) (Table 17).

4.5.10.3. Number of regenerated shoots

A maximum of 3.04 shoots regenerated from callus when supplemented with 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA (T_4), closely followed by 3.0 shoots with 5.0 mgl^{-1} BAP and 1.25 mgl^{-1} NAA (T_5). A medium with 2.0 mgl^{-1} BAP with 0.5 mgl^{-1} NAA (T_2), and 3.0 mgl^{-1} BAP with 0.75 mgl^{-1} NAA (T_3) were on par, producing 1 shoot (Table 17).

4.5.10.4. Length of shoots

The length of regenerated shoots (2.69 cm) was the maximum with 4.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA (T_4) and minimum (1.68 cm) with 2.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA (T_2) (Table 17).

4.6. MICROROOTING

4.6.1. Effect of IBA on rhizogenesis

4.6.1.1. Culture response

A maximum of 84.80 per cent of shoots produced roots when inoculated on ½ MS media supplemented with 0.5 mgl^{-1} IBA (T_2), which was closely followed by a 79.80 per cent response on a media with 0.25 mgl^{-1} IBA (T_1). The lowest response to rhizogenesis (14.80 %) was recorded on a medium supplemented with 1.25 mgl^{-1} IBA (T_5). The shoots did not respond to rhizogenesis in the absence of growth regulators (T_0) (Table 18, Figure 7,Plate 18).

Table 18. Effect of IBA on rhizogenesis in *Centella asiatica* L.

Treatment (Concentration of IBA) (mgl^{-1})	Percentage response to rhizogenesis	Days taken for rooting	Number of roots per shoot	Length of roots (cm)
T_0 (0.0)	00.00 (00.18) f	0.00 e	0.00 f	0.00 e
T_1 (0.25)	79.80 (63.29) b	19.84 c	11.30 b	2.14 b
T_2 (0.50)	84.80 (67.05) a	15.28 d	15.40 a	3.18 a
T_3 (0.75)	45.20 (42.24) c	24.60 b	6.16 c	2.13 b
T_4 (1.00)	20.20 (26.71) d	26.08 a	4.36 d	1.59 c
T_5 (1.25)	14.80 (22.62) e	26.00 a	3.08 e	1.49 d
Mean	**40.80 (37.01)**	**18.63**	**6.71**	**1.76**
SEd	0.258 (0.187)	0.086	0.101	0.016
CD (0.05)	0.533 (0.386)**	0.178**	0.208**	0.034**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

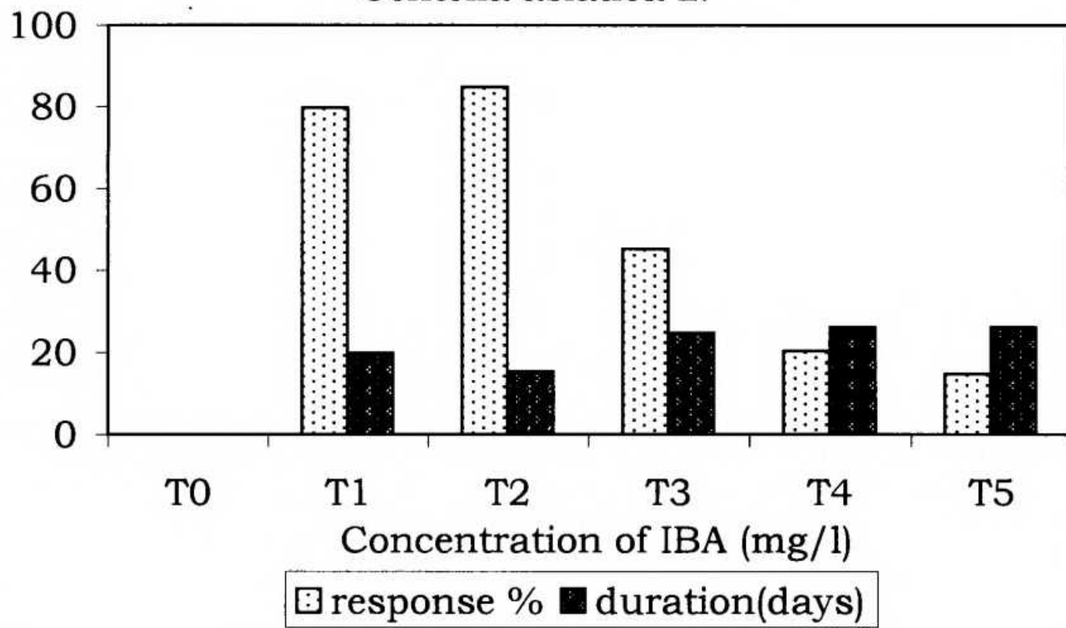
Figure 7(a).Effect of IBA on rhizogenesis of Centella asiatica L.
100
80
60
40
20
0
T0
T1
T2
T3
T4
T5
Concentration of IBA (mg/l)
response %
duration(days)

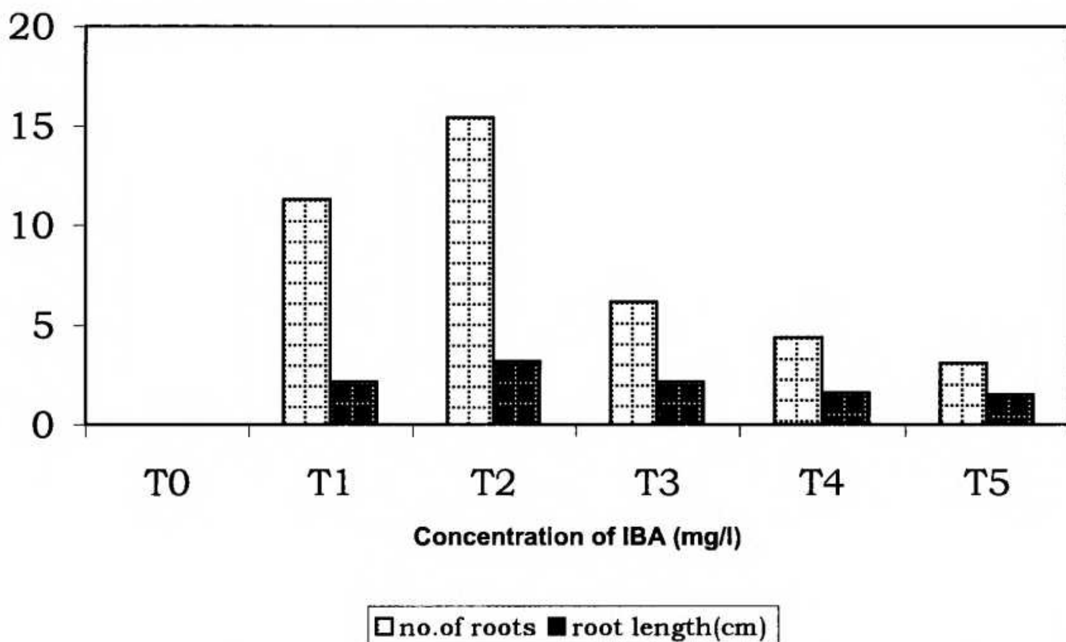
Figure 7b
20
15
10
5
0
T0
T1
T2
T3
T4
T5
Concentration of IBA (mg/l)
no.of roots
root length(cm)

4.6.1.2. Days taken for rooting

Shoots produced roots in 15.28 days on a medium supplemented with 0.5 mgl^{-1} IBA (T_2). The maximum duration of 26.08 and 26.00 days were recorded with 1.0 mgl^{-1} (T_4) and 1.25 mgl^{-1} IBA (T_5) respectively (Table 18, Figure 7).

