UTILISATION OF *IN VITRO* CULTURES OF *Tinospora cordifolia* Miers. (CHITTAMRITHU) FOR BERBERINE

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled **"Utilisation of *in vitro* cultures of *Tinospora cordifolia* Miers. (chittamrithu) for berberine"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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Certified that this thesis, entitled **"Utilisation of *in vitro* cultures of *Tinospora cordifolia* Miers. (chittamrithu) for berberine"** is a record of research work done independently by **Sri. M. KALIMUTHU** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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Dedicated

to my

Loving family

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INTRODUCTION

Plants have been a major source of therapeutic agents for alleviation or cure of human ailments and are used in distinct areas of health management. There are still a large number of conditions for which suitable synthetic drugs are not available and where many indigenous medicinal plants are likely to give good leads. Presently cultivation is restricted to less than 10 per cent of the medicinal plants traded in the country and over 90 per cent are collected from the wild. Due to habitat destruction and indiscriminate exploitation, many valuable medicinal species are getting extinct. Maintenance of homogenous and consistent drug quality is often not possible since collections are made from the wild. Hence suitable biotechnological interventions for optimum product recovery by upgrading the synthesis of therapeutically active principles can provide good prospects.

Hence it is felt that the need of the hour to develop alternate sources of important natural products. There has been considerable interest in investigating the potential of plant cell cultures as an alternative to traditional agriculture for the production of secondary products. Secondary products, though they do not have a role in basic cell metabolism, play ecologically significant roles, performing specific functions. In terms of cellular economy they are metabolically expensive to produce. They accumulate and tend to be biosynthesized in specialized cell types at distinct stages of development. With the observation that plant cell cultures can produce, compounds typical of intact plants, attempts to exploit the technique to supplement or replace the same have been made.

Indian Council of Medical Research has identified 20 diseases for which suitable synthetic drugs are not available, but with effective plant based counterparts. These include tropical diseases like malaria, kala-azar and many viral infections like HIV syndrome. Hence there is a need to prioritise the plants by indepth investigations. *Tinospora cordifolia* is a versatile medicinal plant with therapeutic potential for these refractory diseases. The plant is considered as an effective febrifuge with immuno modulatory action. Often referred to as Indian quinine, *Tinospora* is one of the indigenous sources of berberine containing meagre amounts of the alkaloid (Chopra *et al.*, 1956). Besides the properties attributed to berberine viz., antagonistic to effects of cholera and treatment of diarrhoea and debility, anticarcinogenic activity was also detected for this alkaloid. Also berberine potentiates the therapeutic activity of radiation and hyperthermia (Anis *et al.*, 2000).

Except for a few successful attempts many of our indigenous species have not been exploited fully as primary or secondary sources of emerging plant drugs. It is in this context, that biotechnological interventions are proposed to evolve *Tinospora* as a potential source of berberine. The other indigenous source of this valuable alkaloid, *Coscinium fenestratum* is an endangered species. Therefore, upgrading the content of this alkaloid of undoubted value in *Tinospora* through *in vitro* techniques assumes importance.

Hence the present study on "Utilization of *in vitro* cultures of *Tinospora cordifolia* Miers. (Chittamrithu) for berberine" was taken up at the Department of

Plantation Crops and Spices, College of Horticulture, Vellanikkara from 1999-2001 with the following objectives:

1. To standardize *in vitro* techniques for initiation and proliferation of static and suspension cultures.
2. To screen *in vitro* cultures for synthesis of berberine and quantify it.
3. To enhance the level of product synthesis in *in vitro* cultures.

Review of Literature

REVIEW OF LITERATURE

Higher plants are valuable and irreplaceable sources of extractable organic phytochemicals designated as secondary products. Although they do not have a role in the basic cell metabolism of the plant, secondary products often play ecologically significant roles performing specific functions characteristic of a particular taxa (Farnsworth, 1984). Secondary metabolites provide, to a large degree, basis for adaptation and interaction with the environment. Many of these metabolites find great application as components in pharmaceutical drugs, pesticides, plant growth regulators, food and beverages. The natural products tend to be synthesized in specialized cell types and at distinct developmental stages making their extraction and purification exacting. In addition, the synthesis is significantly influenced by physiological shifts caused by changes in environment, leading to highly fluctuating productivity (Balandrin and Klocke, 1988).

To overcome the stringent biological constraints and environmental uncertainities, plant cell cultures offer attractive possibilities. Available reports pertaining to *in vitro* secondary metabolite production in plants with emphasis on medicinal species are reviewed here.

2.1 Utilising *in vitro* cultures for secondary product synthesis

Production of shikonin from *Lithospermum erythrorhizon* cultures was the first successful case of *in vitro* production of any secondary metabolite on commercial scale. Roots of 3-4 years old *in vivo* plants yielded 1-2 per cent shikonin, but *in vitro* cultures yielded 15-20 per cent shikonin in just 23 days (Fujita, 1988). High metabolite production was also observed from *Coptis* for berberine (Sato and Yamada, 1984) and *Coleus* for rosmarinic acid (Ulbrich *et al.*,

1985). Bhalsing and Maheswari (1998) has listed the plants which give high yield of secondary metabolites in *in vitro* cultures (Table 1).

Table 1. *In vitro* yields of secondary metabolites

Secondary metabolites	Source plant	Yield (% of dry weight)
Alkaloids		
Protoberberine alkaloids	*Berberis stolonifera*	10.00
Berberine	*Coptis japonica*	8.20
	Thalictrum minus	12.10
Steroids and Terpenoids		
Diosgenin	*Dioscorea deltoidea*	7.80
Sterols	*Delphinium ajacis*	8.10
Solasodine	*Solanum khasianum*	2.07
Quinones		
Shikonin	*Lithospermum erythrorhizon*	12.40
Anthraquinones	*Galium* spp.	27.00

Sankar (1998) reported significantly higher yield of ephedrine from *in vitro* cultures (0.02%) than from field grown plants (0.008%) of *Sida* spp. Sindhu (1999) reported higher yield of berberine from *in vitro* cultures (10.079 $\mu g\ g^{-1}$ callus) than from plant parts (0.013 $\mu g\ g^{-1}$) of *Coscinium fenestratum*.

2.1.1 Initiation of callus cultures

2.1.1.1 Selection of explant

Explants are the potential source for *in vitro* cultures. Different explants have been reported to give stable cultures. Meristematic tissues generally have a

high degree of morphogenic competence than older tissues. Anjalikumar (1992) reported the use of young leaf discs of *Thevetia purpurea* for production of callus. In *Coscinium fenestratum*, the explants, except the leaf petiole bases cultured on solid MS medium supplemented with 2,4-D and BAP underwent darkening and died after one week (Nair *et al.*, 1992). Ramashree *et al.* (1994) observed calli on tenth day of culture of young leaves and nodes on MS medium in *Aristolochia bracteolata*. In *Gloriosa superba*, ovaries were cultured on MS medium supplemented with 2,4-D for high degree callus induction (Kalpana *et al.*, 1996). In *Hyoscyamus muticus*, petiole explants gave good callus on medium supplemented with 2,4-D and kinetin (El-Bahr *et al.*, 1997). Suryanarayanan and Pai (1998) observed that among various explants, florets, stem and shootips, florets yielded less callus than others in *Coleus forskohlii*. Agarwal *et al.* (2001) used leaf petiole and immature stem segments as explants for inducing callus in *Ginkgo biloba*. Seeds of *Solanum platanifolium* were germinated *in vitro* and hypocotyl, stem and leaves separated from *in vitro* seedlings were used as explants for callusing (Jaggi and Singh, 2001).

2.1.1.2 Surface sterilization

To reduce the microbes present on the surface of explant, surface sterilization has to be done without damaging the plant tissue. Dodds and Roberts (1982) suggested that use of antibiotics for sterilization may be discouraged since they cause unpredictable results. Sudha and Seeni (1994) reported that treatment with 0.1 per cent $HgCl_2$ for 5 minutes gave contamination free cultures in *Adhatoda beddomeii*.

In *Trigonella foenum-graecum* for the production of diosgenin by hairy root cultures, seeds were surface sterilized with sodium hypochlorite solution (15% w/v available chlorine) supplemented with two drops of Triton X-100 for 6 minutes (Merkli *et al.*, 1997). Kannan and Jasrai (1998) reported that washing of explants in ethanol (90% v/v) followed by soaking in mercuric chloride 0.1 per cent for 1 minute gave contamination free cultures in *Vitex negundo*. Kulkarni and Rao (1999) identified Teepol washing followed by 70 per cent ethanol (2 minutes) and 0.1 per cent mercuric chloride (5 minutes) treatment as an effective surface sterilization method for *Acorus calamus*.

2.1.1.3 Nutrient media for *in vitro* cultures

Different nutrient combinations for successful culture establishment have been standardised and are in use, Murashige and Skoog (MS) (1962), White's medium, Gamborg B5 medium, Linsmaier and Skoog (LS) (1965) medium, Woody Plant medium (WPM) and Nistch's medium are some commonly used media in plant tissue culture (Narayanaswamy, 1997 and Gupta, 1995). Murashige and Skoog medium (MS) is most popular among them.

Fujita (1988) found that calli of *Lithospermum erythrorhizon* on LS medium was good but with less shikonin production, while White's medium produced shikonin, but calli growth was restricted. Hayashi *et al.* (1988) used Limsmaier and Skoog medium to raise *Glycyrrhiza glabra* cultures to produce triterpene glycosides. *Ginkgo biloba* cells could grow on either MS or Gamborg B5 mineral salt medium supplemented with sucrose (3% and 2% respectively) and NAA and kinetin. Best growth and maintenance of callus cultures were achieved using MS medium supplemented with 2 mg l^{-1} NAA and 1 mg l^{-1} kinetin (Carrier *et al.*, 1990).

Anu *et al.* (1994) observed that half strength MS medium gave good results in *Gymnema sylvestre*. Ilahi and Ghauri (1994) induced calli in *Papaver bracteatum* seedlings cultured on half strength MS medium supplemented with NAA 1.0 mgl^{-1} and BAP 0.5 mg l^{-1}. Banerjee *et al.* (1998) reported that half strength MS medium supplemented with growth regulators (BAP, IAA and Kinetin) was found ideal for multiple shoot induction and regeneration in *Piper longum*.

2.1.2 Suspension cultures

2.1.2.1 Initiation of suspension cultures

Cell suspension cultures of *Thalictrum minus* var. *hypoleucum* were established from calli derived from leaf segments cultured on Linsmaier and Skoog (1965) medium containing NAA and BA for 4 weeks.

Bhojwani and Razdan (1983) have enlisted the general protocol for initiating and maintaining cell cultures. Hayashi *et al.* (1988) employed calli obtained from roots of *Glycyrhiza glabra*, to produce cell suspensions to synthesise triterpenoids.

Sindhu (1999) and Gholba (2000) also used calli derived from leaf to initiate suspension cultures in *Coscinium* and *Gymnema* respectively.

2.1.2.2 Utilising suspension cultures in *in vitro* studies

Suspension cultures of *Morinda citrifolia* could be stimulated by appropriate selection methods to produce 20 times more of anthroquinone contents (Zenk *et al.*, 1975.)

Gamborg and Shyluk (1981) have reported in detail the growth stages of cultured plant cells viz., lag phase, exponential phase, stationary phase and retardation phase. The cell suspension cultures of *Coptis japonica* are capable of producing a large amount of protoberberine alkaloids including berberine, coptisine, palmatine and jatrorrhizine (Fukui *et al.*, 1982). Large quantity of berberine was synthesized by suspension cultures of *Thalictrum minus, Thalictrum flavum* and *Berberis* sp.(Sato *et al.*,1990.)

Cell suspension cultures derived from *Ginkgo biloba* leaves produced ginkgolide B. In cell suspension cultures, the production reached a maximum by the 13th day of subculture and was followed by a sharp decrease (Jeon *et al.*, 1995). In *Glycyrrhiza glabra*, suspension culture on MS medium supplemented with 5.0 mg l^{-1} NAA and 0.1 mg l^{-1} BAP resulted in maximum glycyrrhetinic acid content (Tailang and Kharya, 1998). Sindhu (1999) established suspension cultures from calli of *Coscinium fenestratatum* for the production of berberine alkaloid.

2.1.3 Analytical screening techniques for isolating producing lines

Tabata *et al.* (1974) observed that in *Lithospermum* callus cultures certain cells formed small colourless tissue and certain intense red tissue forming a mosaic appearance. The selection of red coloured tissue in later transfer generations could increase shikonin content to 1.5 per cent of cell dry weight.

Hyoscyamus niger cell lines selected by cell aggregate screening method produced increased quantities of hyoscyamine (Hashimoto *et al.*, 1982). Ellis (1985) employed microspectrophotometry wherein the content of a compound in a cell with an absorbance maximum of 300 nm can be monitored.

Analytical screening in cell lines of *Duboisia leichhardtii* resulted in a high alkaloid producing line yielding 0.53 per cent hyoscyamine and 1.16 per cent scopolamine (Endo and Yamada, 1985). Callus tissues from different explants of *Solanum elagnifolium* were cultured on a modified MS medium with 1 mg l^{-1} 2,4-D. The presence of the alkaloid, solasodine was determined by spectrophotometric and TLC methods. Its concentration ranged from 1.00 to 2.5 mg l^{-1} DW (Nigra *et al.*, 1987). Pasqua *et al.* (1991) could derive flavones and isoflavones from calli clones of *Maclura pornifera* after screening out dark coloured clones from light yellow coloured ones.

2.1.4 Factors regulating synthesis of secondary products *in vitro*

2.1.4.1 Culture environment control

2.1.4.1.1 Modifying nutrient media

2.1.4.1.1.1 Manipulating carbon source

Dougall (1980) reported that with increase in concentration of sucrose from 1 to 5 per cent, the yield of shikonin increased in *Lithospermum erythrorhizon.* Wijnsma *et al.* (1986) reported that the anthraquinone yield was maximum at 8 per cent sucrose in Gamborg B5 medium for *Cinchona ledgeriana.* In *Datura stramonium*, highest hyoscyamine production (upto 7.4 mg/litre/day) was recorded in root cultures on full strength B5 medium containing 5 per cent sucrose (Hilton and Rhodes, 1993).

Asaka *et al.* (1994) reported that ginseng embryoids produced maximum amount of saponins when raised in media containing 30 g sucrose and 30 g glucose per litre. Jeon *et al.* (1995) reported that high yield of Ginkgolide B was obtained in MS medium supplemented with 30 g sucrose in *Ginkgo biloba.*

Jaggi and Singh (2001) used 2 per cent sucrose in modified MS medium for solasodine production in cultures of *Solanum platanifolium*.

2.1.4.1.1.2 Effect of inorganic salts

Sasse *et al.* (1983) reported that nitrogen, though it stimulated formation of caffeyl putrescine in *Nicotiana tabacum* and harman alkaloids in *Peganum harmala,* repressed alkaloid synthesis in *Catharanthus roseus*. Ravishankar *et al.* (1988) reported that in cell cultures of *Capsicum annuum,* when nitrates were eliminated from the culture medium, maximum capsaicin production took place. Also elimination of phosphates from the medium greatly improved capsaicin synthesis.

In *Panax notoginseng*, by increasing the initial phosphate concentration in the medium in the range of 0-1.25 mM, both cell growth and saponin accumulation were greatly improved (Zhong and Zhu, 1995). Zhang et al. (1996) reported that the ratio of nitrate to ammonium had a great influence on the cell growth of *Panax notoginseng* and consumption of C and N sources. In the same report, they showed that the dry cell weight increased from 4.9 to 10.9 g/l with an increase of initial nitrate concentration from 0 to 60 mM.

2.1.4.1.1.3 Hormonal regime

The incorporation of GA_3 to the cell suspension cultures of *Coptis japonica* var. *dissectica*, contributed to berberine production by activating primary metabolism of tyrosine biosynthesis (Hara *et al.*, 1994).

Shrivastava and Padhya (1995) reported that increase in the IAA level from 0.5 to 1 and 2 $\mu M\ l^{-1}$ in the medium, reduces the number of roots produced as

well as alkaloid accumulation in the roots (0.05% to 0.15%) in regenerated roots of *Boerhaavia diffusa*. Vander *et al.* (1995) reported that production of anthraquinones was inhibited by addition of 2,4-D whereas NAA induces anthraquinone production in B5 medium, in *Morinda citrifolia*. Content of colchicine was favourably attained in cultures of *Gloriosa* containing 0.2 mg l^{-1} Kin and 0.5 mg l^{-1} 2,4-D (Ramamurthy and Reddy, 1997).

In *Sida* spp. NAA and kinetin at 1.0 mg l^{-1} each, resulted in high ephedrine yield whereas incorporation of 2,4-D resulted in complete inhibition of alkaloid production (Sankar, 1998). Sindhu (1999) observed that MS medium at half strength with phosphate ions reduced to 25 per cent supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1} gave high callus index for immature fruit explants of *Coscinium fenestratum*.

2.1.4.1.1.4 Regulation of temperature

The release of benzyl isoquinoline alkaloid, berberine from cultured cells of *Thalictrum minus* into the medium proved to be temperature dependent showing a marked decrease at low temperature. At 5°C the release was only 37 per cent of that at 25°C during a period of 12 hours incubation (Yamamoto *et al.*, 1987). Levels of alkaloid accumulation in hairy root cultures of *Catharanthus roseus* showed a clear increase with lowering of temperature (Toivonen *et al.*, 1992).