4.6.1.3. Number of roots

A significantly high number of roots (15.40) were recorded with 0.5 mgl^{-1} IBA (T_2) and the lowest number of roots (3.08) was recorded when supplemented with 1.25 mgl^{-1} IBA (T_5) (Table 18, Figure 7).

4.6.1.4. Length of roots

The maximum root length (3.18 cm) was observed at 0.5 mgl^{-1} IBA (T_2), followed by 2.14 cm at 0.25 mgl^{-1} IBA (T_1). The lowest root length (1.49 cm) was observed at 1.25 mgl^{-1} IBA (T_5) (Table 18, Figure 7).

4.6.2. Effect of NAA on rhizogenesis

4.6.2.1. Culture response

A significantly good response (95.00 %) to rooting was recorded on ½ MS medium with 0.25 mgl^{-1} NAA (T_1). Very low response (9.0 %) was observed when a high concentration of NAA (1.25 mgl^{-1}) was used (T_5). The shoots did not respond to rhizogenesis in the absence of growth regulators (T_0) (Table 19,Plate 19).

4.6.2.2. Days taken for rooting

Shoots responded to rooting in 15.12 days when supplemented with 0.25 mgl^{-1} NAA (T_1), followed by 19.76 days with 0.5 mgl^{-1} NAA (T_2). The maximum duration for rooting (27.32 days) was observed with 1.25 mgl^{-1} NAA (T_5) (Table 19).

Table 19. Effect of NAA on rhizogenesis in *Centella asiatica* L.

Treatment (Concentration of NAA) (mgl^{-1})	Percentage response to rhizogenesis	Days taken for rooting	Number of roots per shoot	Length of roots (cm)
T_0 (0.00)	00.00 (00.18) f	0.00 f	0.00 f	0.00 f
T_1 (0.25)	95.00 (77.08) a	15.12 e	14.56 a	2.84 a
T_2 (0.50)	74.00 (59.36) b	19.76 d	11.84 b	2.47 b
T_3 (0.75)	39.00 (38.64) c	21.16 c	6.32 c	2.08 c
T_4 (1.00)	19.00 (25.81) d	25.76 b	5.08 d	1.58 d
T5 (1.25)	9.00 (17.33) e	27.32 a	2.96 e	1.48 e
Mean	**39.33 (36.40)**	**18.18**	**6.79**	**1.74**
SEd	1.154 (0.922)	0.084	0.056	0.043
CD (0.05)	2.383 (1.903)**	0.175**	0.116**	0.089**

Values in parentheses are arcsine – transformed
Column figures followed by different letters are significantly different from each other at 5% level

4.6.2.3. Number of roots

The maximum number of roots (14.56) per shoot was recorded on a medium with 0.25 mgl^{-1} NAA (T_1), closely followed by 11.84 roots with 0.5 mgl^{-1} NAA (T_2). Only 2.96 roots were produced when a high concentration (1.25 mgl^{-1}) of NAA was used (T_5) (Table 19).

4.6.2.4. Length of roots

The media with 0.25 mgl^{-1} NAA (T_1) produced roots with the maximum length (2.84 cm), followed by 2.47 cm with 0.5 mgl^{-1} NAA (T_2). The lowest length of roots (1.48 cm) was recorded on a medium supplemented with 1.25 mgl^{-1} NAA (T_5)(Table 19).

4.7. Hardening

The maximum survival (88.00 %) was recorded on a combination of vermiculite and pot mixture (1: 1)(T_4). It was closely followed by good survival (80.00 %) on pot mixture (1 soil: 1 sand: 1 FYM) (T_1). The lowest survival was recorded on coir compost (28.00 %) (T_3). The hardened plants were then transferred to field conditions. A decrease in survival when the plantlets were covered by polythene bags, for too long was observed (Table 20,Plates 20-21).

4.8.Histological studies

Histological observations revealed that organogenesis took place through *de novo* meristemoid formation. The presence of typical somatic embryos, which passed through normal developmental stages from globular to maturity, was detected (Plates 23-26).

Table 20.Establishment of *Centella asiatica* plantlets *in vivo*

Treatments	No. of plantlets transferred	Survival percentage
T_1	25	80.00
T_2	25	40.00
T_3	25	28.00
T_4	25	88.00
T_5	25	48.00
T_6	25	64.00

Statistically not analyzed

T_1-pot mixture (1 soil: 1sand: 1FYM)

T_2-vermiculite

T_3-coir compost

T_4-pot mixture + vermiculite (1:1)

T_5-vermiculite+ coir compost (1:1)

T_6-coir compost + pot mixture (1:1)

DISCUSSION

CHAPTER V
DISCUSSION

Centella asiatica L. is an important medicinal plant used in several ayurvedic preparations. Owing to its virtues and enhanced demand for the whole plant, the commercial cultivation is being attempted now. Conventional propagation is beset with problems of poor seed germination and unavailability of sufficient planting slips for propagation. Hence there is an urgent need to apply non-conventional propagation methods. Tissue culture plays an important role in the rapid multiplication of elite clones at a much faster rate to meet the demand. Furthermore, there is a wide scope for application of biotechnology for improvement of this important medicinal plant for which standardization of an efficient *in vitro* multiplication protocol is a vital prerequisite.

In vitro culture is a highly complex phenomenon and the success depends on a number of factors. The factors that significantly influence the *in vitro* culture were documented by Murashige (1978). The nature of plant, genotype, culture media, age of explant, season of explant collection, size of explant and hormonal influences are the most important areas of interaction that determine the success of *in vitro* culture. Thus the present study was contemplated to optimize these elements of *in vitro* culture for *Centella asiatica* L.

Surface sterilants and survival of explants

In the present study, using an optimum concentration of mercuric chloride for the right duration significantly reduced the contamination and mortality, thereby increasing the chances of survival. In the case of nodal segments, 1 per cent concentration for 1 minute was found optimum, whereas 0.5 per cent concentration for 1 – 3 minutes was found optimum for leaf bits, petiole bits and stem bits. In all the explants, maximum contamination was observed at very low concentrations exposed to very short durations.

In contrast, very high concentrations for longer durations resulted in mortality. This confirms to the observations of Chandrasekar and Narayanaswamy (1993) and Chitra (2001). But in contrast, 0.1 per cent concentration for 2 – 3 minutes (Suchitra Banerjee *et al.,* 1999) and 0.1 per cent concentration for 20 minutes (Patra *et al.*, 1999) gave good results in *Centella asiatica.*

High contamination at lower concentrations exposed to a lower period is due to the reason that it may not be sufficient to kill the microorganisms, whereas higher concentration for longer durations might be toxic to cells. The difference in concentration between nodal segments and other explants may be due to the difference in physiological maturity of cells (Munavarjan, 2000).

Effect of season on explant survival

The season during which the explants were collected from the mother plant also had a profound influence on the survival of explants *in vitro.* In the present study maximum survival was observed during the months of May – June. The lowest survival was observed during September – October. The highest survival during the months of May – June may be due to the physiologically active state of the plant. In support of this study, the same observation was made in *Beta vulgaris* (Marylise Doctrinal *et al.*, 1989) and *Gerbera* (Cappadocia *et al.*, 1987).

The concentration of endogenous hormones is more during May – June, according to the findings of Wodziki (1978). The least survival during September – October, is due to the higher contamination. During monsoon, the high humidity and dampness provide condusive conditions for microorganisms to proliferate (Malik, 1989).

Type of explant

Of the different types of explants like nodal segments, leaf bits, petiole bits, stem bits, root bits and seeds used in the present study, nodal segments followed by leaf bits gave the highest response. This response might be due to the high meristematic activity (Surya Narmada, 2000). The endogenous auxin content in both these explants is high, which promotes cell division and thereby good regeneration.