2.1.4.1.1.5 Influence of photo period

Yamada and Sato (1981) found that tissue culture of *Coptis japonica* on a solid medium followed by successive liquid cultures produced friable cell lines

with high berberine content and that light inhibited berberine production in cell lines. Treating callus cultures of *Datura* with amino acids and incubating them in dark resulted in alkaloid production (Dattagupta and Datta, 1984). Kim *et al.* (1988) found that maximum dry cell weight was 20.4 g l^{-1} with continuous illumination compared to 17.9 g l^{-1} without light. Berberine production was high in cultures grown in light (216.5 mg l^{-1}) than in those incubated in dark (159.0 mg l^{-1}) in *Thalictrum rugosum* cell suspensions. Nair *et al.* (1992) reported that in *Coscinium fenestratum*, light induced the blackening of callus cultures and retarded their growth in static and suspension cultures. However, light increased the berberine content to 3.5 per cent of dry cell weight. Sankar (1998) and Sindhu (1999) observed higher percentage of callusing under illumination in *Sida* spp and *Coscinium fenestratum* respectively.

2.1.4.1.1.6 Levels of oxygen and carbon dioxide

Hara *et al.* (1987) reported that shikonin production during *Lithospermum erythrorhizon* cell growth on Linsmaier and Skoog agar medium is enhanced by an abundant supply of oxygen. By supplying a mixture of carbon dioxide and ethylene to the air lift system, berberine content was increased two fold in *Thalictrum rugosum* cell suspension in an improved airlift bioreactor (Kim *et al.*, 1991a). Dissolved gaseous metabolic concentrations, especially of carbon dioxide and ethane had a positive influence on ajmalicine production rate by *Catharanthus roseus* cultures (Schlatmann *et al.*, 1997).

2.1.4.1.1.7 Supply of precursors

Biosynthetic precursors are supplied to the medium whenever the productivity of secondary metabolites is limited by lack of precursors. Devadoss *et al.* (1984) could produce morphine alkaloids in callus and suspension cultures of *Papaver sominiferum* by supplementing the culture media with tyrosine and phenyl alanine. Vanadyl sulphate (10-100 mg l^{-1}) when added to cell suspension cultures of *Catharanthus roseus* stimulated increased intra cellular accumulation of catharanthine and ajmalicine (Smith *et al.*, 1987).

Addition of glutamine to the production medium in the cell suspension cultures of *Lithospermum erythrorhizon* proved to be strongly inhibitory to shikonin production (Yazaki *et al.*, 1987). Kim *et al.* (1991b) reported that in *Thalictrum rugosum* cell suspension cultures on MS medium with 2,4-D, maximum enhancement in berberine production was achieved by adding cupric sulphate (200-500 µm) on the 6^{th} day of culture. In *Capsicum annum*, addition of D-limonene and L-ascorbic acid increased the production of capsaicin (Veeresham *et al.*, 1992). Sato and Yamada (1984) reported that addition of 0.1 µM tyrosine did not increase the production of berberine in *Coptis japonica*.

Addition of various concentrations of (0.5-2.0 mM) acetyl-salicylic acid (ASA) to tumour lines of *Catharanthus roseus* cultivated *in vitro* with corn starch as carbon source, produced an increase of 505 per cent total alkaloids per culture, 612 per cent furanocoumarins and 1476 per cent total anthocyanins (Hernandez and Vargas, 1997). Rajkumar *et al.* (1997) initiated callus cultures of *Azadirachta indica* from young leaves on MS medium modified with benzyl adenine (0.5 mg l^{-1}), IAA (1 mg l^{-1}) and glycine (0.3 mg l^{-1}). Supplementation of medium

with precursor sodium acetate (5 mg l^{-1}) stimulated biogenesis of azadirachtin. Sankar *et al.* (2001) reported that between phenylalanine and methionine, phenylalanine at 50.0 and 100 mg l^{-1} induced synthesis of the alkaloid ephedrine in *Sida* spp. whereas methionine was not effective.

2.1.4.2 Biological control

2.1.4.2.1 Morphological differentiation

Artemisin, an antimalarial drug isolated from *Artemisia annua* was not accumulated in undifferentiated *in vitro* cultures, possibly because the expression of secondary metabolism was closely correlated with morphological differentiation (Paniego *et al.*, 1995).

Punarnavine profile of regenerated roots of *Boerhaavia diffusa* was studied by Shrivastava and Padhya (1995). In the presence of 2,4-D, leaf segments produced callus which regenerated roots that contained trace amounts of punarnavine. Callus obtained from cotyledonary leaves differentiated to shoots when adenine was added to the culture (Purohit *et al.*, 1997). Amount of xanthotoxin in such regenerating cultures was more than that in undifferentiated callus. Jaggi and Singh (2001) observed that differentiated leaf calli have higher potential for producing solasodine (0.101% dry weight) in *Solanum platanifolium.*

2.1.4.2.1.1 Somatic organogenesis

Plant regeneration through direct or indirect organogenesis has been reported in many medicinal plants. Vincent *et al.* (1991) obtained maximum regeneration from callus cultures of *Kaempferia galanga* in MS medium supplemented with BAP 1.5 mg l^{-1}. In *Piper longum*, plant regeneration through callus mediated organogenesis was achieved (Bhat *et al.*, 1992). In *Gomphrena*

officinalis, shoot regeneration could be obtained from callus derived from leaf and stem segments in MS medium supplemented with BAP and NAA (Mercier *et al.*, 1992).

Kumari and Saradhi (1992) identified cotyledonary explants as the best source for compact and nodulated calli which showed shoot induction for further subculture in *Origanum vulgare.* Roots were regenerated from one year old callus cultures of *Atropa accuminata* in the presence of kinetin at 1.0 mg l^{-1} (Akram *et al.*, 1994). Adventitious shoots were obtained from callus when cultured on half strength MS medium supplemented with kinetin and silver thiosulphate in *Panax notoginseng* (Lem *et al.*, 1997).

Similar reports on organogenesis were obtained in a number of medicinal plants like *Tylophora indica* (Mhatre *et al.*, 1984; Keshavachandran *et al.*, 1997), *Papaver somniferum* (Jaiswal and Narayan, 1985) and *Chrysanthemum morifolium* (Bhattacharya *et al.*, 1990).

2.1.4.2.1.2 Somatic embryogenesis

Shanthamma *et al.* (1991) produced somatic embryos by subculturing the calli of *Emilia sonchifolia* on MS medium having 2 mg l^{-1} BA and 1 mg l^{-1} kinetin. Embryogenesis from callus cultures of *Kaempferia galanga* was observed by Vincent *et al.* (1992) in MS medium supplemented with 2,4-D 1.0 mg l^{-1} and BAP 0.5 mg l^{-1}. Induction of somatic embryogenesis by the addition of thidiazuron to the culture medium was reported in leaf disc cultures of *Nicotiana tabacum* (Gill and Saxena, 1993).

Jasrai *et al.* (1993) induced calli from 10-12 days old leaf explants cultured on solid medium supplemented with 1 μM 2,4-D and 2 μM kinetin. Somatic embryogenesis occurred in cell suspensions after two subcultures in liquid medium with same concentration of growth regulators. Raychaudhuri (1998) reported the development of somatic embryos of *Plantago ovata* from different explants on MS medium supplemented with 2,4-D and kinetin or NAA and BAP. When the somatic embryos were subcultured to hormone free MS medium, plantlets regenerated from somatic embryos.

Similarly, somatic embryogenesis was reported in *Digitalis* spp. (Kuberski *et al.*, 1984), *Tylophora indica* (Mhatre *et al.*, 1984), *Rauvolfia caffra* (Upadhyay *et al.*, 1992), *Aconitum* spp. (Giri *et al.*, 1993), *Azadirachta indica* (Hwang *et al.*, 1997) and *Panax ginseng* (Choi *et al.*, 1998).

2.1.5 Employing special techniques to induce secondary product synthesis *in vitro*

2.1.5.1 Effect of stress inducing compounds in secondary metabolite production

For manipulating the growth and metabolite production, many chemicals are added to the media, which are collectively grouped as media additives. Activated charcoal in finely powdered form reduces growth and create stress condition which triggers synthesis of secondary metabolites (Fridborg and Eriksson, 1975). Funk *et al.* (1987) used yeast extract to enhance production of secondary metabolites, which gave a steep rise in glyceollin production followed by a fall, in *Glycine max* cultures, while in *Thalicturm rugosum*, they found a continuous rise in berberine production.

Addition of spermidine to the suspension culture of *Thalictrum minus* showed enhanced production of berberine while other polyamines like cadaverine, putrescine and spermine were ineffective (Hara *et al.*, 1991). Zhang *et al.* (1995) observed that, in *Panax notoginseng*, addition of mannitol increased the initial osmotic pressure of the culture medium, thereby increasing saponin production. In *Datura stramonium, Atropa belladonna* and *Hyoscyamus muticus*, reduced cell growth was noticed due to stimulating effect of osmotic stress caused by mannitol, which resulted in rapid accumulation of alkaloids (Saker *et al.*, 1997). Accumulation of the alkaloid curine was more in the callus cultures of *Cissampelos pareira* on MS medium supplemented with 2 mg/l NAA + 0.5 mg/l abscisic acid (ABA) compared to those cultures on MS + 2 mg/l NAA (Gokul *et al.*, 1998).

2.1.5.2 Elicitation

Addition of autoclaved mycelia of fungi to the medium may cause variation in the synthesis of secondary metabolites by the cells. Elicitor solution prepared from *Pythium vexans*, which was added to a *Catharanthus roseus* cell suspension culture at low concentration, was found to stimulate alkaloid production (Nef-Campa *et al.*, 1994).

Elicitor treatment of dill (*Anethum graveolens*) cultures with chitosan did not result in increased carvone production (Doernenburg *et al.*, 1990). Hairy roots of *Catharanthus roseus* when elicited with mycelia of *Aspergillus* exhibited increased production of ajmalicine (Vazquez-Flota *et al.*, 1994). Culture filtrate of

Penicillium minioluteum, *Botrytis cinera* and *Verticillium dahliae*, which when added to cell suspension of *Taxus* sp., improved the production of Taxol (Ciddi *et al.*, 1995).

Catharanthus roseus cell cultures elicited with different enzymes (*Rhizopus* sp. cellulase, *Trichoderma viride* macerozyme and *Aspergillus niger* Polygalacturonase) exhibited increased accumulation of alkaloids (Garnier *et al.*, 1996). Moreno *et al.* (1996) observed that, cell cultures elicited with autoclaved mycelia of *Pythium aphanidermatum* showed high accumulation of indole alkaloids in *Catharanthus roseus*.

Elicitating the production media with autoclaved mycelia of *Pythium aphanidermatum* at levels of 500 mg l^{-1}, 2.0 g l^{-1} and 5.0 g l^{-1} yielded encouraging results with respect to synthesis of ephedrine *in vitro* by 4 to 5 week old leaf and stem calli of *Sida cordifolia* (Sankar, 1998).

2.1.5.3 Immoblization

Physical restraining of cells on a fixed support exerts stress on plant cells leading to restricted growth which is a pre-requisite for metabolite production (Choi *et al.*, 1996). They achieved 20 fold increase in taxol production from *Taxus brevicola* by immobilization.

2.1.5.3.1 Immobilization by gel entrapment

Nakazima *et al.* (1985) found that cells immobilized in calcium alginate retained greatest degree of viability while gelatin resulted in reduced cell viability due to harsh chemical environment. They obtained earlier synthesis of the pigment in immobilized cell cultures of *Lavendula sera*.

2.1.5.3.1.1 Utilising immobilization techniques for secondary product synthesis

Ayabe *et al.* (1986) investigated the biosynthetic activity of alginate trapped cells of 4 week old *Glycyrrhiza echinata*. In comparison to freely suspended cells, a high and more prolonged accumulation of echinatins in both cells and medium occurred as a result of gel immobilization. Immobilized cell cultures of *Catharanthus roseus* showed retention of respiratory activity and biosynthetic capacity over an extended period compared to free cells probably due to cell stabilization as observed by Majerus and Parcilleux (1986).

Immobilization of *Datura innoxia* cells in calcium alginate beads resulted in increased secondary metabolite production (Gontier *et al.*, 1994). Sankar (1998) observed failure of immobilized cells to synthesis any alkaloid as revealed by qualitative and chromatographic tests in *Sida* spp.

2.1.6 Separation, purification and quantification of alkaloids *in vitro*

Callus derived from *Taxus wallichiana*, after methanolic extracts were concentrated under reduced pressure, treated with deionized water, then partitioned with $CHCl_3$ and $CH_3COOC_2H_5$ respectively. (+) Catechin was isolated from the $CH_3COOC_2H_5$ extract by column chromatography over silica gel. The identification of (+) catechin was based on comparison of its spectral characteristics by NMR (300 MH_2 and FAB-MS) with those reported for the authentic sample (Miketova *et al.*, 1998).

Methanolic extracts of *Coscinium fenestratum* calli were subjected to column chromatography using butanol, acetic acid glacial and water (7:1:2) as running solvent mixture. The concentrated fractions were further separated by thin

layer chromatography (TLC). The alkaloid spot gave yellow colour identical to standard berberine were scrabbed and dissolved in 4.5 ml methanol and centrifuged at 1500 rpm for 18 minutes. Absorbance (nm) of the supernatent solution was noted from UV spectrophotometer at 228 nm and respective concentration was estimated from the standard curve of standard berberine hydrochloride (Sindhu *et al.*, 2000).

2.1.7 Separation and purification of alkaloids in *ex vitro* conditions

Kunitomo *et al.* (1983) isolated menisphorphine from the rhizome of *Menispermum dauricum.* Bowen and Motawe (1985) isolated alkaloid from *Tinospora malabarica* belonging to Menispermaceae family. Three alkaloids were isolated from the stem methanol extract by flash column chromatography and were further purified by TLC. Alkaloids such as palmatine, isotetrandrine, aromoline, jatrorrhizine, berberine, columbamine and magnoflorine were isolated from the root bark of *Berberis cartagena* (Basher *et al.*, 1996). Sankar (1998) extracted ephedrine alkaloid from four species of *Sida.* The alkaloid content varied with leaf, stem and root as well as with the stage of harvest.

2.2 Investigations in *Tinospora cordifolia*

2.2.1 Description of the plant

Tinospora cordifolia Miers. or Amritu, of family Menispermaceae is a tropical glabrous climber with succulent stems and long filiform, aerial roots arising from branches. Bark is warty and creamy white with soft wood. Leaves are membranous, cordate with broad sinus and long petiole. Flowers are unisexual, greenish, male, fascicled and female usually solitary. Drupes are ovoid, succulent,

red, pea sized with curved seeds. The plant flowers during summer and produces fruits during winter (Chatterjee and Pakrashi, 1991).

The plant is distributed throughout tropical India, Andaman, Myanmar and Sri Lanka. Apart from the above species, *T. crispa* and *T. sinensis* are also available in India (Singh *et al.*, 1983).

2.2.2 Therapeutic uses of *T. cordifolia*

The officinal part of the plant i.e. mature stem is reported to be acrid, bitter and restorative with curative effects on fever (antipyretic), jaundice and diabetes (Jain, 1968). The starch prepared from the aqueous extract of dried stem 'Palo' or 'Giloe-ka-sat' is considered to have antacid, antidiarrhoeal and antidysenteric properties (Ambasta, 1986). Leaves are rich in protein, calcium and phosphorus, their decoction is given in gout. The plant is a constituent of Ayurvedic preparations like Amritarishtam and Dhanwantaram tailam (Sivarajan and Balachandran, 1994).

Siddique and Zafar (1995) reported that the plant is used in several formulations for the treatment of diabetes, jaundice (Phalatrikadi kasaya) and bacterial infection (Septillin). Maurya *et al.* (1995) have identified clerodane diterpenoides in the plant. It is also one of the constituents of the crude drug formulation exhibiting immunomodulatory action (Sohni and Bhatt, 1996). The alkaloid, berberine possesses antagonistic properties to the effects of cholera and is employed in the treatment of diarrhoea and debility. It is also reported to have anticarcinogenic activity. It potentiates the therapeutic activity of radiation and hyper thermia as well (Anis *et al.*, 2000).

2.2.3 Biochemical investigations

Chopra *et al.* (1956) identifies berberine as one of the alkaloid present in the officinal part of *Tinospora* which gives its antipyretic property. Baquar and Tasnif (1984) reported that *T. cordifolia* stem contains a bitter substance, berberine, while the fresh stem contains crude giloin and giloinin. Chatterjee and Pakrashi (1991) reported that the plant contains tinosporin, columbin, chasmanthin, berberine, tinopsoric acid, tinosporol, giloin, giloinin and substituted pyrrolidine.

Materials and Methods

MATERIALS AND METHODS

The study entitled "Utilisation of *in vitro* cultures of *Tinospora cordifolia* Miers. (Chittamrithu) for berberine" was carried out at Plant Tissue Culture Laboratory and the Biochemistry Laboratory of the College of Horticulture, Kerala Agricultural University, Vellanikkara, Thrissur from 1999 to 2001. The objectives of the study were to standardize *in vitro* techniques for initiation and proliferation of static and suspension cultures of *Tinospora cordifolia* Miers., to screen *in vitro* cultures for synthesis of berberine and quantify it and to enhance the product synthesis in *in vitro* cultures. The details regarding the experimental materials and the methodology adopted for conducting various aspects of the study are presented in this chapter.

3.1 Materials

3.1.1 Source of explants

Mature plants of two ecotypes of the experimental species viz., Vellanikkara ecotype and Madurai ecotype, formed the source plants. Nodal cuttings were prepared from source plants of the two ecotypes maintained at the Ayurvedic herbal garden of the Department of Plantation Crops and Spices, Vellanikkara (Plate 1) and from Madurai, Tamilnadu. The cuttings were planted in pots which were maintained in the net house of the College of Horticulture. Prophylatic sprays were given to source plants with 0.1 per cent mancozeb at fortnightly intervals to control fungal pathogens. Leaf, petiole and stem segments taken from mature plants were used as explants for the study.

Plate 1. *Tinospora cordifolia* Miers.

3.1.2 Culture medium

3.1.2.1 Chemicals

The major and minor nutrients required for the preparation of media were of analytical grade and procured from M/s.Sisco Research Laboratories (SRL), British Drug House (BDH) and Merck India. The amino acids, vitamins, plant growth regulators and other media additives were obtained from M/s.Merck, SRL and Sigma Chemicals, USA.

3.1.2.2 Glasswares

Borosilicate glassware of Corning/Borosil brand were used for the study. The glasswares were cleaned by soaking in potassium dichromate solution for 12 hrs followed by thorough washing with tap water to remove all traces of dichromate solution. They were then soaked in detergent solution (Teepol 0.1%), thoroughly washed with tap water and rinsed twice with distilled water. The glassware were then dried in hot air oven at 100°C for 24 hrs and stored in cupboards away from contaminants and dust until use.

3.1.2.3 Composition of media

Murashige and Skoog (1962) (MS) medium, half strength MS medium and Woody plant medium were used for the study. The composition of these media are given in Appendix-I.

3.1.2.4 Preparation of medium

The stock solutions for macro and micro nutrients, vitamins, iron EDTA and growth regulators were prepared with sterile distilled water.

Required quantity of each stock solution, sucrose and inositol were added to distilled water and pH of the solution was adjusted between 5.6 and 5.8 using 0.1 N HCl/NaOH. Agar (0.75% w/v) was added to the medium and melted by keeping the solution in a microwave oven for 10 min. The medium was transferred to test tubes (15 cm x 25 cm) at the rate of 15 ml per tube. The test tubes were plugged with non-absorbent cotton and autoclaved at 121°C at 15 psi (1.06 kg/cm^2) for 30 min (Dodds and Roberts, 1982). The medium was allowed to cool at room temperature and stored in culture room.

3.1.3 Growth regulators

Auxins (2,4-D, NAA, IAA, IBA) and cytokinins (BA, BAP, Kin) were incorporated into the basal media for callus induction and proliferation.

3.1.4 Carbon source

Sucrose was used as the source of carbon for the establishment of *in vitro* systems and was added to the medium at the rate of 1.5 and 3.0 per cent. For enhancing synthesis of the target alkaloid, sucrose was substituted with lactose at the rate of 1.5 per cent.

3.1.5 Transfer area and aseptic manipulation

All the aseptic manipulations such as surface sterilization of explants, preparation and inoculation of explants and subsequent subculturing were carried out under the hood of a clean laminar air flow cabinet.

3.1.6 Culture room

The cultures were incubated at 26 ± 2°C in an air conditioned room with 16 hrs photoperiod (1000 lux) from fluorescent tubes. Humidity in the culture

room varied between 60 to 80 per cent. Cultures were also incubated under dark to study the effect of light on culture establishment, callus growth, proliferation and alkaloid accumulation.

3.2 Methods

3.2.1 Preparation of explants

Mature green leaves, leaf petioles and semi mature stems were used as explants. Explants were trimmed into small pieces of 1.0 x 1.0 cm^2. Petiole and stem explants were excised into pieces of 2.5 cm length each.

3.2.2 Standardisation of surface sterilization

The excised explants were thoroughly washed in running tap water and then with distilled water. The explants were subjected to mercuric chloride ($HgCl_2$) at 0.1 per cent and 0.2 per cent for varying periods ranging from 2 to 10 min. After the treatments, explants were washed free of the sterilants with sterile water, drained over blotting paper and inoculated to the media prepared under the hood of laminar air flow cabinet. The incubated cultures were observed for the levels of contamination and percentage of survival.

3.2.3 Effect of season on culture establishment

The explants were inoculated after surface sterilization using 0.1 per cent $HgCl_2$. The extent of contamination as affected by season was studied. Observations on percentage of contamination, survival and establishment were recorded to find out the optimum period for culture initiation.

3.2.4 Identifying best basal medium for culture establishment

Various basal media like MS medium, MS medium at half strength and Woody Plant medium were employed for initial culture. Best basal medium for establishing *in vitro* cultures was identified based on survival within 3 weeks, and used for further studies.

3.3 Callus initiation and proliferation

3.3.1 Effect of auxins on callusing

Leaf segments, petiole and stem segments were cultured in MS medium supplemented with auxins, NAA, IAA and 2,4-D, each at 0.5, 1.0 and 2.0 mg l^{-1} to study their relative effects on callus induction and growth. Observations were recorded on percentage of cultures initiating calli, callus growth rate and period taken for the calli to initiate. Numerical scores (1-4) were given for assessing callus growth rate. Callus index (CI) was calculated as CI = P x G

Where P = Percentage of cultures initiating calli

G = Growth score

Scoring was done based on the spread of calli and a maximum growth score of 4 was given to those that occupy the entire media surface within 4-5 weeks culture period. The proliferated calli were subscultured to the very same medium at 4-5 weeks interval for further proliferation. The variable responses of the explants used for initiating and proliferating calli were recorded.

3.3.2 Effect of cytokinins on callusing

Major cytokinins, benzyl adenine (BA) and kinetin (Kin) at varying concentrations (each at 0.5, 1.0 and 2.0 mg l^{-1}) were incorporated singly and in

combination with auxins (NAA and IAA), to the basal media. Response of leaf, petiole and stem explants to varying combination of auxins and cytokinins were evaluated with respect to percentage of cultures initiating calli and days taken for callus initiation. Callus indices were worked out as described in 3.3.1. The best among them were subcultured for further studies.

3.3.3 Effect of other media additives on callusing

Media additives like phloroglucinol (100 and 300 mg l^{-1}), activated charcoal (0.25 and 0.50 mg l^{-1}) and casein hydrolysate (100 and 300 mg l^{-1}) were supplemented to media containing IAA (2 mg l^{-1}) and NAA (2 mg l^{-1}), each, in combination with BA (2 mg l^{-1}), to assess their relative effects on callusing of leaf and petiole segments. The percentage of cultures initiating calli and days taken for callus initiation were observed.

3.3.4 Influence of culture environment on callusing

Effect of culture conditions on callus initiation and proliferation was observed by incubating the cultures in dark and under illumination. The incubated cultures were observed for callus initiation and proliferation.

3.4 Regeneration of organoids

Calli produced in MS medium supplemented with NAA and IAA were used to initiate organoids.

3.4.1 Rhizogenesis

Four week old calli produced from leaf, petiole and stem segments were subcultured into media supplemented with various combinations of growth regulator to initiate root (Table 2). Cultures were incubated in light for 10 hrs per

day. Observations on percentage of cultures initiating roots and days to rooting were recorded under each treatment.

Table 2. Media supplements employed for inducing rhizogenesis in *Tinospora cordifolia*

Sl.No	Basal media	Media supplements	Concentrations (mg l^{-1})
1	MS	NAA	1.00, 2.00, 4.00, 5.00
2	„	IAA	1.00, 2.00, 4.00, 5.00
3	„	IBA	1.00, 2.00
4	„	NAA + IAA	1.00 + 1.00, 1.00 + 2.00, 1.00 + 3.00
5	„	NAA + 2,4-D	1.00 + 1.00
6	„	IAA + 2,4-D	1.00 + 1.00
7	„	NAA + Kin	1.00 + 1.00, 2.00 + 2.00
8	½ MS	NAA	2.00, 4.00
9	„	IAA	2.00, 4.00

3.4.2 Shizogenesis

Different concentrations of auxins and cytokinins were incorporated to MS media to evaluate their effects singly and in combination, on callus mediated shoot organogenesis (Table 3). Cultures were maintained in light and dark. Observations on percentage of cultures initiating shoots, period taken for shoot initiation were recorded.

Table 3. Growth regulators incorporated for inducing shizogenesis in *Tinospora cordifolia*

Basal medium – MS

Sl.No	Growth regulators	Concentrations (mg l^{-1})
1	BA	2.00, 3.00
2	NAA + BA	1.00 + 1.00, 1.00 + 2.00, 2.00 + 2.00, 2.00 + 3.00, 2.00 + 4.00
3	NAA + Kin	1.00 + 1.00, 1.00 + 2.00, 2.00 + 2.00
4	IAA + BA	1.00 + 1.00, 1.00 + 2.00, 2.00 + 2.00, 2.00 + 1.00
5	IAA + Kin	1.00 + 1.00, 2.00 + 2.00, 1.00 + 2.00
6	IAA + NAA + BA	1.00 + 1.00 + 1.00

3.4.3 Somatic embryogenesis

Calli maintained on MS medium supplemented with NAA and IAA were subcultured to media incorporated with various auxins (NAA and IAA) and cytokinins (BA and KIN) for the production of somatic embryos.

Effects of varying the carbon sources on regeneration and embryoid production were also evaluated (Table 4).

Table 4. Carbohydrate sources employed for initiating and regenerating embryoid production in *Tinospora cordifolia*

Source of carbohydrates	Concentration (per cent)
Sucrose	5.00
Lactose	3.00
Sucrose + lactose	3.00 + 1.50

3.5 Screening calli for berberine

Leaf, petiole and stem calli obtained from various treatments as detailed subsequently were screened for berberine content.

3.5.1 Preparation of callus extract from solid medium

One gram of fresh callus from each treatment was homogenized with mortar and pestle and repeatedly extracted with methanol till the extract was colourless. The extract was concentrated to dryness and stored for qualitative and quantitative analysis.

3.5.2 Tests for detection of alkaloids

Methanol extracts of calli were subjected to the following qualitative tests to confirm the presence of alkaloids (O'Dowd *et al.*, 1993).

3.5.2.1 Test employing Dragendorff's reagent

Two ml of extract was taken in a test tube and acidified with H_2SO_4 to which 1 ml of Dragendorff's reagent was added. The presence of alkaloid was indicated by the formation of an orange precipitate.

3.5.2.2 Test employing Mayer's reagent

Two ml of the test solution was made solvent free and acidified with H_2SO_4. Then 1 ml of Mayer's reagent was added and observed for a white precipitate which indicates the presence of alkaloids.

3.5.2.3 Detection of alkaloids by chromatograms

The methodology followed was as per Harbone (1973).

3.5.2.3.1 Preparation of gel plate

Thin layer chromatography was employed for detecting berberine. Sixty grams of silica gel G 160-250 mesh size was taken in a flat bottomed flask and mixed with 120 ml distilled water and the slurry was spread on glass plates of 20 x 20 cm size with an applicator to provide 0.25 mm thick gel layer. The plates were

allowed to set for 10 minutes at room temperature and then placed in chromatographic oven maintained at 120-150°C for an hour to dry and activate the same and stored.

3.5.2.3.2 Application of sample

Methanol extracts of test calli along with the standard berberine were applied to the TLC plate. Five microlitres of the sample was applied with a capillary tube on a pre-coated silica plate at a distance of 2 cm from the base. Samples and standard berberine hydrochloride were applied in the same plate at a distance of 2 cm.

The plate was transferred to a chromatographic glass chamber, saturated with the solvent systems as given in the Table 5. The chamber was closed with a lid and the solvent was allowed to run upto two-third portion of the plate. The plates were then taken out and dried for further analysis.

Table 5. Solvent systems employed for developing chromatograms of *in vitro* and *ex vitro* culture extracts of *Tinospora cordifolia*

Spray reagent	Solvent system	Proportion
Dragendorff's reagent	n-BuOH:HOAC:H_2O	7:1:2
Dragendorff's reagent	$CHCl_3$:MeOH:HOAC	25:10:1
Dragendorff's reagent	PrOH:HCO_2H:H_2O	90:1:9
Dragendorff's reagent	n-BuOH: H_2O	5:14

3.5.2.3.3 Detection of berberine

For the detection of berberine, Dragendorff's reagent (prepared by mixing 0.6 g bismuth subnitrate and 6 g potassium iodide in 9 mg of conc. HCl and 10 ml water and made up to 400 ml) was sprayed and visualized under visible and

UV light (365 nm). The standard berberine gave a yellow spot at 365 nm with the appropriate solvent system. Colours of characteristic main zones were described, Rf values were calculated and compared with that of the standard berberine hydrochloride.

$$\text{Rf value} = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent}}$$

3.6 Optimisation of *in vitro* metabolite production

3.6.1 Standardisation of production medium

Leaf, petiole and stem calli were subcultured to basal medium (MS) supplemented with various combinations of growth regulators as detailed in Table 6 and incubated in light. The incubated cultures were analysed for berberine after 3-4 weeks to identify the best growth regulator combination for alkaloid production which was identified as production medium (medium P).

Table 6. Growth regulators incorporated for inducing synthesis of berberine in *in vitro* cultures of *Tinospora cordifolia*

Sl.No	Growth regulators	Concentrations (mg l^{-1})
1	NAA + BA	1.00 + 1.00, 2.00 + 2.00, 1.00 + 3.00, 2.00 + 3.00
2	NAA + Kin	1.00 + 1.00, 2.00 + 2.00, 1.00 + 3.00, 2.00 + 3.00
3	IAA + BA	1.00 + 1.00, 2.00 + 2.00, 1.00 + 3.00, 2.00 + 3.00
4	IAA + Kin	1.00 + 1.00, 2.00 + 2.00, 1.00 + 3.00, 2.00 + 3.00
5	BA	1.00, 2.00
6	Kin	1.00, 2.00

3.6.1.1 Modification of carbon source

Basal media (MS) supplemented with the best hormonal combinations (NAA with BA and Kin and IAA with BA and Kin, each at 2 mg l^{-1}) was modified by increasing levels of sucrose to four and five per cent. Sucrose was substituted with lactose (3%). Combination of sucrose with lactose (2% and 1.0% respectively) was also tried for inducing berberine synthesis.

3.6.1.2 Withdrawal of inorganic nutrients

Nitrate and phosphate ion supply of basal MS medium were reduced to 50 per cent singly, test calli grown on them for 4 to 5 weeks and screened for the presence of berberine.

3.6.1.3 Precursor feeding

Phenyl alanine, the identified amino acid precursor of berberine was added the medium supplemented with NAA and BA and IAA and BA, each at 2 mg l^{-1}, at 100, 150 and 200 mg l^{-1} concentrations, in which 1 to 2 month old calli were subcultured for subsequent detection of the alakaloid.

3.6.1.4 Inducing morphological differentiation

Organised differentiation of experimental calli into roots and shoots through indirect organogenesis was attempted as detailed in 3.4.1 and 3.4.2. Calli at the stage of differentiation was subjected to screening of the alkaloid. The differentiated organoids (shoots and roots) were extracted with methanol for chromatographic studies for alkaloid screening.

3.6.1.5 Regulation through cell growth kinetics

Leaf, petiole and stem calli subcultured on medium P were screened for synthesis of berberine at varying stages of growth from one month to seven months

at 15 days interval. Observations were made on the influence of age of calli in the synthesis of the secondary metabolite.

3.6.1.6 Regulation of photoperiod

The effect of photoperiod on alkaloid synthesis was observed by incubating the cultures in dark and under illumination.

3.6.2 Employing special techniques for synthesis of berberine in *in vitro* cultures

3.6.2.1 Creating stress conditions in the culture media

3.6.2.1.1 Addition of osmoregulants

Osmoregulants like mannitol at 1.5 per cent and polyethylene glycol at 2 and 3 per cent were added singly to the basal MS medium supplemented with NAA and BA at 2 mg l^{-1} each and NAA and Kin at 2 mg l^{-1} each to which the experimental calli were subcultured for subsequent screening.

3.6.2.1.2 Increasing concentration of agar

Concentration of gelling agent agar was increased to 1.0 and 1.5 per cent and added to production medium for subculturing the experimental calli.

3.6.2.1.3 Elicitation

Calli from leaf, petiole and stem segments were subcultured to the production medium supplemented with NAA and BA each at 2 mg l^{-1} and NAA and Kin each at 2 mg l^{-1} to which autoclaved mycelia of *Pythium aphanidermatum* was incorporated.

0.5 g, 1.0 g and 1.5 g of autoclaved fungal mycelia were homogenized with pestle and mortar and added to the production medium before sterilization in to which the test calli were subcultured.

3.6.2.1.4 Gel entrapment

Uniform bits of calli (0.2 g) of leaf, petiole and stem segments of experimental ecotypes were used for conducting gel entrapment studies.

MS basal salt solutions containing the gel forming chemical sodium alginate at concentrations of 3.0, 5.0, 7.0, 9.0, and 11.0 per cent were autoclaved. Calli bits were thoroughly mixed with the alginate gel and dropped into autoclaved calcium chloride ($CaCl_2$) solution prepared at varying levels of 50, 75 and 100 mM. Sterilized glass tubes were employed for dropping calli bits. Observations were recorded as to the degree of firmness of beads in various concentrations of sodium alginate and calcium chloride solution employed. Optimum concentrations of sodium alginate and calcium chloride were standardised.