Direct organogenesis

In the present study, the best response to direct shoot regeneration was observed at 2 mgl^{-1} of BAP. The response decreased as the concentration of cytokinin increased. Similar results were evident in *Coleus forskohlii* (Jayanthi Sen and Sharma, 1991a). The decrease in shoot production at higher concentration of BAP may be due to the inhibition of shoot initiation or induction of callusing.

When Kinetin was supplemented with BAP in equal concentrations, the best results were obtained at 1 mgl^{-1} each of Kinetin and BAP. Though results from both BAP alone, and using Kinetin and BAP were almost similar, BAP showed more activity than Kinetin for shoot proliferation. Shoot elongation was marked in Kinetin. Basal MS medium containing 2 mgl^{-1} BAP produced the maximum number of shoots per explant, whereas kinetin was less effective. Combined cytokinins produced longer shoots but with callusing at the base. Similar findings were reported in *Chlorophytum borivilianum* (Purohit *et al.*, 1994).

Multiple shoots were obtained on a medium supplemented with a higher cytokinin concentration of 3 mgl^{-1} BAP and lower auxin concentration of 0.3 mgl^{-1} NAA. Similar results were observed in *Vitex negundo* (Thiruvengadam and Jayabalan, 2001), *Artemisia annua* (Anamika Gulati *et al.*, 1996) and *Aristolochia indica* (Manjula *et al.*, 1997). Relatively high levels of cytokinins are required to encourage the growth of axillary buds,

and reduce apical dominance of one main shoot. Incorporation of NAA along with BAP was stimulatory for axillary shoot multiplication. This observation is consistent with that of Sudha and Seeni (1996) in *Rauwolfia micrantha.*

To induce shoot elongation, 0.5 mgl^{-1} of GA_3 supplemented with 2 mgl^{-1} BAP gave the best response. A 1.5 – 1.6 cm increase in shoot length was observed with GA_3, than with cytokinin alone or in combination with an auxin. GA stimulates cell elongation and cell wall plasticity. Cell division is stimulated in the shoot apex especially in the more basal meristematic cells, from which develop the long files of cortex and pith cells (Sachs, 1965). They also promote cell growth because they increase hydrolysis of starch, fructans and sucrose into glucose and fructose molecules (Salisbury and Ross, 1986). In the case of bean (*Phaseolus vulgaris*), exogenous GA stimulated both cell division and cell enlargement, but the largest effect was thought to be on cell elongation (Mitchell *et al.*, 1951). Enhancement of cell enlargement has also been reported to be the major or exclusive response to exogenous GAs by Tradescantia stamen hairs of Peaches (Jackson, 1968) and intercalary meristems of excised internodal segments of *Avena sativa* stems (Adams *et al.*, 1975). In general, the stem elongation response manifested by whole plants to treatment is correlated with effects of the hormones on both cell division and cell enlargement, the former effect preceding the latter in time.

Callogenesis from explants

Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium, and the growth substances produced endogenously by cultured cells.

Callus was induced from nodal segments and leaf bits on a media supplemented with 3 mgl^{-1} NAA and 0.3 mgl^{-1} BAP, whereas a higher concentration of NAA and BAP (4mgl^{-1} and 0.4 mgl^{-1} respectively) was needed for callus induction in stem and petiole

bits. In contrast to this observation, it was reported in *Aristolochia bracteolata* that callusing took place in a high cytokinin and low auxin combination (Remeshree *et al.*, 1994). An auxin is generally required to be incorporated into the nutrient medium for the induction of callus from explants. Irvine *et al.* (1983) reported having tested 79 compounds for the ability to initiate callus from immature sugarcane leaf tissue. Of the effective compounds, 96 per cent had structures known to be associated with auxin activity. Skoog and Miller (1957) reported in tobacco pith tissue that if only auxin was supplied, the pith tissue exhibited pronounced cell enlargement, entirely unaccompanied by cell division, and cytokinin was inactive in promoting cell division in the absence of auxin.

The requirement of lesser concentration of growth regulators for nodal segments may be attributed to the presence of an associated organized meristem, which can cause callus to grow more vigorously. This suggests that meristematic cells are particularly active sites for the biosynthesis and / or the release of natural growth factors favouring cell growth. The duration for callusing ranged from 20 – 40 days on various combinations of NAA and BAP. Similar findings were reported in *Trichopus zeylanicus* (Rose George and Joseph, 2000). The best response to callusing was observed in leaf bits. The same was observed in *Artemisia absinthium* (Stefania Nin *et al.*, 1996).

Callus from leaf bit explants was greenish white in colour and a slight degree of root formation took place in the callus. This observation could be attributed to the high levels of endogenous auxins in young leaves, which get triggered on exogenous application of auxins. Similar findings were reported in *Valeriana wallichii* (Pradeep Mathur and Paramvir Singh, 1991). The callus from stem bits and petiole bits was homogenous, whereas from that of leaf bits and nodal segments was heterogenous, thereby producing roots. Yeoman (1970) proposed that an explant of simple cell

composition such as pith tissue would form homogenous callus, while leaves, consisting of several cell types, would form heterogenous callus. Homogenous versus heterogenous callus formation might be a consequence of the proportion of explant cells competent to form a specific callus type and their proliferative capacity. Besides competency, other factors such as site of callus formation and explant age and size are involved in determining the type of callus formed.

Callus formation in leaves and nodal segments was found at the excision site and veins, whereas in stem and petiole bits, callus was produced on the entire surface of explant. Generally, it appears that only certain cells and tissues are targeted for callus formation, however, in some explant callus seemed to form everywhere. This may be attributed to the type of cells in each explant. Similar finding was reported in tomato (Zankowski and Rost, 1990).

In the present study the development age of the explant played an important role in callus type induction. The youngest explants, stem and petiole bits, formed homogenous callus. Both explants were excised from regions that consisted of differentiated epidermal and ground tissue, and relatively undifferentiated vascular tissue. Ketel *et al.* (1985) found differences in callus morphology in *Tagetes* leaf explants of different ages.

Explant size may also influence the type of callus, which forms. Small explants in general as in the case of stem bits and petiole bits, tend to have a more uniform cell composition, hence any cell involved in callus formation is similar to neighbouring cells. These results suggest that small explants have greater potential to form homogenous callus. Explant size has been shown to influence bud and shoot formation in *Solanum* (Chandler *et al.*, 1982) and *Chrysanthemum* (Roest and Bokelmann, 1975).

Subculture and callus proliferation

Callus from all the four explants were subcultured on a media supplemented with 3.0 mgl^{-1} NAA and 0.3 mgl^{-1} BAP for nodal segments and leaf bits, and 4.0 mgl^{-1} NAA with 0.4 mgl^{-1} BAP for stem and petiole bits. The callus from all explants sources showed cent per cent proliferation in the first two subcultures, which gradually declined on subsequent subculturing. A decrease in weight of callus was also observed on subsequent subculturing. Chitra (2001) reported similar observations in *Phyllanthus amarus*. Some tissues become habituated during repeated cell cultures and hence loose the requirement for hormones in the growth medium. Subculturing over a very long period makes the callus hormone autonomous.

A variation in morphology and proliferation potential was noticed in callus from different explant sources. The explant source can imprint structural and biochemical characteristics on callus, even after explant remnants have been removed by repeated subculture (Zankowski and Rost, 1990). This behaviour was observed by Bassiri and Carlson (1978) when subcultured calli from different plant parts continued to express dissimilar isozyme patterns.

The proliferative capacity of cells of the explant may be governed by several factors including endogenous concentration of growth regulator at the time of excision and capacity to synthesize growth regulators and essential metabolites (George and Sherrington, 1984).