The round firm beads were washed in sterile distilled water, thrice, cultured in basal solid MS medium devoid of any supplements and incubated at 26±1°C. The stored beads were subjected to biochemical analysis to detect and estimate berberine.

3.7 Establishing suspension cultures

MS basal medium without agar supplemented with NAA with BA or Kin each are 2 mg l^{-1} was distributed to 100 ml conical flask @ 30 ml in each flask. The flasks were plugged with cotton and autoclaved as described in 3.1.2.4.

Calli from leaf, petiole and stem segments, of 1 g each were inoculated to the liquid medium. The conical flasks were incubated at 100 rpm at 28±2°C

with a 16 hour photoperiod in an orbital shaker. Subculturing intervals and cell density were established after observing the cell count at specific intervals for each of the varying fresh weight employing a haemocytometer.

3.7.1 Subculture of suspensions

For subculturing, the supernatant liquid was poured into fresh liquid media at the ratio of 1:4 (v/v) and cultured in an orbital shaker.

3.7.2 Determination of packed cell volume

Suspension cultures were shaken thoroughly. Five ml of suspension was pipetted out to a graduated centrifuge tube. The samples were centrifuged at 2000 rpm for 5 minutes and sedimented mass expressed as ml pellet per ml culture.

3.8 Screening of berberine in liquid medium

3.8.1 Preparation of extracts

The liquid media were separated from the cells by filtration and concentrated by vacuum evaporator. Adsorption chromatography was carried out for separation of berberine (Nakagawa *et al.*, 1984).

The cation exchanger Amberlite x AD 20 x 1.6 cm column was prepared for the purpose. It was made neutral by washing three times with distilled water followed by drying in hot air oven at 70°C for 15 minutes.

The concentrated liquid media was poured on top of column without disturbing the top layer. The sample was allowed to percolate through the column and the flow stopped when the alkaloid was fully adsorbed in the column. The alkaloid was eluted with methanol at the rate of 3 ml per minute. The fractions were concentrated by evaporating the solvent.

3.8.2 Detection of berberine by qualitative methods

The fractions collected by adsorption chromatography as detailed above from different liquid media were screened for the presence of berberine by thin layer chromatography. The fractions collected were spotted in pre-coated plate along with the standard berberine hydrochloride. The fractions giving spots identical to the authentic sample at 365 nm were recorded and Rf value calculated. Qualitative tests employing Dragendorff's reagent and Mayer's reagent were also carried out for the detection of berberine.

3.9 Screening of *ex vitro* samples for berberine

3.9.1 Preparation of extracts

Samples of young and mature stem (20 g each), tender and mature leaves (10 g each) were ground separately by using pestle and morter. Methanol was used to extract the sample. The pulp was filtered through filter paper till the extract was colourless. It was concentrated to dryness and stored.

3.9.2 Detection of berberine by qualitative analysis

The qualitative analysis for the detection of berberine was carried out as per the method described by Harbone (1973).

3.10 Quantification of berberine from static and suspension cultures

3.10.1 Estimation of Lambda max

Lambda (λ) max of berberine was identified by using UV spectrometer. Natural and standard berberine hydrochloride were used for this purpose. The extracts were prepared from *ex vitro* plants as detailed in 3.7.1. Purification of

berberine from the extract was carried out by column chromatography followed by thin layer chromatography.

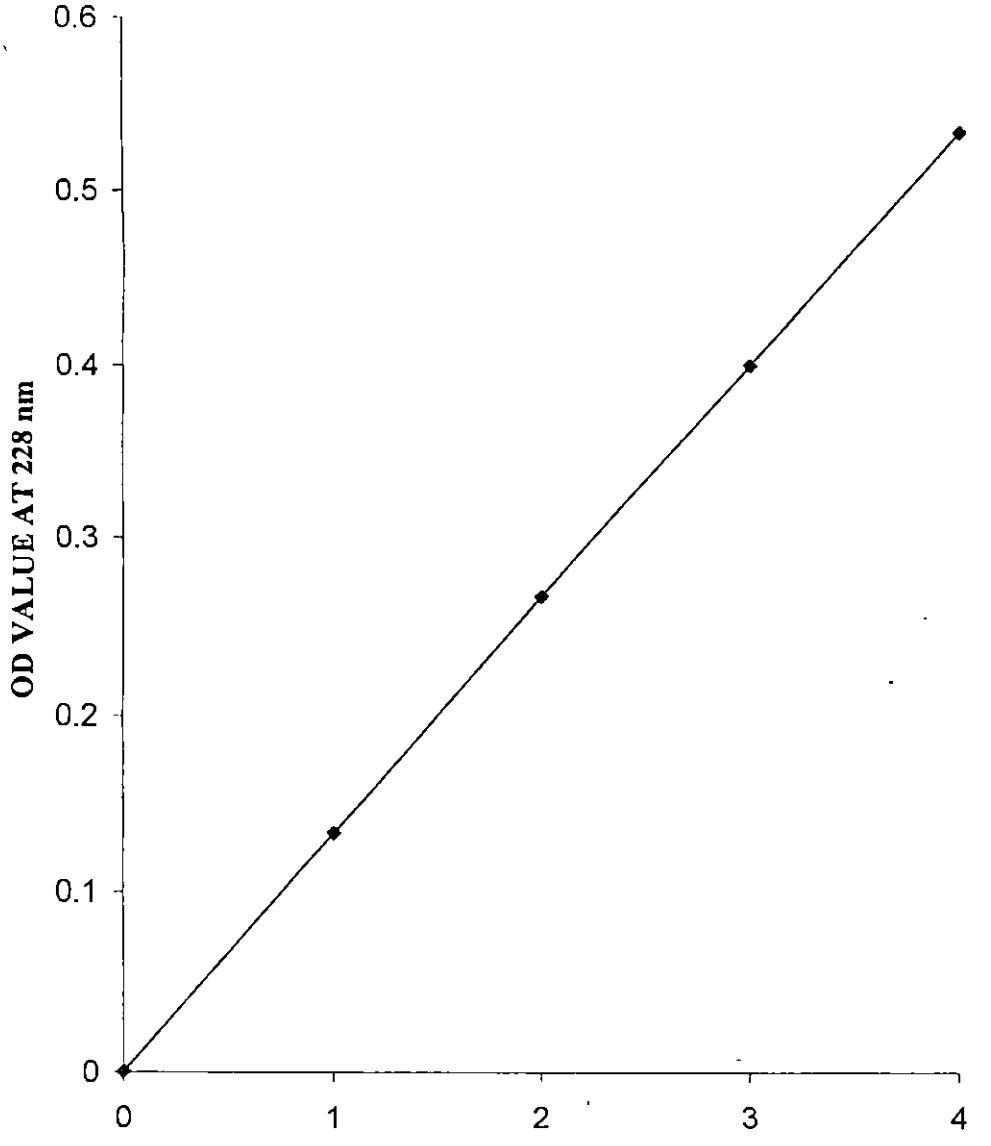
Silica (30 x 1.6 cm) column was saturated with solvent mixture (Butanol:aceticacid:water – 7:1:2). Concentrated 2 ml sample was applied on top of the column without disturbing the top player. The flow rate was adjusted to 3 ml/2 minute.

The fractions were concentrated under air flow and TLC was performed against standard berberine hydrochloride. The spot that gave yellow colour identical to that of standard berberine, was scraped and dissolved in 4.5 ml methanol and centrifuged at 1500 rpm for 18 minutes.

The standard berberine at 0.001 per cent concentration gave 4 peaks at 228 nm, 264 nm, 348 nm and 429 nm. The supernatants of centrifuged samples were scanned in the UV spectrometer. Peak at 228 nm was common for all samples, including standard berberine. The peaks at 264 nm, 348 nm and 429 nm of standard berberine hydrochloride were not recorded in the samples. So 228 nm was selected as the lambda max of berberine in the present study.

The absorbance of 2, 4, 6 8, 10, 12, 14, 16, 18 and 20 µg standard berberine hydrochloride was recorded at 228 nm. Methanol was used as the solvent as in the case of *ex vitro* and *in vitro* extracts. The different concentrations of standards were read in the UV spectrometer at 228 nm against the reagent blank. A standard curve of berberine hydrochloride was plotted using absorbance (nm) Vs concentration of the berberine hydrochloride (µg/ml) (Fig. 1).

Fig.1. STANDARD GRAPH OF BERBERINE, OD AT 228 nm



3.10.2 Estimation of berberine from *in vitro* static and suspension cultures and *ex vitro* sources

The methanol extracts of calli, suspension cultures, stem and leaves that gave positive test for the presence of berberine were subjected to column chromatography as described earlier.

Collected fractions were spotted in a thin layer chromatogram against the standard berberine. The fraction giving yellow spots identical to the standard were noted at 365 nm in the UV chamber. They were concentrated to dryness under constant air flow, scraped the same and dissolved in methanol and centrifuged at 1500 rpm for 18 minutes.

The supernatant was taken and read in the UV spectrometer at 228 nm against reagent blank (methanol). The absorbance of berberine from different extracts were recorded. The standard graph was used for estimating the concentration of berberine from samples of various treatments.

3.11 Statistical analysis

The data obtained from the different treatments were subjected to statistical analysis as per Panse and Sukhatme (1978). The treatments were grouped into homogeneous groups using Duncan's multiple range test.

Results

RESULTS

The results of the studies on "Utilization of *in vitro* cultures of *Tinospora cordibolia* Miers. (Chittamrithu) for berberine" carried out at the Plant Tissue Culture and Biochemistry Laboratories, College of Horticulture, Vellanikkara are presented in this chapter.

4.1 Standardisation of surface sterilisation of explants

Effect of surface sterilants at varying levels on survival of explants of the ecotypes under study is presented in Table 7. Among the treatments tried, $HgCl_2$ at 0.1 per cent concentration for 8 min was most effective in reducing the levels of contamination of leaf, petiole and stem explants registering 73.74 per cent survival (Table 7). Though treatment with $HgCl_2$ at 0.1 per cent concentration for 8 and 10 min registered same levels of contamination (11.26%) the percentage of survival was less (43.29) in the latter due to scorching of explants. Increasing the concentration of $HgCl_2$ to 0.2 per cent reduced percentage of surviving cultures. Higher duration of treating with surface sterilants reduced microbial interference, but resulted in substantial scorching of explants.

4.2 Influence of season on survival of cultures

The data on seasonal influence on culture establishment and sustained growth of explants are presented in Table 8. Observations over a period of nine months revealed marked difference in the survival of cultures. Substantial contamination was observed for the explants inoculated in the months of December to March while cultures established better during the period from June to August, registering maximum survival percentage in July (90.35%) followed by August (88.10%).

Table 7. Effect of surface sterilization on survival of leaf, petiole and stem explants of *Tinospora cordifolia*

Surface sterilants	Duration of treatment (min.)	Cultures contaminated 15 days after inoculation (%)	Culture survival without contamination 15 days after inoculation (%)	
			Scorched	Healthy
$HgCl_2$ 0.1 %	2.0	55.55	3.25	41.25
$HgCl_2$ 0.1 %	3.0	41.66	4.17	54.17
$HgCl_2$ 0.1 %	5.0	25.35	12.55	62.10
$HgCl_2$ 0.1 %	8.0	11.26	15.00	73.74
$HgCl_2$ 0.1 %	10.0	11.26	45.45	43.29
$HgCl_2$ 0.1 %	2.0	27.77	53.75	18.48
$HgCl_2$ 0.1 %	3.0	22.22	61.00	16.78
$HgCl_2$ 0.1 %	5.0	11.11	77.45	11.40

4.3 Standardisation of basal media for inducing calli

Response of the ecotypes to different plant tissue culture basal media in initiating calli is given in Table 9. Marked variation was observed with respect to basal media for leaf, petiole and stem cultures in initiating calli in both Vellanikkara and Madurai ecotypes. Highest mean percentage of cultures initiated calli in MS medium at full strength for Vellanikkara and Madurai ecotypes, the mean values being 73.15, 75.39 and 83.56 per cent respectively for cultures derived from leaf, petiole and stem explants (Table 9). In MS medium at half strength, only 62.96 per cent leaf explants, 63.14 per cent petiole explants and 70.21 per cent stem explants callused. WPM medium registered lowest percentage for callusing in all the cultures. Regardless of the basal medium employed, stem cultures of both the ecotypes responded well registering highest mean callus initiation percentage (72.31 per cent).

4.4 Induction and proliferation of calli

4.4.1 Effect of auxins in inducing and proliferating calli

Response of leaf, petiole and stem cultures of the experimental ecotypes to auxins in initiating and proliferating calli are presented in Table 10a, 10b and 10c (Plate 2). From the results, it is inferred that the three auxins employed in the study, NAA, IAA and 2,4-D at varying levels exerted significant influence on the performance of leaf, petiole and stem cultures of the experimental ecotypes with respect to the percentage of cultures initiating calli, callus growth score and mean callus index. Days to callus induction did not vary significantly in stem

Table 8. Effect of season of collection of explants on *in vitro* culture establishment in *Tinospora cordifolia*

Treatment	Month of culture establishment	Percentage of culture establishment
1.	December	28.50
2.	January	38.45
3.	February	42.55
4.	March	48.75
5.	April	50.45
6.	May	58.15
7.	June	84.20
8.	July	90.35
9.	August	88.10

Table 9 . Standardisation of basal medium for callus induction in *Tinospora cordifolia*

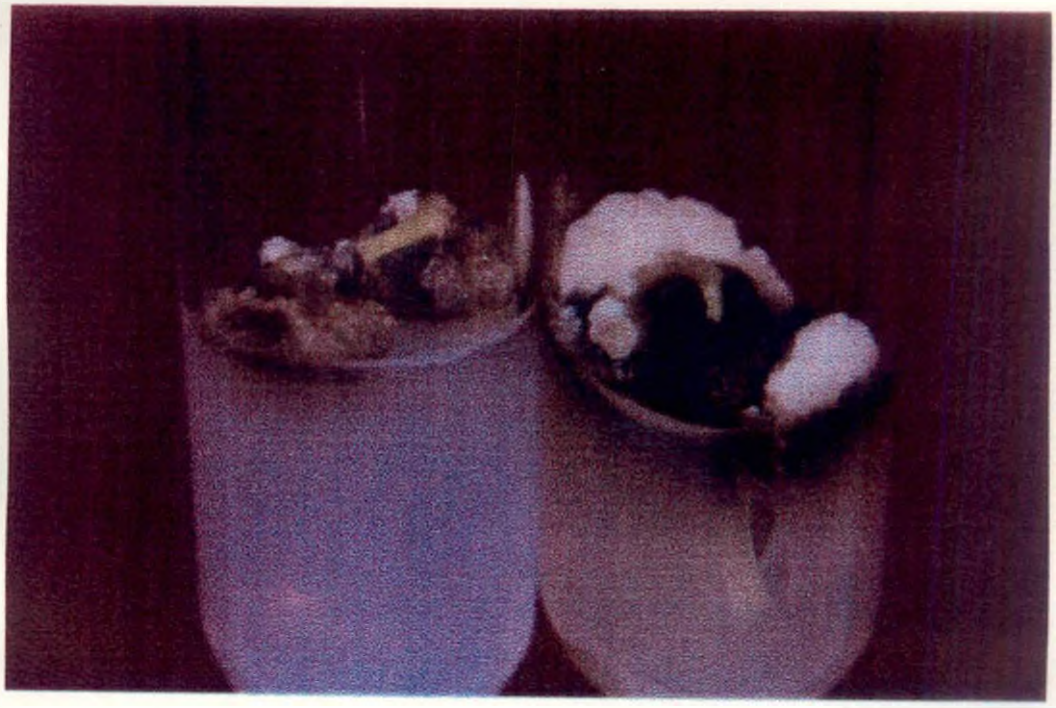
Basal media	Percentage of cultures initiating calli								
	Vellanikkara ecotype			Madurai ecotype			Mean		
	Leaf	Petiole	Stem	Leaf	Petiole	Stem	Leaf	Petiole	Stem
MS	72.22	75.81	82.59	74.08	74.98	84.54	73.15	75.39	83.56
½ MS	61.45	62.78	71.08	64.47	63.50	69.35	62.96	63.14	70.21
WPM	58.16	60.08	62.18	57.28	57.56	64.16	57.72	58.82	63.17
Mean	63.94	66.22	71.95	65.27	65.34	72.01	64.61	65.73	72.31

cultures under the influence of the above auxins. MS medium enriched with NAA at 4.0 mg l^{-1} recorded highest percentage of callus initiation registering 91.428 per cent (Table 10a) for leaf cultures and 91.012 per cent for stem cultures (Table 10c). NAA at 2.0 mg l^{-1} recorded the highest percentage (94.394) of callus initiation for petiole cultures. In leaf cultures, NAA at 1 mg l^{-1} in combination with IAA at 1 mg l^{-1} or 2 mg l^{-1} or 3 mg l^{-1} respectively registered values on par with the above (Table 10a).