Callus regeneration

Ratio of cytokinin and auxin is important in determining the fate of the callus. Callus with low cytokinin / auxin ratio results in the formation of roots and a high cytokinin / auxin, results in shoots. Skoog and Miller (1957) pointed out, that a balance between the relative levels of auxin and cytokinin played a good role in the initiation of

shoots and roots. Cytokinins seem to increase the endogenous auxin levels. Apart from a possible effect on levels of endogenous auxin, cytokinins appear to be implicated in sugar metabolism. Conditions favouring bud formation, including the availability of cytokinins, seem to enhance starch metabolism in cells. Callus, which produces shoots, has been noted to have high specific activities of enzymes involved in both starch accumulation and breakdown

In the present study, a high cytokinin concentration of 4 mgl^{-1} BAP along with a relatively low auxin concentration of 1 mgl^{-1} NAA was ideal for regenerating shoots from callus, irrespective of the explant source. A maximum of 4 shoots per callus was obtained. Callus from leaf bits and nodal segments showed good regeneration potential. Similar response was reported in *Centella asiatica* by Patra *et al.* (1999). Shoots have been regenerated from callus, using high cytokinin and low auxin concentration in a number of medicinal plants like *Mandevilla velutina* (Handro *et al.*, 1988), *Kaempferia galanga* (Vincent *et al.*, 1992) and *Aristolochia indica* (Manjula *et al.*, 1997) .The callus on regeneration turned dark green in colour. Cytokinins tend to promote the formation of chlorophyll in callus and auxins are inhibitory. Similar reports in *Oxalis dispar* where the callus was found to turn green only when the auxin content in the medium was reduced to $1/10^{th}$.i.e.NAA from 10 to 1 mgl^{-1} (George and Sherrington, 1984).

The reason for the low plantlet conversion frequency could be attributed to high cytokinin levels. High cytokinin concentrations are capable of converting callus into dark green nodal masses, which fail to develop further. This was also reported in *Valeriana wallichi* (Pradeep Mathur and Paramvir Singh Ahuja, 1991).

In many studies morphology and composition of callus have been related to regenerative potential (Yeoman and Forsche, 1980). Changes in callus morphology on subculturing often indicate endogenous change within the callus and frequent alteration of morphogenetic potential.

Rooting

Low concentration of auxin promotes the growth of intact root, but higher concentration of auxin inhibits growth. Synthetic auxins such as NAA and IBA are usually more effective than IAA, apparently because they are not destroyed by IAA oxidase or other enzymes and therefore persist longer (Salisbury and Ross, 1986).

In the present study, good response to rooting was observed with 0.5 mgl^{-1} IBA and 0.25 mgl^{-1} NAA. As many as 13–15 roots / shoot were produced in a very short duration of 15–18 days. The response to rooting gradually decreased with increasing concentrations of auxins. The reason could be attributed to the inhibition of root growth by an exogenous supply of an auxin. Part of this inhibition is caused by ethylene, because auxins of all types stimulates many kinds of plant cells to produce ethylene, especially when relatively large amounts of auxin are added. Ethylene retards elongation of both roots and stems. Growth promotion by extremely low concentration of auxin when the auxin cannot cause production of inhibitory ethylene confirms that ethylene production nullifies what otherwise would be a more general growth enhancement by exogenous auxins (Salisbury and Ross, 1986).

The results of the present investigation on rooting are supported by similar findings in *Centella asiatica* (Patra *et al.*, 1999), *Vitex negundo* (Thiruvengadam and Jayabalan, 2001), *Helianthus annuus* (Srinivasan and Mullin, 1978) and *Chlorophytum borivilianum* (Purohit *et al.*, 1994). But in contrast, rooting has been achieved with cytokinins in *Kaempferia galanga* (Vincent *et al.*, 1992). Similar role of cytokinin in rooting has also been reported in *Zingiber officinale* (Sharma and Singh, 1995).

Hardening

Maximum survival was observed in vermiculite and pot mixture (1 : 1) than on potmixture alone. Similar observations were reported in *Phyllanthus amarus* (Chitra, 2001).

The better establishment where vermiculite was used could be attributed to the good water holding capacity of vermiculite. *Centella asiatica* grows well in natural habitats where good water supply is available continuously, viz., bunds of paddy fields, irrigation canals and rivers. Hence a potting mixture that could hold ample quantities of water is well suitable. Vermiculite also increases the air exchange within the media and thereby better growth of fragile roots from *in vitro* conditions. A decrease in survival when the plantlets were covered by polythene bags for too long was observed. The reason could be, high humidity within the bags can cause putrification of the plantlets, especially in the wounded tissues. This observation is in support of similar findings in *Agave* by Binh *et al.* (1990).

Histological observations

The histological sections clearly revealed that the mode of regeneration is through organogenesis, as in *Solanum nigrum* (Anwar Shahzad *et al.,* 1999) and *Trachyspermum ammi* (Sehgal and Syed Abbas, 1994)

SUMMARY

CHAPTER VI

SUMMARY

The present study on micro propagation techniques in *Centella asiatica* was undertaken at the Tissue culture Laboratory of the Horticultural College and Research Institute, Coimbatore. The salient findings of the study are summarized here under.

1. Of the various explants, nodal segment gave the best response (90.18 %) followed by leaf bits (81.45 %), petiole bits (77.22 %) and stem bits (62.56 %). Seeds did not respond under *in vitro* conditions.
2. The best season for collection of explants for maximum survival *in vitro* was found to be May – June (74.00 %), followed by January – April (66.00 %). The lowest survival was recorded in September –October (29.00%).
3. Surface sterilization at 1 per cent concentration of $HgCl_2$ for 1 minute proved to be optimum for recording the maximum survival (83.00 %) in nodal segments.
4. For leaf bits, petiole bits and stem bits, 0.5 per cent concentration of $HgCl_2$ for 1–3 minutes was found to be optimum in combating contamination.
5. For direct shoot regeneration from nodal segments, BAP 2.0 mgl^{-1}, and BAP 1.0 mgl^{-1} with Kinetin 1.0 mgl^{-1} was found optimum. Shoots regenerated in 6–10 days of inoculation.
6. Multiple shoots were induced in nodal segments when BAP 3.0 mgl^{-1} along with NAA 0.3 mgl^{-1} was used.
7. GA_3 0.5 mgl^{-1} used along with 2.0 mgl^{-1} BAP increased the shoot length to about 3.72 cm.
8. Callusing was observed in nodal segments and leaf bits when 3.0 mgl^{-1} NAA along with 0.3 mgl^{-1} BAP was used.

9. Callusing in both nodal segments and leaf bits was initiated from 16–20 days of inoculation.
10. NAA 4.0 mgl^{-1} with BAP 0. 4 mgl^{-1} gave the best response in petiole and stem bits for callusing.
11. Callusing in stem and petiole bits was initiated in 22 – 25 days.
12. All the four explants,viz., nodal segments, leaf bits, petiole bits and stem bits recorded 100.00 per cent response to sub culturing in the initial two subcultures (15 days interval), which gradually declined on subsequent subculture.
13. A high concentration of BAP 4 mgl^{-1} along with a relatively lower concentration of NAA 1 mgl^{-1} induced regeneration of shoots from callus of all explant sources.
14. Regeneration of shoots was observed in 28–40 days from inoculation of callus.
15. ½ MS medium supplemented with IBA 0.5 mgl^{-1} and NAA 0.2 mgl^{-1} induced rhizogenesis of the regenerated shoots.
16. Rooting was observed from 15 days onwards. A maximum of up to 15 roots / shoot was recorded on using both IBA and NAA.
17. Rooted plantlets survived well in a combination of pot mixture (1 sand: 1 soil: 1 FYM) and Vermiculite in the ratio of 1: 1.

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ANNEXURES

ANNEXURE – I

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

Sl.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
	$Na_2MoO_4.2H_2O$	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

Sl.No.	Constituents	Amount (mg/lit)	Volume of stock / L of medium (ml)
I.	**Stock – A (X200)**		
	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)**		
	KI	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)**		
	Myo - inositol	20000	
	Nicotinic acid	100	
	Pyridoxine HCl	100	
	Thiamine HCl	20	
	Glycine	400	

ANNEXURE - III

Sl. 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-naphthalene 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

Plate 1. Whole plant view of *Centella asiatica* L.

Plate 2. Explant - nodal segment

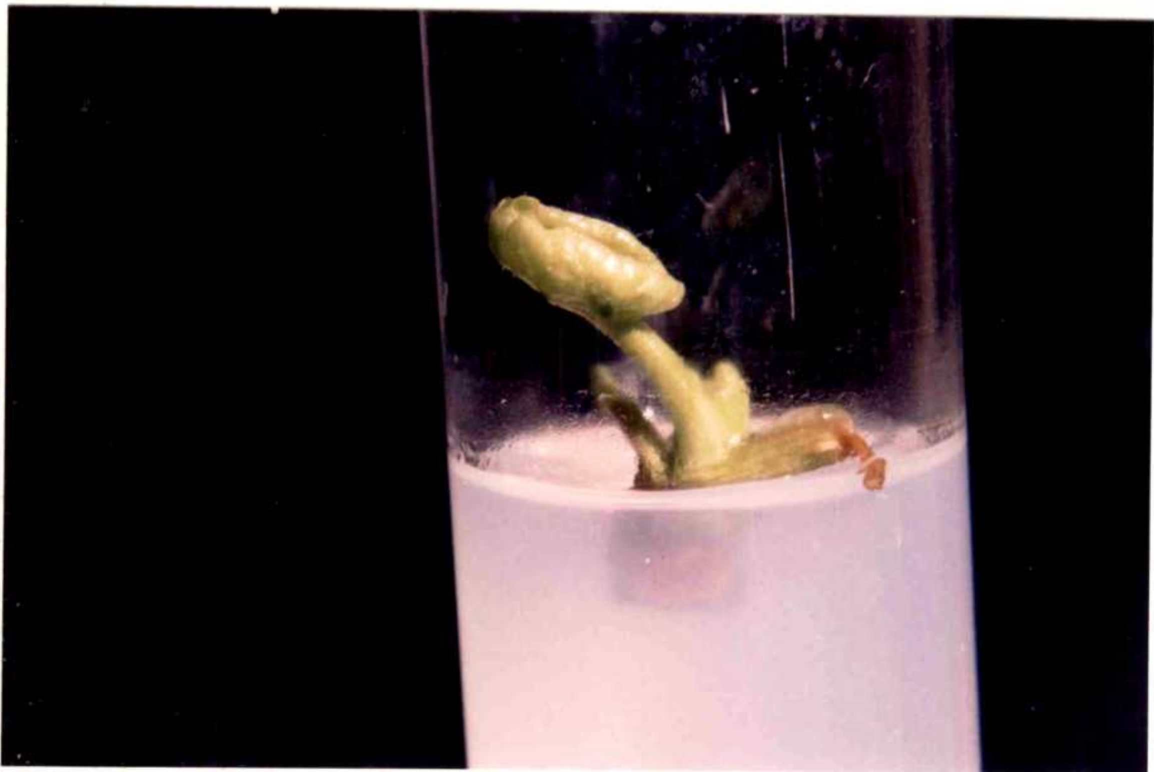

Plate 3. Direct oganogenesis from nodal segment (MS+BAP 2.0 mgl^{-1})

- 4 th day from date of inoculation

Plate 4. Direct organogenesis from nodal segment (MS+BAP 2.0 mgl^{-1})

-7 th day from date of inoculation

Plate 5. Effect of Kinetin – increased shoot length in nodal segment

(MS + BAP 1.0 mgl^{-1} + Kinetin 1.0 mgl^{-1})

Plate 6. Multiple shoot induction from nodal segment

(MS + BAP 3.0 mgl^{-1} + NAA 0.3 mgl^{-1})

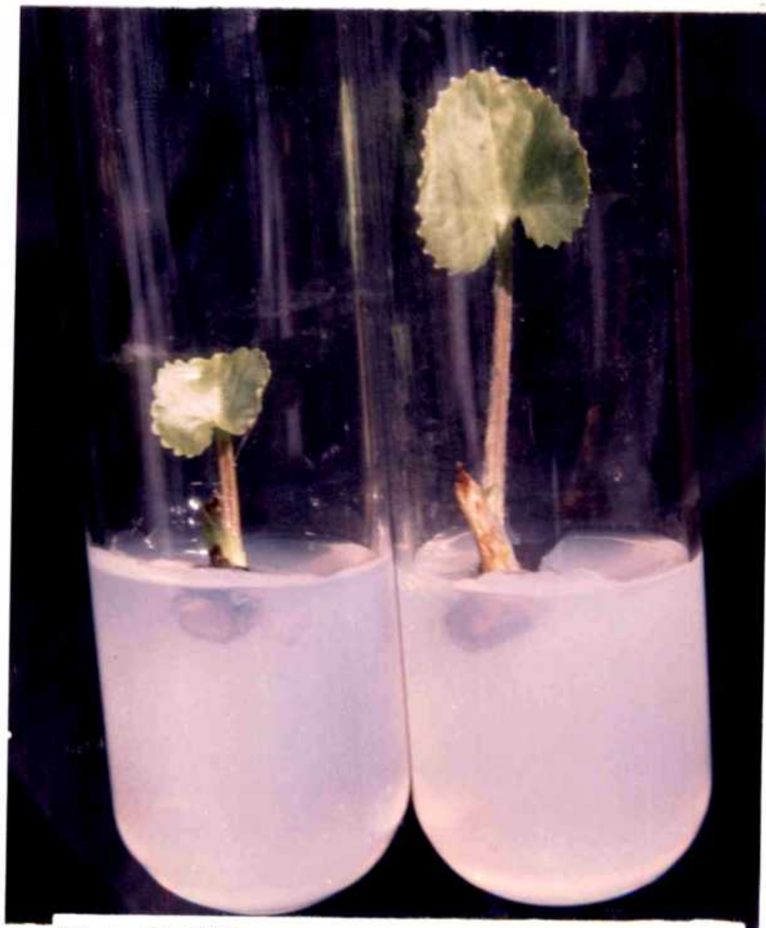

Plate 7. Effect of GA_3 on shoot elongation

Plate 8. Callus induction from leaf bit

(MS+ NAA 3.0 mgl^{-1}+ BAP 0.3 mgl^{-1})