Among the three explants used, leaf explants callused earlier in media to which NAA was incorporated at levels of 5.0 mg l^{-1} (9.699 days of incubation) or 4.0 mg l^{-1} (9.808 days of incubation). The same auxin at 4.0 mg l^{-1} recorded significantly early callusing in petiole (11.037 days of culture) cultures. For stem cultures, all the treatments responded equally, without any significant difference (Table 10c). Callus growth rate as measured by callus growth score was significantly higher in media supplemented with IAA at 2.0 mg l^{-1} for petiole cultures (2.906) and stem cultures (3.113). For leaf cultures, NAA at 1 mg l^{-1} in combination with IAA at 3 mg l^{-1} registered highest growth score (3.096). Uniform callus growth score values were obtained for leaf explants with IAA and NAA, each at 4 mg l^{-1} and 5 mg l^{-1}.

Highest mean callus index values of 267.194 and 280.977 respectively were obtained on incubating petiole and stem explants on media supplemented with NAA at 4 mg l^{-1}. NAA at 1 mg l^{-1} with IAA at 3 mg l^{-1} recorded highest callus index value (286.583) for leaf cultures. The treatments wherein the basal medium was supplemented with IAA at 1.0, 2.0, 4.0 and 5.0 mg l^{-1} recorded callus index

Plate 2. Initiation and proliferation of callus from stem and leaf explants of *Tinospora cordifolia* Miers.



values on par with one other without any significant difference for leaf and petiole cultures. Uniform mean callus index values on par with each other were obtained for stem explants with NAA at 4 mg l^{-1} and 5 mg l^{-1} and IAA at 4 mg l^{-1}, incorporated singly to the basal medium. Irrespective of the levels tried, the auxin 2,4-D was less effective in improving callusing in *Tinospora cordifolia* recording lowest values for the parameters studied (Table 10a, 10b, 10c). However, a positive effects of this auxin was observed in stem cultures, wherein days to callus induction were on par with other treatments.

4.4.1.1 Relative performance of experimental ecotypes in callusing

The two ecotypes did not vary significantly between each other for most of the parameter observed. The Vellanikkara ecotype recorded significantly superior performance with respect to cultures initiating callus, when leaf (85.168%) and petiole (89.788%) segments were incubated for culture initiation (Fig. 2b). Both Vellanikkara and Madurai ecotypes registered values on par with each other for mean callus index in leaf, petiole and stem cultures. Early induction of calli was noticed in petiole cultures (11.525 days) of Madurai ecotype while Vellanikkara ecotype showed early induction in stem cultures (11.654 days). Both the ecotypes responded in a similar manner with respect to earliness in callus induction, in leaf cultures.

Petiole cultures of Madurai ecotype registered high callus growth score (2.478) as compared to Vellanikkara ecotype (2.353). No significant variation was observed between ecotypes in callus growth score when leaf and stem explants were brought to culture.

Fig. 2a. Relative response of *T.cordifolia* ecotypes to auxins with respect to callus initiation period.

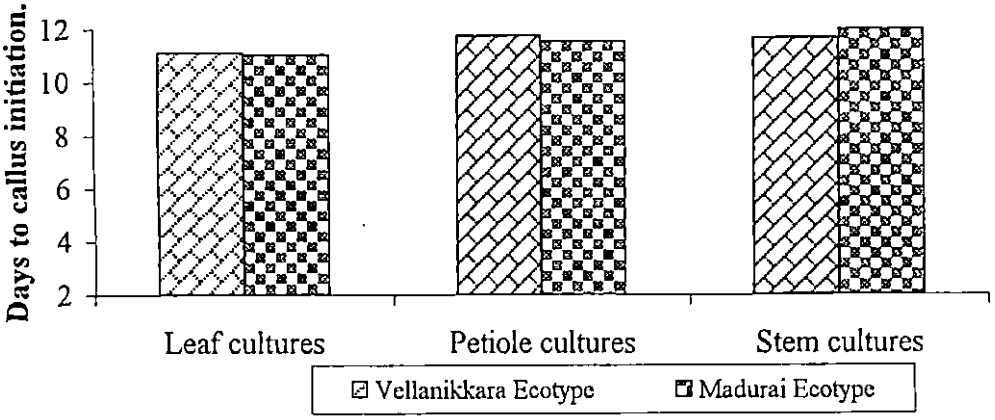


Fig.2b. Relative response of *T.cordifolia* ecotypes to auxins with respect to callus initiation percentage.

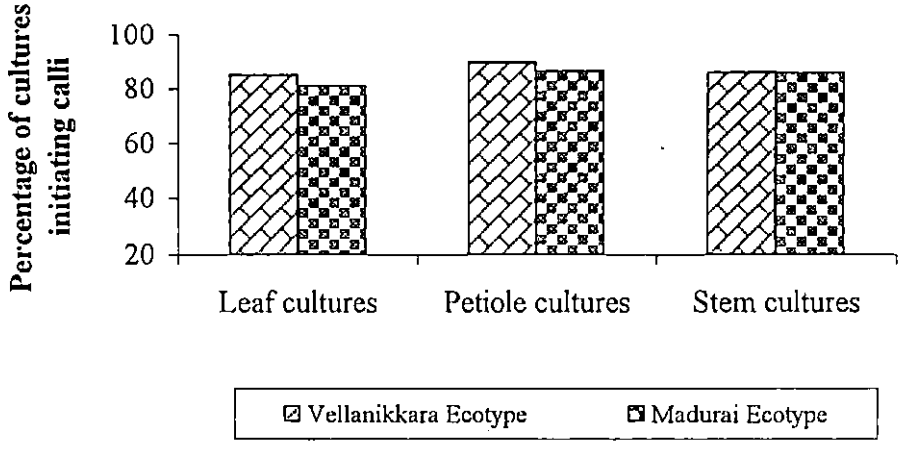


Table 10a. Effect of auxins on callusing in leaf cultures of *Tinospora cordifolia*

Basal medium - MS

Sl. No.	Auxins (mg l^{-1})	Days to initiate callus			Percentage of cultures initiating callus in 30 days			Callus growth score in 30 days			Callus index		
		1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
1	NAA 0.5	11.530	11.566	11.548^{bc}	76.389	78.333	77.361^{cde}	1.761	1.658	1.710^{de}	134.449	130.666	132.557^{ef}
2	NAA 1.0	12.207	11.849	12.028^{a}	90.972	84.523	87.748^{ab}	2.724	2.718	2.721^{abc}	248.195	229.921	239.058^{bc}
3	NAA 2.0	10.794	10.656	10.725^{de}	85.640	90.417	88.028^{ab}	2.939	2.595	2.767^{abc}	251.634	235.456	243.545^{ab}
4	NAA 4.0	9.658	9.959	9.808^{g}	93.333	89.523	91.428^{a}	2.765	2.901	2.833^{ab}	257.725	259.651	258.688^{ab}
5	NAA 5.0	9.622	9.777	9.699^{g}	93.750	86.607	90.178^{ab}	2.872	2.865	2.868^{ab}	269.600	248.263	258.932^{ab}
6	IAA 0.5	12.274	11.758	12.016^{a}	74.167	76.667	75.417^{cde}	2.025	1.764	1.895^{d}	150.061	135.400	142.730^{e}
7	IAA 1.0	11.816	11.658	11.737^{abc}	90.000	90.000	90.000^{ab}	2.700	2.871	2.786^{abc}	243.044	258.364	250.704^{ab}
8	IAA 2.0	11.223	11.790	11.507^{bc}	89.204	86.607	87.906^{ab}	2.938	2.800	2.869^{ab}	262.144	242.321	252.233^{ab}
9	IAA 4.0	10.738	10.766	10.752^{d}	92.857	86.190	89.524^{ab}	2.556	3.063	2.809^{ab}	239.085	264.042	251.564^{ab}
10	IAA 5.0	10.775	10.564	10.669^{de}	90.000	87.121	88.560^{ab}	2.862	2.897	2.879^{ab}	257.518	252.507	255.012^{ab}
11	2, 4D 0.5	11.608	11.320	11.464^{bc}	74.598	69.047	71.823^{de}	1.683	1.605	1.644^{de}	125.761	110.568	118.164^{ef}
12	2, 4D 1.0	11.694	11.666	11.680^{abc}	78.567	72.381	75.474^{cde}	1.616	1.258	1.437^{e}	128.551	91.028	109.789^{ef}
13	2, 4D 2.0	11.793	11.766	11.780^{ab}	81.250	63.333	72.292^{de}	1.206	1.524	1.365^{e}	97.881	95.540	96.710^{f}
14	2, 4D 4.0	11.773	11.916	11.844^{ab}	74.999	61.904	68.451^{e}	1.524	1.365	1.444^{e}	115.348	83.856	99.602^{f}
15	IBA 1.0	10.672	10.816	10.744^{d}	70.417	72.381	71.399^{de}	2.375	2.737	2.556^{bc}	169.371	198.272	183.822^{d}
16	NAA1+2,4D1	11.641	11.086	11.363^{c}	85.000	75.714	80.357^{bcd}	2.360	2.480	2.420^{c}	199.999	187.892	193.945^{d}
17	IAA1+2,4D1	11.777	11.766	11.771^{ab}	82.222	85.000	83.611^{abc}	2.288	2.558	2.423^{c}	187.180	217.941	202.561^{cd}
18	NAA1+IAA1	10.591	10.323	10.457^{def}	96.667	90.417	93.542^{a}	2.857	3.000	2.929^{ab}	276.652	271.660	274.156^{ab}
19	NAA1+IAA2	10.358	10.215	10.286^{f}	95.454	89.903	92.679^{a}	2.711	2.946	2.828^{ab}	258.318	264.979	261.649^{ab}
20	NAA+IAA3	10.347	10.346	10.347^{ef}	96.875	87.762	92.318^{a}	3.140	3.053	3.096^{a}	305.063	268.104	286.583^{a}
21	Mean	11.145^{a}	11.078^{a}	11.111	85.168^{a}	81.191^{b}	83.405	2.395^{a}	2.433^{a}	2.414	202.879^{a}	202.322^{a}	205.600

1. Vellanikkara ecotype 2. Madurai ecotype

Table 10b. Effect of auxins on callusing in petiole cultures of *Tinospora cordifolia*

Basal medium - MS

Sl. No.	Auxins (mg l^{-1})	Days to initiate callus			Percentage of cultures initiating callus in 30 days			Callus growth score in 30 days			Callus index		
		1	2	Mean	1	2	Mean	1	2	Mean	.1	2	Mean
1	NAA 0.5	11.788	12.089	11.938 ^{abc}	76.389	85.000	80.694 ^{fg}	1.656	1.658	1.657 ^{c}	126.715	140.927	133.821 ^{cde}
2	NAA 1.0	11.491	11.947	11.719 ^{abcd}	83.974	87.083	85.528 ^{def}	2.911	2.720	2.816 ^{ab}	244.433	236.844	240.639 ^{ab}
3	NAA 2.0	11.208	11.265	11.237 ^{bcd}	96.667	92.121	94.394 ^{a}	2.813	2.750	2.781 ^{ab}	272.546	253.29	262.788 ^{ab}
4	NAA 4.0	11.158	10.916	11.037 ^{d}	92.820	89.204	91.012 ^{abcd}	2.964	2.910	2.937 ^{a}	275.136	259.251	267.194 ^{a}
5	NAA 5.0	11.161	11.064	11.112 ^{cd}	89.903	84.523	87.213 ^{bcde}	2.724	3.054	2.889 ^{a}	244.705	257.900	251.302 ^{ab}
6	IAA 0.5	11.966	11.564	11.765 ^{abcd}	67.500	76.667	72.083 ^{h}	1.736	1.773	1.755 ^{c}	117.750	136.119	126.935 ^{de}
7	IAA 1.0	11.957	12.145	12.051 ^{ab}	86.190	91.608	88.899 ^{abcde}	2.911	2.840	2.876 ^{ab}	250.984	260.178	255.581 ^{ab}
8	IAA 2.0	11.059	11.289	11.174 ^{cd}	91.608	89.204	90.406 ^{abcde}	2.875	2.938	2.906 ^{a}	263.455	261.932	262.693 ^{ab}
9	IAA 4.0	11.831	12.258	12.044 ^{ab}	90.417	83.216	86.816 ^{cde}	2.813	2.961	2.887 ^{a}	254.887	246.606	250.747 ^{ab}
10	IAA 5.0	11.839	10.995	11.417 ^{abcd}	95.454	92.121	93.788 ^{ab}	2.424	2.964	2.694 ^{ab}	231.043	273.003	252.023 ^{ab}
11	2, 4D 0.5	11.819	11.166	11.492 ^{abcd}	85.000	73.214	79.107 ^{g}	1.250	1.256	1.253 ^{d}	105.833	91.521	98.677 ^{e}
12	2, 4D 1.0	12.058	11.786	11.922 ^{abc}	93.750	85.000	89.375 ^{abcde}	2.000	1.775	1.887 ^{c}	190.625	150.832	170.728 ^{c}
13	2, 4D 2.0	12.239	11.845	12.042 ^{ab}	90.000	89.486	89.743 ^{abcde}	1.450	1.909	1.679 ^{c}	130.665	171.086	150.876 ^{cd}
14	2, 4D 4.0	12.420	11.793	12.107 ^{a}	88.311	79.370	83.841 ^{efg}	1.445	1.503	1.474 ^{cd}	128.168	118.626	123.397 ^{de}
15	IBA 1.0	12.066	11.314	11.690 ^{abcd}	95.434	88.311	91.883 ^{abcd}	2.333	2.938	2.635 ^{ab}	224.242	259.253	241.747 ^{ab}
16	NAA1+2,4D1	11.516	11.264	11.390 ^{abcd}	97.307	88.974	93.141 ^{abc}	2.258	2.616	2.437 ^{b}	206.446	233.406	219.926 ^{b}
17	IAA1+2,4D1	12.361	11.766	12.064 ^{ab}	98.250	87.063	92.656 ^{abc}	2.550	2.558	2.554 ^{ab}	238.750	224.320	231.535 ^{ab}
18	NAA1+IAA1	12.089	11.516	11.802 ^{abcd}	92.140	88.311	90.226 ^{abcde}	2.429	2.911	2.670 ^{ab}	223.802	257.348	240.575 ^{ab}
19	NAA1+IAA2	11.865	11.208	11.536 ^{abcd}	88.715	90.417	89.566 ^{abcde}	2.661	2.811	2.736 ^{ab}	244.434	254.172	249.303 ^{ab}
20	NAA+IAA3	11.415	11.316	11.365 ^{abcd}	95.909	88.461	92.185 ^{abcde}	2.890	2.722	2.806 ^{ab}	262.448	241.582	252.015 ^{ab}
21	Mean	11.765^{a}	11.525^{b}	11.645	89.788^{a}	86.468^{b}	88.128	2.355^{b}	2.478^{a}	2.417	211.853^{a}	216.397^{a}	214.125

1. Vellanikkara ecotype 2. Madurai ecotype

Table 10c. Effect of auxins on callusing in stem cultures of *Tinospora cordifolia*

Basal medium - MS

Sl. No.	Auxins (mg l^{-1})	Days to initiate callus			Percentage of cultures initiating callus in 30 days			Callus growth score in 30 days			Callus index		
		1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
1	NAA 0.5	11.566	11.966	11.766[a]	77.500	84.524	81.012[e]	2.375	2.739	2.557[efg]	184.375	231.674	208.025[efg]
2	NAA 1.0	11.786	11.814	11.800[a]	85.000	86.190	85.595[abcde]	2.711	2.801	2.756[cdef]	230.661	241.453	236.057[cde]
3	NAA 2.0	11.315	12.367	11.841[a]	86.667	91.608	89.137[abc]	2.911	3.080	2.996[abc]	252.919	282.208	267.564[ab]
4	NAA 4.0	11.791	12.316	12.053[a]	91.608	90.417	91.012[a]	3.081	3.089	3.085[ab]	282.142	279.813	280.977[a]
5	NAA 5.0	11.206	11.758	11.482[a]	89.903	87.083	88.493[abc]	3.063	3.104	3.083[ab]	275.826	270.269	273.047[a]
6	IAA 0.5	11.663	11.916	11.790[a]	82.307	78.974	80.641[e]	2.919	2.921	2.920[abcd]	240.519	230.685	235.602[cde]
7	IAA 1.0	11.817	11.996	11.906[a]	86.607	85.000	85.803[abcde]	2.849	2.777	2.813[bcde]	246.719	236.168	241.444[bce]
8	IAA 2.0	11.230	11.666	11.448[a]	88.311	91.608	89.960[ab]	3.166	3.061	3.113[a]	279.736	280.324	280.030[bce]
9	IAA 4.0	11.758	12.039	11.898[a]	91.608	89.204	90.406[ab]	2.945	3.108	3.027[abc]	269.834	277.432	273.633[a]
10	IAA 5.0	11.788	11.614	11.701[a]	86.190	86.190	86.190[abcde]	2.946	3.088	3.017[abc]	253.906	266.119	260.012[abc]
11	2, 4D 0.5	11.889	11.762	11.825[a]	81.667	82.576	82.121[de]	1.480	1.765	1.623[i]	121.249	145.779	133.514[i]
12	2, 4D 1.0	11.806	12.258	12.032[a]	86.607	80.909	83.758[cde]	1.761	1.499	1.630[i]	152.469	121.307	136.888[i]
13	2, 4D 2.0	12.116	11.516	11.816[a]	85.164	86.607	85.886[abcde]	2.118	2.106	2.112[h]	180.476	182.178	181.477[gh]
14	2, 4D 4.0	11.089	12.139	11.614[a]	81.667	82.211	81.939[de]	2.031	2.083	2.057[h]	165.499	171.156	168.328[h]
15	IBA 1.0	11.589	12.315	11.952[a]	83.974	83.766	83.870[cde]	2.165	2.667	2.416[g]	181.589	222.993	202.291[fg]
16	NAA1+2,4D1	11.820	11.991	11.906[a]	85.000	85.417	85.208[abcde]	2.661	2.314	2.488[fg]	226.495	197.266	211.881[ef]
17	IAA1+2,4D1	11.792	12.051	11.921[a]	82.857	88.311	85.584[abcde]	2.613	2.525	2.569[efg]	216.468	223.225	219.846[def]
18	NAA1+IAA1	11.440	12.366	11.903[a]	84.523	85.164	84.844[bcde]	2.410	2.945	2.678[defg]	203.517	250.846	227.181[def]
19	NAA1+IAA2	12.015	11.990	12.003[a]	95.454	84.659	90.057[ab]	2.865	2.944	2.905[abcd]	272.790	249.395	261.093[abc]
20	NAA+IAA3	11.714	11.742	11.728[a]	87.063	87.762	87.412[abcd]	3.046	3.006	3.026[abc]	264.215	263.793	264.004[abc]
21	Mean	11.654[b]	11.979[a]	11.819	85.984[a]	85.909[a]	85.946	2.606[a]	2.681[a]	2.643	225.070[a]	231.219[a]	228.145

1. Vellanikkara ecotype 2. Madurai ecotype

Fig.2c. Relative response of *T.cordifolia* ecotypes to auxins with respect ot callus growth score.