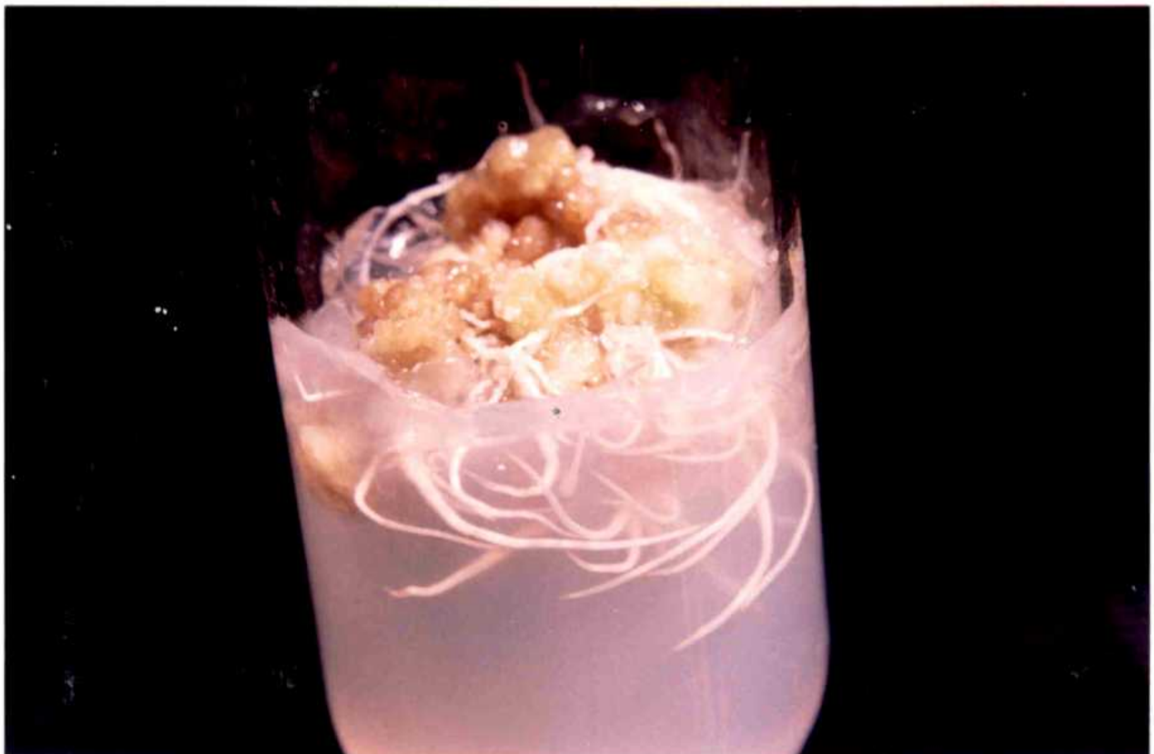

Plate 9. Rhizogenic callus from leaf bit

(30 days from date of inoculation)

Plate 10. Callus induction from petiole bit

(MS+ NAA 4.0 mgl^{-1}+ BAP 0.4 mgl^{-1})

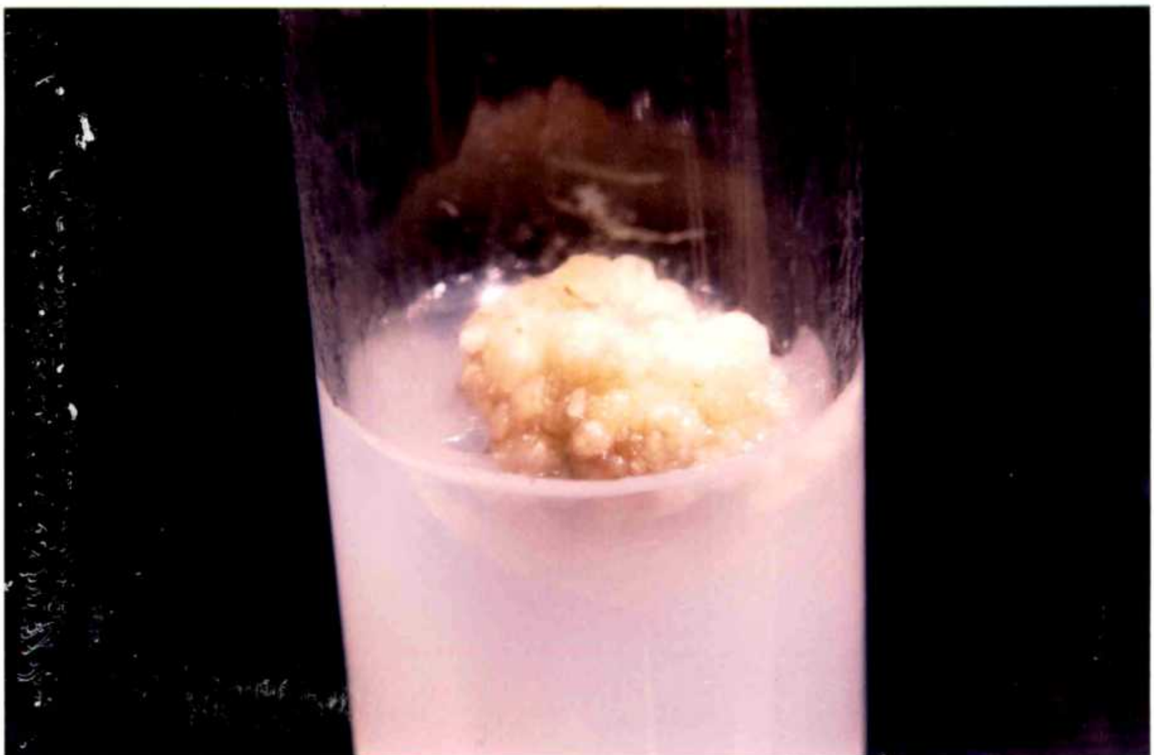

Plate 11. Callus induction from stem bit

(MS+ NAA 4.0 mgl^{-1}+ BAP 0.4 mgl^{-1})

Plate 12. Callus on regeneration medium

(MS+ BAP 4.0 mgl^{-1}+ NAA 1.0 mgl^{-1}) - 8th day

Plate 13. Spontaneous embryoid proliferation all over the callus derived from leaf bits