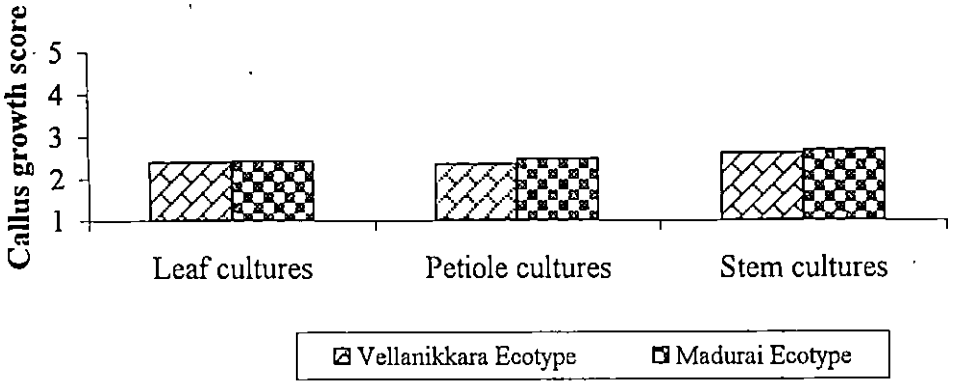
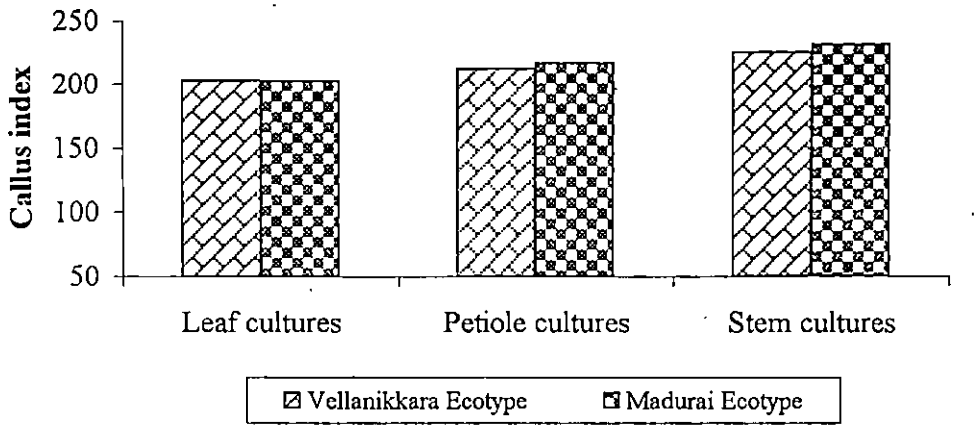


Fig.2d. Relative response of *T.cordifolia* ecotypes to auxins with respect to callus index.



4.4.1.2 Suitability of explants for initiating and proliferating calli

Stem explants surpassed leaf and petiole cultures registering a mean callus index value of 228.145 (Fig. 2d) with a callus growth score of 2.643 (Fig. 2c). Next to stem explants, petiole explants performed better wherein 88.128 per cent cultures callused with a callus growth score of 2.417 and a mean callus index value of 214.125. Superior performance of leaf explants was evident only for early induction of callus (11.11 days) (Fig. 2a).

4.4.2 Effect of cytokinins in inducing and proliferating calli

The influence of cytokinins in inducing and proliferating calli are presented in the Table 11a, 11b and 11c. The major cytokinins, BA and kinetin at varying levels exerted significant influence on all the parameters studied. The explants when cultured in MS medium with Kin at 2 mg l^{-1} callused earlier for leaf (11.316 days), petiole (11.535 days) and stem (11.249 days) cultures. Next to this, BA at 1 mg l^{-1} and 2 mg l^{-1} registered early callusing.

MS medium enriched with BA at 2 mg l^{-1} showed higher percentage of callusing for leaf (75.425%) and stem (81.098%) cultures whereas for petiole cultures, Kin 2 mg l^{-1} registered higher percentage of callusing (72.559%). Maximum callus growth score of 2.743 and 2.845 was observed for leaf and stem explants, when cultured in the basal media supplemented with BA at 2 mg l^{-1}. A growth score of 2.406 was achieved in basal medium enriched with Kin at 2 mg l^{-1} for petiole cultures. Highest mean callus index values of 206.616 and 228.206 were obtained on incubating leaf and stem explants on basal media supplemented with BA 2 mg l^{-1}. Among the treatments, BA and kinetin each at 0.5 mg l^{-1} registered lowest values for all the parameters studied in both the ecotypes.

Fig.3a. Comparative response of *T.cordifolia* ecotypes to cytokinins with respect to callus initiation period.

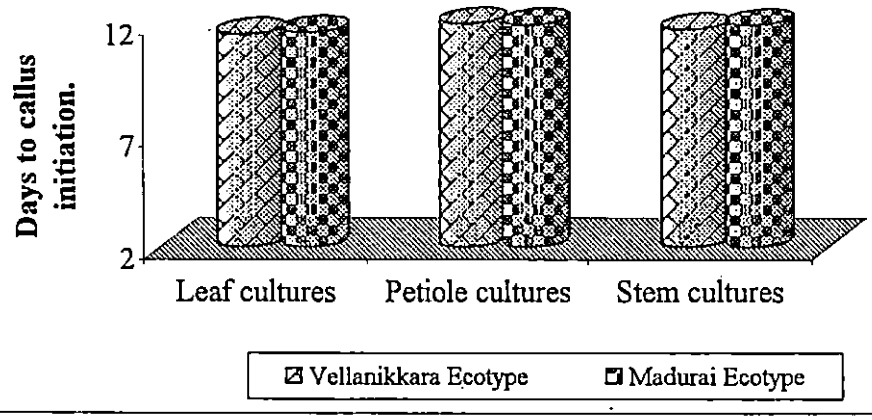


Fig.3b. Comparative response of *T.cordifolia* ecotypes to cytokinins with respect to callus initiation percentage.

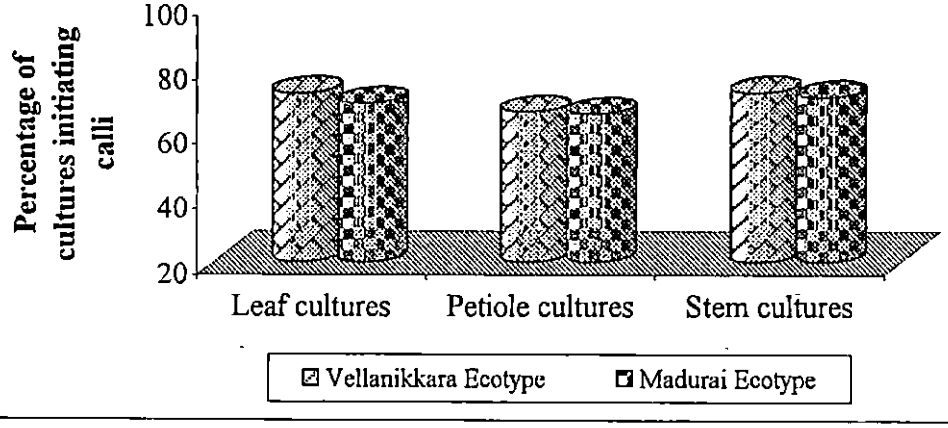


Table 11 a . Effect of cytokinins on callusing in leaf cultures of *Tinospora cordifolia*

Basal medium – MS

Treatment (mgl^{-1})	Days to initiate callus			Percentage of cultures initiating calli in 30 days			Callus growth score in 30 days			Callus index		
	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
BA 0.5	11.875	11.582	11.728 ^{ab}	74.422	61.441	67.932 ^{d}	1.643	1.244	1.444 ^{d}	121.864	76.843	99.354 ^{f}
BA 1.0	11.452	11.548	11.500 ^{bc}	69.441	74.455	71.935 ^{b}	2.249	2.542	2.395 ^{b}	156.549	189.788	173.169 ^{c}
BA 2.0	11.411	11.215	11.313 ^{c}	78.412	72.438	75.425 ^{a}	2.543	2.944	2.743 ^{a}	199.294	213.937	206.616 ^{a}
KIN 0.5	11.524	12.409	11.967 ^{a}	72.549	64.418	68.483 ^{cd}	1.640	1.639	1.640 ^{e}	118.991	105.778	112.384 ^{e}
KIN 1.0	11.465	11.496	11.480 ^{bc}	68.458	69.470	68.964 ^{c}	2.344	2.440	2.392 ^{b}	160.863	169.912	165.387 ^{d}
KIN 2.0	11.315	11.316	11.316 ^{c}	70.500	72.458	71.489 ^{b}	2.353	2.641	2.497 ^{b}	165.965	191.978	179.971 ^{b}
Mean	11.507 ^{a}	11.595 ^{a}	11.551	72.293 ^{a}	69.113 ^{b}	70.703	2.129 ^{b}	2.242 ^{a}	2.185	153.921 ^{b}	158.039 ^{a}	155.980

1-Vellanikkara ecotype , 2 - Madurai ecotype

Table 11 b . Effect of cytokinins on callusing in petiole cultures of *Tinospora cordifolia*

Basal medium – MS

Treatment (mgl^{-1})	Days to initiate callus			Percentage of cultures initiating calli in 30 days			Callus growth score in 30 days			Callus index		
	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
BA 0.5	11.847	12.456	12.152 ^{ab}	62.685	60.853	61.769 ^{f}	2.257	2.069	2.163 ^{cd}	141.706	125.901	133.804 ^{f}
BA 1.0	11.849	12.078	11.964 ^{ab}	66.487	65.397	65.942 ^{c}	2.088	2.163	2.126 ^{d}	138.702	141.852	140.277 ^{e}
BA 2.0	11.517	11.538	11.538 ^{b}	70.515	67.460	68.988 ^{d}	2.261	2.367	2.314 ^{ab}	159.689	159.918	159.804 ^{b}
KIN 0.5	12.539	12.347	12.443 ^{a}	62.589	63.569	63.079 ^{e}	2.061	2.522	2.292 ^{abc}	129.004	160.615	144.810 ^{c}
KIN 1.0	12.495	11.649	12.072 ^{ab}	64.498	65.493	64.995 ^{d}	2.149	2.237	2.193 ^{bcd}	138.565	146.793	142.679 ^{d}
KIN 2.0	11.573	11.497	11.535 ^{b}	72.520	72.598	72.559 ^{a}	2.378	2.434	2.406 ^{a}	172.547	176.857	174.702 ^{a}
Mean	11.970 ^{a}	11.927 ^{a}	11.949	66.549 ^{a}	65.895 ^{b}	66.222	2.199 ^{b}	2.299 ^{a}	2.249	146.702 ^{b}	151.990 ^{a}	149.346

1 - Vellanikkara ecotype , 2 - Madurai ecotype

Table 11 c . Effect of cytokinins on callusing in stem cultures of *Tinospora cordifolia*

Basal medium – MS

Treatment (mgl^{-1})	Days to initiate callus			Percentage of cultures initiating calli in 30 days			Callus growth score in 30 days			Callus index		
	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
BA 0.5	12.465	12.819	12.642[a]	72.849	64.409	68.629[d]	2.164	2.253	2.209[e]	159.051	145.779	152.415[e]
BA 1.0	11.580	11.404	11.492[c]	70.635	72.483	71.559[c]	2.543	2.345	2.444[c]	179.467	170.159	174.813[c]
BA 2.0	11.159	11.948	11.554[c]	89.091	73.106	81.098[a]	2.643	3.047	2.845[a]	233.001	223.410	228.206[a]
KIN 0.5	12.401	11.911	12.156[b]	65.991	66.423	66.207[e]	2.265	2.104	2.184[e]	148.648	140.491	144.570[f]
KIN 1.0	11.401	11.918	11.660[c]	64.663	72.681	68.672[d]	2.277	2.365	2.321[d]	147.122	172.733	159.928[d]
KIN 2.0	11.213	11.285	11.249[d]	72.818	78.365	75.592[b]	2.375	2.745	2.560[b]	173.255	215.553	194.404[b]
Mean	11.703[b]	11.881[a]	11.792	72.674[a]	71.245[b]	71.960	2.378[b]	2.477[a]	2.427	173.424[b]	178.021[a]	175.723

1 - Vellanikkara ecotype , 2 - Madurai ecotype

Between the two ecotypes, Madurai ecotype performed well with a high mean callus index and callus growth score, whereas Vellanikkara ecotype showed higher percentage of callusing with early induction. Among the explants, stem explants showed higher percentage of callusing (Fig. 3b) with maximum growth score (Fig. 3c) and mean callus index (Fig. 3d). Leaf explants registered early callus induction (11.551 days) than others (Fig. 3a). For all the parameters studied petiole cultures showed lowest values.

4.4.3 Effect of cytokinin in combination with auxin on callusing in *Tinospora cordifolia*

The incorporation of major cytokinins, BA and kin to basal media fortified with auxins influenced significantly all parameters tested to assess the efficiency in callusing in leaf, petiole and stem cultures (Tables 12a, 12b and 12c). The results revealed that the most favourable and consistent response was obtained with Kin, which at 2 mg l^{-1} favoured callusing of petiole and stem cultures when supplemented to media containing IAA 2 mg l^{-1}. On an average, 96.060 per cent in petiole cultures and 98.373 per cent stem cultures initiated calli. In leaf cultures, higher percentage (96.410) of cultures initiated calli, when incubated in MS medium supplemented with BA 2 mg l^{-1} and IAA 1 mg l^{-1}.

High mean callus index values 294.001 and 323.400 were recorded for petiole and stem cultures respectively, when cultured in IAA 2 mg l^{-1} with Kin 2 mg l^{-1}. For leaf cultures, IAA with Ki, each at 1 mg l^{-1} registered the highest (347.166) mean callus index value (Table 12a). Significant difference was observed for days to callus induction in all the cultures. BA 4 mg l^{-1} and Kin 3 mg l^{-1} each with NAA 2 mg l^{-1} registered early induction of callus in leaf (9.898

Fig.3c. Comparative response of *T.cordifolia* ecotypes to cytokinins with respect to callus growth score.

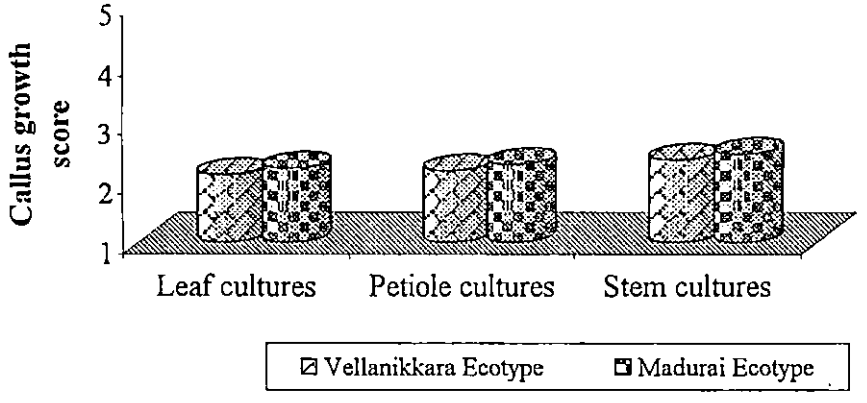
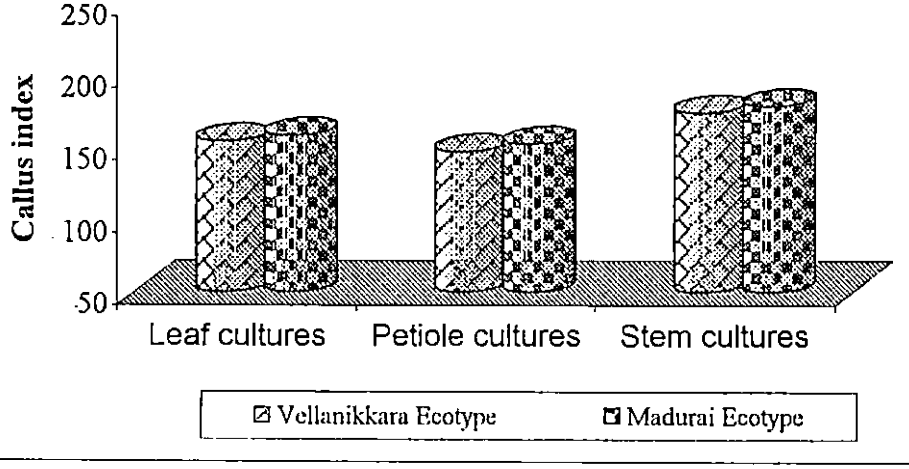


Fig.3d. Comparative response of *T.cordifoia* ecotypes to cytokinins with respect to callus index.



days) and petiole (10.832 days) cultures respectively, whereas stem cultures initiated callus in 10.825 days in the media enriched with IAA 2 mg l^{-1} and BA 3 mg l^{-1}.

Callus growth score values of leaf cultures did not vary significantly for all the treatments whereas NAA 1 mg l^{-1} with Ki 2 mg l^{-1} registered maximum growth score (3.189) for petiole cultures. Maximum growth score of 3.316 was observed for stem cultures in basal media containing NAA 1 mg l^{-1} with BA 1.0 mg l^{-1} (Table 12c). BA or kin along with 2,4-D was ineffective in accelerating callusing of leaf, petiole and stem cultures of both the ecotypes.

4.4.3.1 Variable performance of ecotypes in media enriched with auxins and cytokinins

Between the Velanikkara and Madurai ecotypes there was no significant difference for percentage of cultures initiating callus in leaf, petiole and stem cultures. Stem cultures of Vellanikkara ecotype showed early induction of calli (11.256 days) while petiole and leaf cultures of both the ecotypes recorded equal response with respect to days to callus induction.

Madurai ecotype performed better with high callus growth score and mean callus index than Vellanikkara ecotype for petiole and leaf derived cultures. But stem cultures of both the ecotypes did not exhibit significant difference for callus growth score and mean callus index.

4.4.3.2 Comparative efficiency of explants in initiating and proliferating calli under the influence of auxins and cytokinins

Leaf explants performed better than stem and petiole cultures wherein 84.172 per cent cultures callused in 10.481 days (Fig. 4a) registering mean callus growth score of 3.524, with mean callus index value of 297.893 (Table 12a). Next

Fig.4a. Relative response of *T.cordifolia* ecotypes to auxin and cytokinin combinations with respect to callus initiation period.

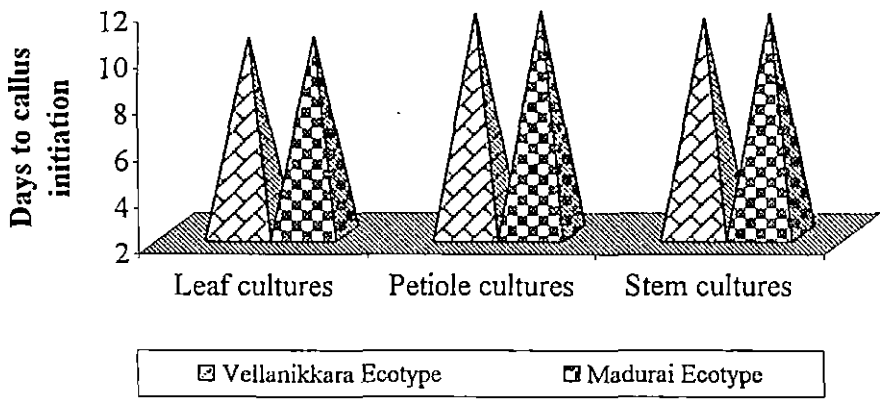


Fig.4b. relative response of *T.cordifolia* ecotypes to auxin and cytokinin combinations with respect to callus initiation percentage.

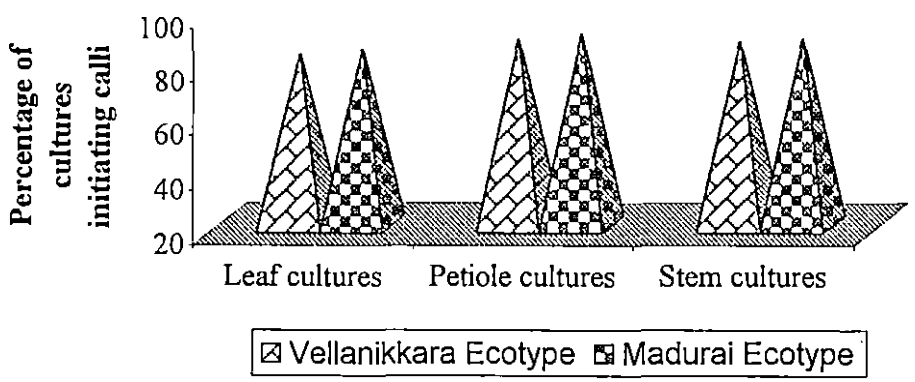


Table 12a. Effect of auxins + cytokinins on callusing in leaf cultures of *Tinospora Cordifolia*

Basal medium - MS

Treatment (mg l^{-1})	Days to callus initiation			% cultures initiating callus in 30 days			Callus growth score in 30 days			Callus index		
	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
NAA1+BA 0.5	11.131	11.588	11.359^{bc}	74.598	88.311	81.455^{defg}	3.729	3.665	3.697^{a}	278.548	324.090	301.319^{abc}
NAA1+BA 1.0	10.558	10.441	10.500^{efg}	80.357	87.500	83.928^{cdef}	3.594	3.929	3.761^{a}	287.351	342.850	315.101^{abc}
NAA1+BA 2.0	10.017	10.093	10.055^{gh}	93.333	90.417	91.875^{abcd}	3.500	3.929	3.714^{a}	326.656	354.992	340.824^{ab}
NAA1+Ki 0.5	11.125	10.966	11.046^{cd}	85.417	88.689	87.053^{abcdef}	3.594	3.665	3.629^{a}	306.395	326.992	316.693^{abc}
NAA1+Ki 1.0	10.220	10.021	10.120^{gh}	88.311	85.147	86.864^{abcdef}	3.115	3.650	3.382^{a}	276.947	312.702	294.824^{abc}
NAA1+Ki 2.0	10.014	10.072	10.043^{gh}	82.857	92.307	87.582^{abcdef}	3.460	3.665	3.563^{a}	286.570	335.896	311.233^{abc}
NAA2+BA 1.0	9.965	9.942	9.954^{gh}	85.000	85.164	85.082^{bcdef}	3.665	3.929	3.797^{a}	311.248	334.531	322.889^{abc}
NAA2+BA 2.0	9.948	9.966	9.957^{gh}	83.750	87.500	85.625^{bcdef}	3.605	3.780	3.692^{a}	301.525	330.750	316.137^{abc}
NAA2+BA 3.0	10.018	9.970	9.994^{gh}	79.167	79.167	79.167^{efgh}	3.745	3.580	3.663^{a}	296.833	283.083	289.958^{abc}
NAA2+BA 4.0	10.299	9.496	9.898^{h}	96.667	65.710	81.188^{defg}	3.915	3.854	3.884^{a}	378.732	253.366	316.049^{abc}
NAA2+Ki 1.0	10.013	10.129	10.071^{gh}	79.167	85.640	82.403^{def}	3.173	3.762	3.467^{a}	250.541	321.930	286.260^{abc}
NAA2+Ki 2.0	9.958	10.040	9.999^{gh}	83.750	86.607	85.178^{bcdef}	3.331	3.580	3.455^{a}	278.294	309.678	293.986^{abc}
NAA2+Ki 3.0	10.117	10.029	10.073^{gh}	74.999	87.121	81.060^{defg}	3.750	3.679	3.714^{a}	280.352	322.400	301.376^{abc}
IAA1+BA 0.5	11.278	11.053	11.165^{cd}	75.714	85.000	80.357^{efg}	3.340	3.429	3.384^{a}	254.984	290.706	272.845^{c}
IAA1+BA 1.0	11.067	11.241	11.154^{cd}	85.417	69.043	77.230^{fgh}	3.555	3.500	3.528^{a}	303.979	241.650	272.814^{c}
IAA1+BA 2.0	10.538	10.487	10.152^{efg}	96.153	96.667	96.410^{a}	3.415	3.625	3.520^{a}	328.691	349.166	338.928^{ab}
IAA1+Ki 0.5	11.062	10.543	10.802^{def}	84.523	89.204	86.864^{abcdef}	3.315	3.759	3.537^{a}	280.808	333.705	308.257^{abc}
IAA1+Ki 1.0	10.539	11.308	10.924^{cde}	96.667	92.857	94.762^{ab}	3.666	3.625	3.646^{a}	354.999	339.333	347.166^{a}
IAA1+Ki 2.0	10.150	10.316	10.233^{gh}	95.454	84.500	89.977^{abcde}	3.605	3.750	3.678^{a}	343.636	319.105	331.400^{abc}
IAA2+BA 1.0	10.014	10.584	10.299^{fgh}	77.376	87.083	82.230^{def}	3.455	3.362	3.409^{a}	268.855	292.354	280.604^{bc}
IAA2+BA 2.0	10.115	10.275	10.195^{gh}	96.153	92.121	94.137^{abc}	3.600	3.450	3.525^{a}	345.383	317.757	331.570^{abc}
IAA2+BA 3.0	10.231	9.952	10.092^{gh}	69.040	89.204	79.122^{efgh}	3.133	3.583	3.358^{a}	216.626	319.478	268.052^{c}
IAA2+Ki 1.0	10.375	10.264	10.319^{fgh}	82.307	88.787	85.547^{bcdef}	3.330	3.500	3.415^{a}	274.845	311.817	293.331^{abc}
IAA2+Ki 2.0	10.265	10.501	10.383^{fgh}	83.333	82.857	83.095^{def}	3.580	3.775	3.678^{a}	299.998	312.714	306.356^{abc}
IAA2+Ki 3.0	10.016	10.317	10.167^{gh}	80.909	85.417	83.163^{ef}	3.601	3.436	3.519^{a}	291.146	292.164	291.880^{abc}
2,4D1+BA 1.0	11.622	11.788	11.705^{ab}	69.043	73.210	71.126^{gh}	2.725	2.911	2.818^{a}	188.439	213.725	201.082^{d}
2,4D1+Ki 1.0	11.997	11.945	11.971^{a}	65.710	74.598	70.154^{h}	2.644	2.801	2.723^{a}	175.130	209.226	192.178^{d}
Mean	10.469^{a}	10.494^{a}	10.481	83.155^{a}	85.189^{a}	84.172	3.450^{b}	3.599^{a}	3.524	288.426^{b}	307.360^{a}	297.893

1. Vellanikkara ecotype 2. Madurai ecotype

Table 12b. Effect of auxins + cytokinins on callusing in petiole explants of *Tinospora Cordifolia*

Basal medium - MS

Treatment (mg l^{-1})	Days to callus initiation			% cultures initiating callus in 30 days			Callus growth score in 30 days			Callus index		
	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
NAA1+BA 0.5	12.282	12.833	12.558^{a}	90.000	88.311	89.156^{ab}	2.500	2.875	2.688^{bcd}	224.165	253.570	238.868^{fghi}
NAA1+BA 1.0	11.416	12.278	11.847^{bcdef}	89.204	96.667	92.935^{ab}	3.000	2.928	2.964^{ab}	267.613	282.446	275.030^{abcde}
NAA1+BA 2.0	11.853	11.896	11.875^{dcde}	96.667	90.417	93.542^{ab}	2.875	3.150	3.012^{ab}	277.499	284.374	280.937^{abcde}
NAA1+Ki 0.5	11.853	12.608	12.231^{ab}	88.787	93.750	91.269^{ab}	2.675	2.893	2.784^{abc}	237.437	270.950	254.149^{abcde}
NAA1+Ki 1.0	11.151	12.415	11.783^{bcdefg}	88.311	100.000	94.156^{a}	3.166	2.911	3.039^{ab}	279.077	291.150	285.113^{abc}
NAA1+Ki 2.0	11.853	12.010	11.932^{bcd}	86.607	96.667	91.637^{ab}	3.165	3.214	3.189^{a}	273.964	311.654	292.809^{ab}
NAA2+BA 1.0	11.018	11.500	11.259^{ghi}	86.607	88.311	87.459^{ab}	2.500	2.929	2.714^{bcd}	216.294	258.805	237.550^{ghi}
NAA2+BA 2.0	10.853	11.059	10.956^{hi}	87.762	93.750	90.756^{ab}	3.000	3.166	3.083^{ab}	263.286	295.688	279.487^{abcde}
NAA2+BA 3.0	10.836	11.187	11.011^{hi}	92.121	90.417	91.269^{ab}	2.500	3.017	2.758^{bc}	230.302	272.527	251.414^{cdefg}
NAA2+BA 4.0	11.354	10.690	11.022^{hi}	89.903	86.607	88.255^{ab}	2.875	2.724	2.800^{abc}	258.773	236.004	247.388^{cdefg}
NAA2+Ki 1.0	11.119	11.735	11.427^{defgh}	86.190	89.204	87.697^{ab}	2.875	2.833	2.854^{abc}	247.736	253.001	250.369^{cdefg}
NAA2+Ki 2.0	10.956	11.251	11.103^{hi}	90.000	93.333	91.666^{ab}	2.375	2.714	2.544^{cd}	212.498	254.732	233.615^{hi}
NAA2+Ki 3.0	10.719	10.946	10.832^{i}	85.417	92.875	89.137^{ab}	2.500	2.926	2.713^{bcd}	213.020	272.271	242.646^{defgh}
IAA1+BA 0.5	11.750	11.128	11.439^{defgh}	92.121	86.607	89.364^{ab}	2.625	3.061	2.843^{abc}	242.272	264.934	253.603^{abcde}
IAA1+BA 1.0	11.209	11.423	11.316^{fghi}	93.750	88.311	91.031^{ab}	3.000	2.750	2.875^{abc}	281.250	243.506	262.378^{abcde}
IAA1+BA 2.0	11.022	10.926	10.974^{hi}	85.640	95.454	90.547^{ab}	2.375	2.964	2.669^{bcd}	198.915	282.763	240.839^{efgh}
IAA1+Ki 0.5	11.853	11.877	11.865^{bcdef}	92.121	88.787	90.454^{ab}	2.688	3.114	2.901^{abc}	247.348	276.089	261.718^{abcde}
IAA1+Ki 1.0	11.329	11.323	11.326^{efghi}	92.307	89.523	90.915^{ab}	3.168	2.803	2.985^{ab}	291.010	249.779	270.395^{abcde}
IAA1+Ki 2.0	10.806	11.092	10.949^{hi}	93.750	93.333	93.542^{ab}	2.964	3.053	3.008^{ab}	278.100	285.299	281.700^{abcd}
IAA2+BA 1.0	12.296	11.346	11.821^{bcdef}	93.751	96.665	95.208^{a}	2.813	2.875	2.844^{abc}	264.212	277.789	271.001^{abcde}
IAA2+BA 2.0	12.086	11.244	11.665^{cdefg}	95.454	89.204	92.329^{ab}	2.750	2.926	2.838^{abc}	261.363	261.182	261.273^{abcde}
IAA2+BA 3.0	11.776	10.896	11.336^{efghi}	85.000	85.417	85.208^{bc}	2.964	2.964	2.964^{ab}	251.998	253.100	252.549^{bcdef}
IAA2+Ki 1.0	12.118	11.819	11.968^{bcd}	85.460	96.667	91.153^{ab}	3.053	2.897	2.975^{ab}	261.514	280.146	270.830^{abcde}
IAA2+Ki 2.0	11.813	11.895	11.854^{bcdef}	96.667	95.454	96.060^{a}	2.964	3.150	3.057^{ab}	286.639	301.363	294.001^{a}
IAA2+Ki 3.0	10.531	11.309	10.920^{hi}	85.714	92.121	88.917^{ab}	2.875	2.926	2.901^{abc}	246.427	269.503	257.965^{abcde}
2,4D1+BA 1.0	12.190	11.919	12.054^{abc}	78.889	76.385	77.637^{d}	2.461	2.926	2.694^{bcd}	194.585	223.449	209.017^{ij}
2,4D1+Ki 1.0	12.042	12.377	12.209^{abc}	76.785	82.537	79.661^{cd}	2.225	2.464	2.345^{d}	171.248	202.834	187.041^{j}
Mean	11.485^{a}	11.592^{a}	11.538	89.080^{a}	90.991^{a}	90.036	2.775^{b}	2.932^{a}	2.853	247.350^{b}	266.967^{a}	257.174

1. Vellanikkara ecotype 2. Madurai ecotype

Table 12c. Effect of auxins + cytokinins on callusing in stem cultures of *Tinospora Cordifolia*