Plate 14. Callus on regeneration medium

(MS+ BAP 4.0 mgl^{-1}+ NAA 1.0 mgl^{-1}) - 13 th day

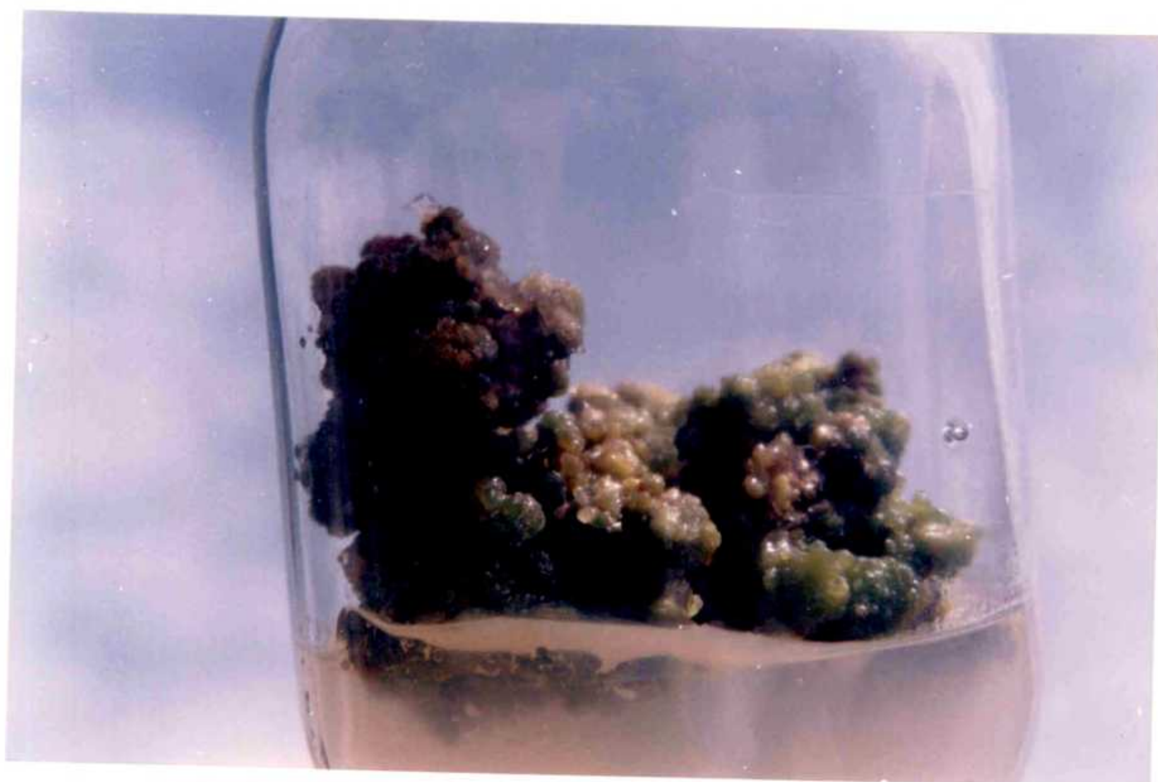

Plate 15. Callus on regeneration medium

(MS+ BAP 4.0 mgl^{-1}+ NAA 1.0 mgl^{-1})- 17 th day

Plate 16. Initial stage of shoot regeneration from callus

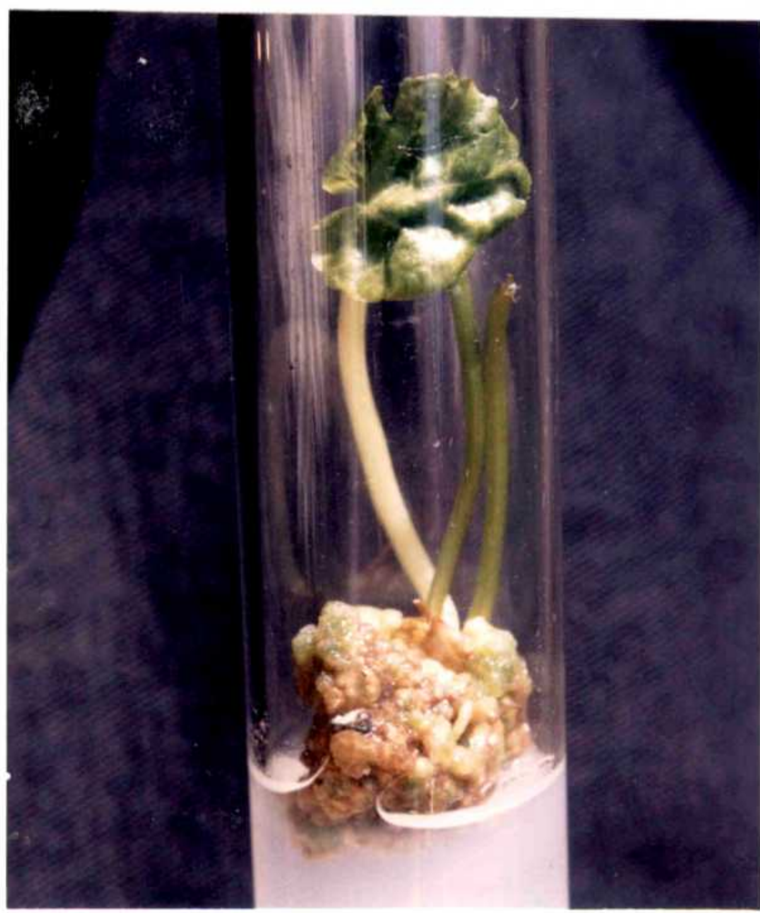

Plate 17. Complete regeneration of shoots from callus

Plate 18.

Rhizogenesis in shoot

(½ MS +IBA 0.5 mgl^{-1})

Plate 19.

Rhizogenesis in shoot

(½ MS +NAA 0.2 mgl^{-1})

Plate 20. Hardening of *in vitro* derived plantlets

Plate 21. Hardening of plantlets using perforated polythene bags

Plate 22. Culture incubation under controlled conditions

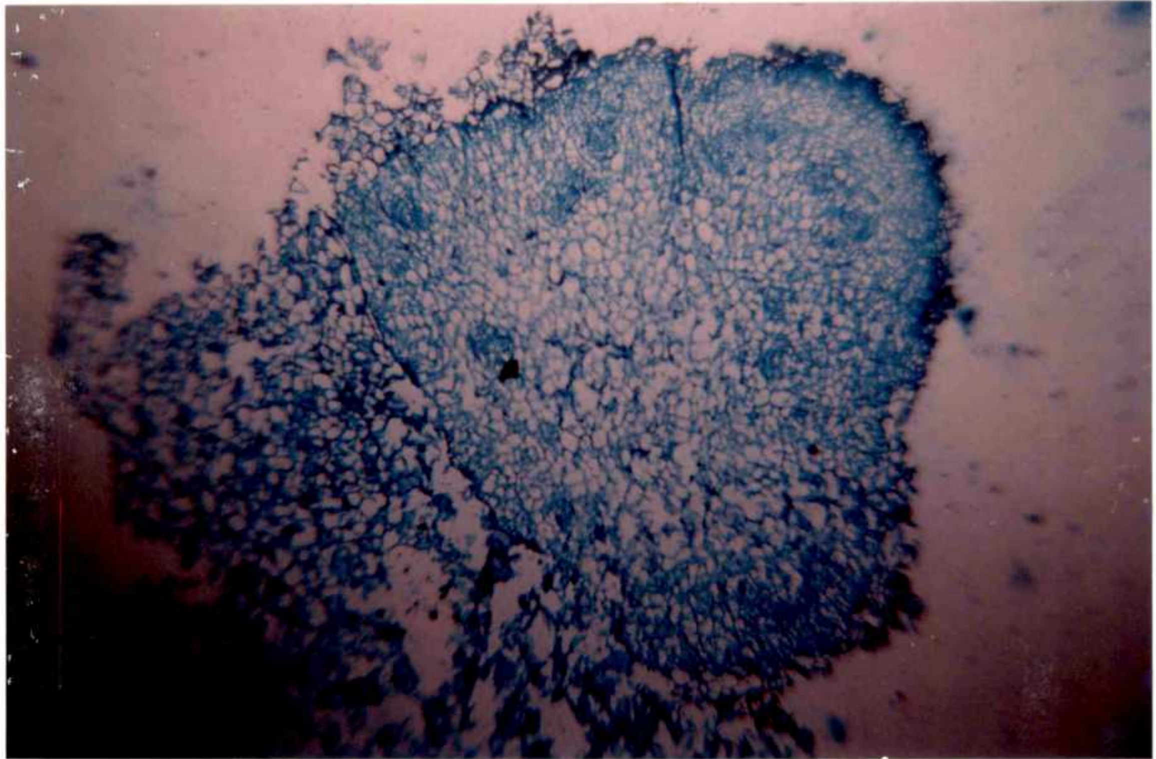

Plate 23. Callus section showing meristemoid development from superficial cells

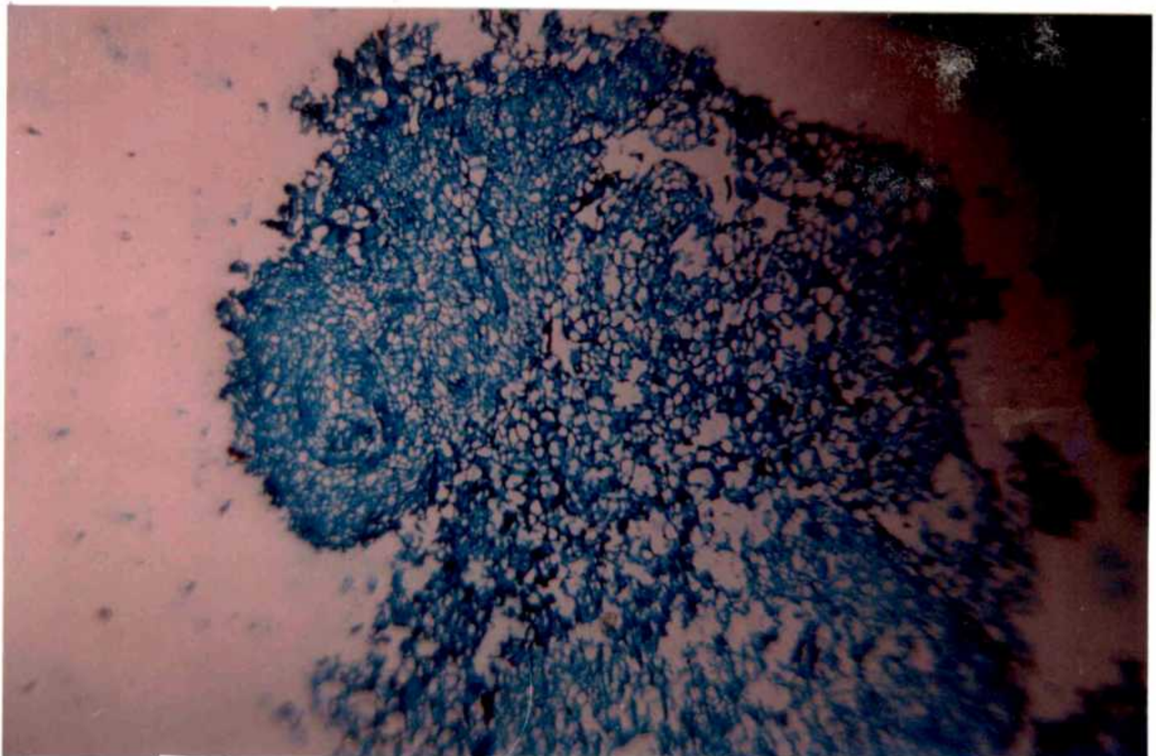

Plate 24. Callus section showing shoot bud primordia

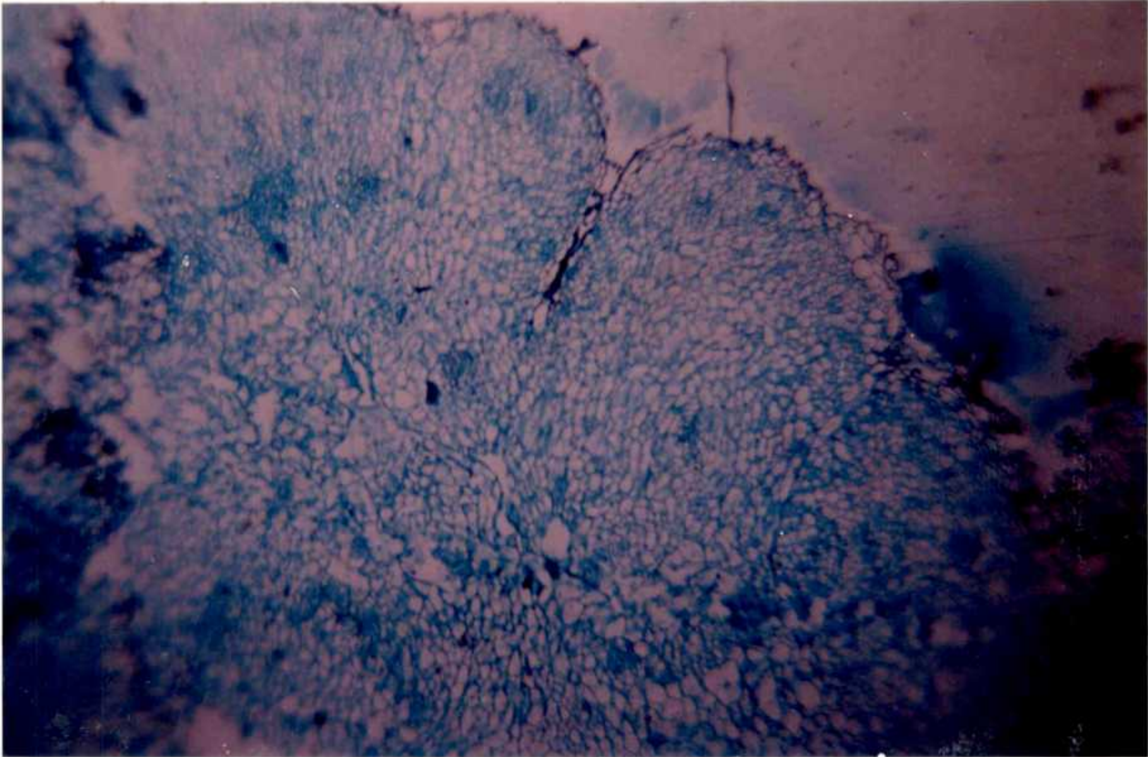

Plate 25. Callus section showing initial stage of embryoid development

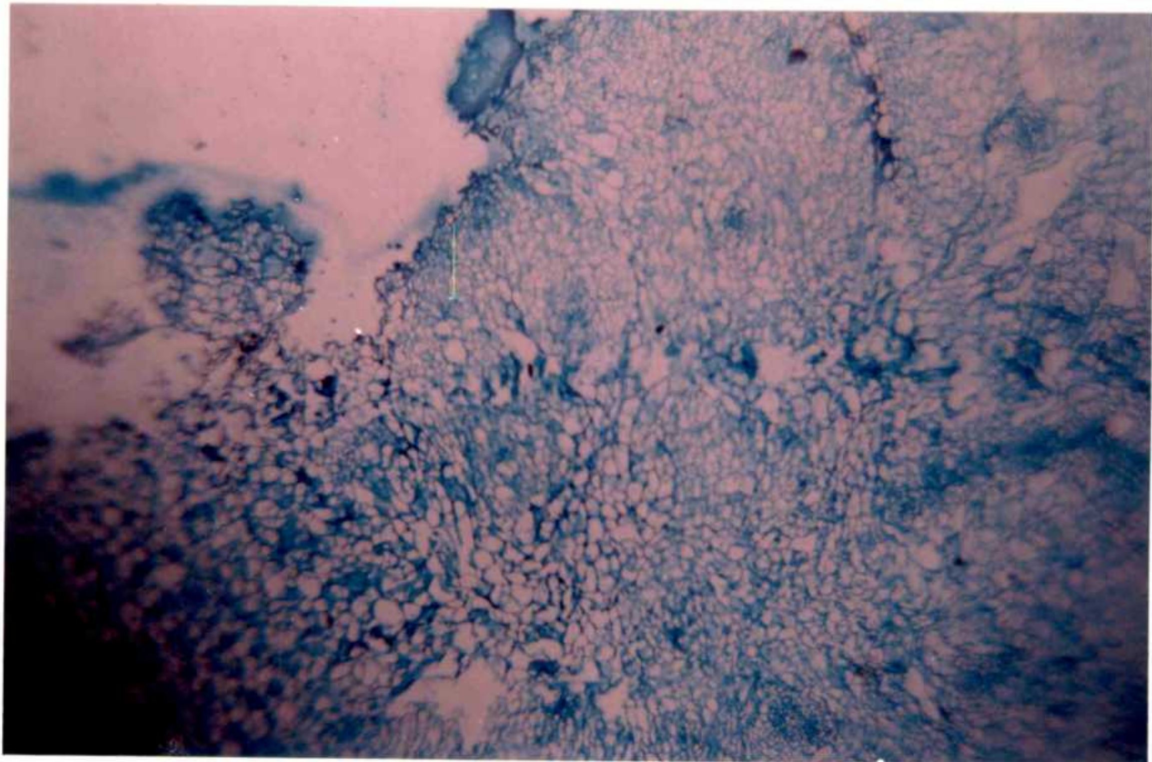

Plate 26. Callus section showing development of somatic embryo