Basal medium - MS

Treatment (mg l^{-1})	Days to callus initiation			% cultures initiating callus in 30 days			Callus growth score in 30 days			Callus index		
	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
NAA1+BA 0.5	11.578	11.964	11.771^{cd}	85.417	86.607	86.012^{def}	2.783	2.890	2.837^{efg}	238.824	250.371	244.598^{def}
NAA1+BA 1.0	11.251	11.286	11.268^{efgh}	89.204	89.204	89.204^{bcd}	3.465	3.166	3.316^{a}	308.863	282.748	295.806ab
NAA1+BA 2.0	10.609	11.000	10.804^{h}	92.121	92.121	92.121^{bc}	3.150	2.964	$3.057^{abcdefg}$	290.362	273.017	281.690^{bcdef}
NAA1+Ki 0.5	11.398	12.216	11.807^{c}	89.523	84.523	87.023^{cdef}	2.686	2.926	2.806^{fg}	242.074	247.445	244.760^{def}
NAA1+Ki 1.0	10.986	11.609	11.298^{defgh}	86.057	86.607	86.332^{def}	3.081	2.879	2.980^{bcdefg}	265.216	250.671	257.944^{bcdef}
NAA1+Ki 2.0	10.789	11.261	11.025^{gh}	88.787	85.523	87.155^{cdef}	3.275	3.000	3.138^{abcde}	290.875	253.570	272.223^{bcdef}
NAA2+BA 1.0	11.148	11.291	11.219^{efgh}	87.762	89.204	88.483^{bcd}	2.964	2.964	2.964^{bcdefg}	260.239	264.464	262.351^{bcdef}
NAA2+BA 2.0	11.021	11.149	11.085^{fgh}	86.607	58.523	88.065^{bcd}	3.291	3.200	3.246^{abc}	284.973	287.237	286.105^{abcd}
NAA2+BA 3.0	10.836	11.069	10.953^{gh}	92.119	89.204	90.662^{bcd}	3.000	3.114	$3.057^{abcdefg}$	276.363	278.100	277.231^{bcdef}
NAA2+BA 4.0	10.957	11.011	10.984^{gh}	85.164	89.204	87.184^{cdef}	3.088	2.964	$3.026^{abcdefg}$	262.905	264.464	263.684^{bcdef}
NAA2+Ki 1.0	11.240	12.056	11.648^{cde}	84.523	89.204	86.864^{cdef}	2.928	2.849	2.885^{defg}	246.942	254.099	250.521^{cdef}
NAA2+Ki 2.0	11.194	11.960	11.577^{cdef}	87.083	90.909	88.996^{bcd}	3.038	3.311	3.174^{abcd}	264.639	300.954	282.796^{bcde}
NAA2+Ki 3.0	10.862	11.878	11.370^{cdefg}	89.204	89.204	89.204^{bcd}	2.025	3.072	$2.998^{abcdefg}$	261.051	271.790	267.421^{bcdef}
IAA1+BA 0.5	10.945	11.513	11.229^{efgh}	88.311	85.417	86.864^{cdef}	2.625	2.913	2.769^{fg}	230.843	248.999	239.921^{f}
IAA1+BA 1.0	11.005	11.049	11.027^{gh}	87.500	88.311	87.906^{bcd}	3.174	2.911	$3.043^{abcdefg}$	277.725	256.889	267.307^{bcdef}
IAA1+BA 2.0	10.936	10.909	10.922^{gh}	92.121	87.500	89.810^{bcd}	3.008	3.166	3.087^{abcdef}	277.044	276.938	276.991^{bcdef}
IAA1+Ki 0.5	11.639	11.529	11.584^{cde}	88.311	89.204	88.758^{bcd}	2.714	2.764	2.739^{g}	240.233	246.282	243.257^{ef}
IAA1+Ki 1.0	11.859	11.383	11.621^{cde}	90.417	86.607	88.512^{bcd}	3.331	3.083	3.207^{abcd}	300.637	266.935	283.786^{bcde}
IAA1+Ki 2.0	11.036	10.792	10.914^{gh}	87.083	88.311	87.697^{bcde}	3.061	2.775	2.918^{cdefg}	266.572	245.231	255.887^{bcdef}
IAA2+BA 1.0	11.064	11.701	11.383^{cdefg}	86.607	92.857	89.732^{bcd}	3.130	2.964	$3.047^{abcdefg}$	271.317	274.975	273.146^{bcdef}
IAA2+BA 2.0	11.055	11.750	11.402^{cdefg}	89.204	96.667	92.935^{b}	3.250	3.285	3.267^{ab}	290.356	286.639	288.498^{abc}
IAA2+BA 3.0	10.688	10.962	10.825^{h}	84.524	96.667	90.595^{bcd}	3.130	3.000	$3.065^{abcdefg}$	264.359	289.999	277.179^{bcdef}
IAA2+Ki 1.0	11.872	11.851	11.861^{bc}	87.121	89.204	88.163^{bcd}	2.926	2.878	2.901^{defg}	254.681	256.598	255.639^{bcdef}
IAA2+Ki 2.0	11.463	11.916	11.689^{cde}	100.000	96.667	98.333^{a}	3.358	3.210	3.284^{ab}	335.800	310.999	323.400^{a}
IAA2+Ki 3.0	11.276	11.339	11.307^{defgh}	88.787	92.121	90.454^{bcd}	3.416	2.964	3.190^{abcd}	303.165	273.104	288.134^{abc}
2,4D1+BA 1.0	12.589	11.914	12.251^{ab}	82.857	81.667	82.262^{f}	2.964	2.964	2.964^{bcdefg}	245.691	241.999	243.845^{def}
2,4D1+Ki 1.0	12.609	12.101	12.355^{a}	81.667	83.330	82.498^{ef}	2.537	2.330	2.433^{h}	207.274	195.005	201.140^{g}
Mean	11.256^{b}	11.498^{a}	11.377	88.077^{a}	89.095^{a}	88.586	3.048^{a}	2.982^{a}	3.015	268.852^{a}	264.871^{a}	266.861

1. Vellanikkara ecotype 2. Madurai ecotype

Fig.4c. Relative response of *T.cordifolia* ecotypes to auxin and cytokinin combinations with respect to callus growth score.

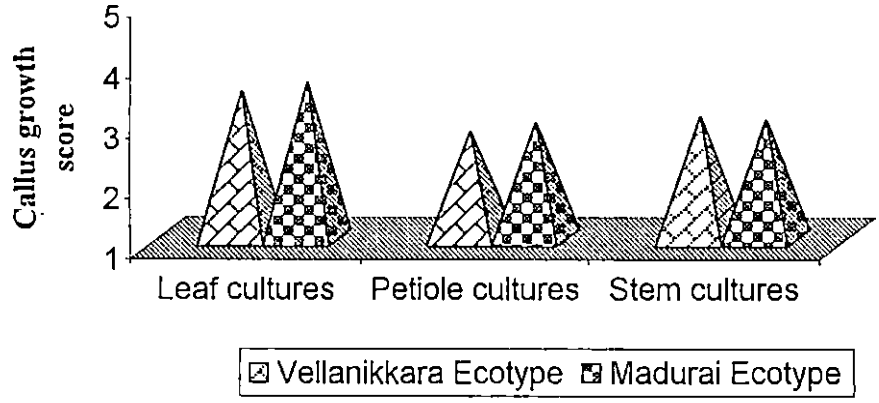
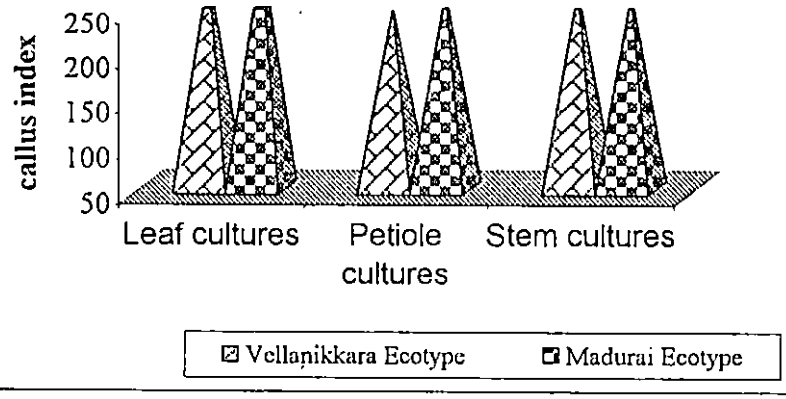


Fig.4d. Relative response of *T.cordifolia* ecotypes to auxin and cytokinin combinations with respect to callus index.



to leaf explants, stem cultures registered mean callus index of 266.861 (Fig. 4d) with a callus growth score of 3.015 (Fig. 4c). Petiole explants were inferior in all the parameters studied, as compared to leaf and stem cultures except for percentage cultures initiating callus wherein 90.036 per cent cultures initiated callus (Fig. 4b).

4.4.4 Effect of media supplements on callusing

Data pertaining to the effect of media additives on callusing in leaf, petiole and stem cultures of *Tinospora cordifolia* are presented in Tables 13a, 13b and 13c. From the Tables, it can be inferred that the response of incorporated media additives varied significantly for all parameters tested in leaf, petiole and stem cultures. Among the growth additives supplemented to basal medium, the phenolic hormone synergist, phloroglucinol at 100 and 125 mg l^{-1} and casein hydrolysate at 100, 200 and 300 mg l^{-1} responded equally, without any significant difference for percentage of cultures initiating calli in leaf, petiole and stem cultures. Activated charcoal at 0.25 and 0.5 per cent concentration showed poor response with respect to percentage of cultures initiating calli in leaf, petiole and stem cultures (Tables 13a, 13b and 13c). Casein hydrolysate at 100 mg l^{-1} was effective in early induction of callus in leaf, petiole and stem cultures. Casein hydrolysate at 200 mg l^{-1} and 300 mg l^{-1} did not vary significantly between each other in earliness for inducing calli in petiole and stem cultures. In all the cultures, irrespective of the explant, activated charcoal, both at 0.25 and 0.5 per cent concentration resulted in inferior performance for all the parameters studied (Tables 13a, 13b and 13c).

Casein hydrolysate at 300 mg l^{-1} registered highest callus growth score in petiole cultures, whereas for stem cultures, the same supplement at all levels

Table 13a. Effect of media additives on callusing in leaf cultures of *Tinospora cordifolia*

Basal medium - MS + NAA 2 mg l^{-1} + BA 2 mg l^{-1}

Treatments	Days to initiate callus			% of cultures initiating calli			Callus growth score			Callus index		
	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
1. Phloroglucinol 100 mg l^{-1}	10.238	10.488	10.343^{b}	92.895	91.717	92.306^{a}	3.047	3.722	3.384^{b}	284.231	349.211	316.721^{b}
2. Phloroglucinol 125 mg l^{-1}	10.136	10.302	10.219^{bc}	90.581	94.747	92.664	3.249	3.468	3.369^{b}	290.537	334.054	312.295^{b}
3. Caseinhydro-lysate 100 mg l^{-1}	10.011	9.792	9.901^{d}	88.361	93.703	91.032^{a}	3.403	3.550	3.477^{ab}	299.278	330.704	314.991
4. Caseinhydro-lysate 200 mg l^{-1}	9.905	9.998	9.952^{cd}	93.361	94.747	94.054^{a}	3.592	3.791	3.691^{a}	330.346	355.605	342.975^{a}
5. Caseinhydro-lysate 300 mg l^{-1}	10.172	10.592	10.382^{b}	93.123	92.895	93.009^{b}	3.581	3.640	3.610^{a}	339.057	355.472	347.265^{a}
6. Activated charcoal 0.2%	10.883	11.345	11.114^{a}	82.023	83.508	82.766^{b}	2.913	2.831	2.872^{c}	226.297	218.835	222.566^{c}
7. Activated charcoal 0.5%	11.141	11.383	11.262^{a}	85.149	84.720	84.935^{b}	2.746	2.403	2.574^{d}	227.899	190.395	209.197
Mean	10.355^{b}	10.551^{a}	10.453	89.356^{a}	90.836^{a}	90.109	3.129^{b}	3.344^{a}	3.281	285.378^{b}	304.897^{a}	295.137

1. Vellanikkara ecotype 2. Madurai ecotype

Table 13b. Effect of media additives on callusing in petiole cultures of *Tinospora cordifolia*

Basal medium - MS + NAA 2 mg l^{-1} + BA 2 mg l^{-1}

Treatments	Days to initiate callus			% of cultures initiating calli			Callus growth score			Callus index		
	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
1. Phloroglucinol 100 mg l^{-1}	11.067	11.128	11.097[b]	95.525	92.213	93.869[a]	3.067	2.658	2.868[b]	277.871	270.429	274.150[a]
2. Phloroglucinol 125 mg l^{-1}	10.725	10.899	10.812[b]	96.296	92.895	94.596[a]	3.091	3.089	3.090[b]	303.079	284245	293.662[a]
3. Caseinhydro-lysate 100 mg l^{-1}	10.348	10.544	10.446[c]	92.803	95.926	94.364[a]	3.207	2.875	3.041[b]	299.357	289.946	294.651[a]
4. Caseinhydro-lysate 200 mg l^{-1}	10.310	10.726	10.518[c]	90.253	95.926	93.089[a]	3.148	3.047	3.097[b]	283.710	291.831	287.770[a]
5. Caseinhydro-lysate 300 mg l^{-1}	10.091	10.746	10.419[c]	95.584	95.525	94.054[a]	3.408	3.417	3.418[a]	289.938	288.997	289.467[a]
6. Activated charcoal 0.2%	11.744	11.612	11.678[a]	80.470	74.444	77.457[b]	2.209	2.481	2.345[c]	208.265	173.049	190.657[b]
7. Activated charcoal 0.5%	11.683	11.414	11.549[a]	76.031	73.809	74.920[b]	2.401	2.669	2.535[c]	168.689	188.844	178.766[b]
Mean	10.852[b]	11.010[a]	10.931	89.138[a]	88.667[a]	88.907	2.933[a]	2.891[a]	2.912	261.559[a]	255.334[a]	258.446

1. Vellanikkara ecotype 2. Madurai ecotype

Table 13c. Effect of media additives on callusing in stem cultures of *Tinospora cordifolia*

Basal medium - MS + NAA 2 mg l^{-1} + BA 2 mg l^{-1}

Treatments	Days to initiate callus			% of cultures initiating calli			Callus growth score			Callus index		
	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
1. Phloroglucinol 100 mg l^{-1}	11.774	11.506	11.640^{bc}	95.213	94.747	94.980^{a}	3.199	3.194	3.196^{a}	311.652	307.354	309.503^{a}
2. Phloroglucinol 125 mg l^{-1}	11.726	11.530	11.628^{bc}	92.895	94.747	93.821^{a}	3.096	3.262	3.179^{a}	293.262	296.491	294.877^{a}
3. Caseinhydro-lysate 100 mg l^{-1}	11.176	11.677	11.427^{c}	93.939	94.747	94.343^{a}	3.379	3.235	3.307^{a}	307.994	311.152	309.573^{a}
4. Caseinhydro-lysate 200 mg l^{-1}	11.329	12.149	11.739^{bc}	94.747	92.087	93.417^{a}	3.392	3.387	3.390^{a}	321.552	311.969	316.761^{a}
5. Caseinhydro-lysate 300 mg l^{-1}	11.234	12.117	11.676^{bc}	94.747	91.717	93.232^{a}	3.183	3.593	3.388^{a}	314.536	311.161	312.849^{a}
6. Activated charcoal 0.2%	11.894	12.150	12.022^{ab}	83.611	85.00	84.306^{b}	2.815	2.790	2.802^{b}	225.056	233.157	228.606^{b}
7. Activated charcoal 0.5%	12.218	12.172	12.195^{a}	84.682	85.833	85.258^{b}	2.599	2.772	2.686^{b}	215.060	227.397	221.228^{b}
Mean	11.622^{b}	11.900^{a}	11.761	91.405^{a}	91.268^{a}	91.337	3.095^{a}	3.176^{a}	3.135	284.016^{a}	285.526^{a}	284.771

1. Vellanikkara ecotype 2. Madurai ecotype

responded on par with phloroglucinol at 100 or 125 mg l^{-1}, in proliferating calli. Phloroglucinol at 100 and 200 mg l^{-1} registered callus growth score values which are on par with growth score values registered for casein hydrolysate at 100 and 200 mg l^{-1} for petiole cultures. In leaf cultures, casein hydrolysate at 200 and 300 mg l^{-1} were equally effective in proliferating calli registering the highest mean callus index values (Table 13a).

Phloroglucinol and casein hydrolysate at all levels furnished mean callus index values on par with one another in stem and petiole derived callus cultures. Madurai ecotype was significantly superior to Vellanikkara ecotype for days to callus induction in all the cultures and callus growth score and mean callus index values in leaf cultures. For all other parameters both the ecotypes responded without any significant difference. Activated charcoal both at 0.25 and 0.5 per cent concentration furnished lowest values for all parameters studied in leaf, petiole and stem cultures.

4.5 Effect of culture environment on callusing in *Tinospora cordifolia*

Influence of modified culture environment (culture in dark) as compared to control (culture at 26±1°C under 10 hours illumination) is given in Tables 14a and 14b.

Marked difference between culture conditions was exhibited with respect to days to callus induction, percentage cultures initiating calli, callus growth score and mean callus index values, when leaf and stem segments were cultured *in vitro*. Incubating the cultures in 10 hr photoperiod at 26±1°C was superior with respect to all parameters observed with respect to callusing (Tables 14a and 14b).

Table 14a. Effect of culture environment on callusing in leaf cultures of *Tinospora cordifolia*

Basal medium - MS + NAA 2 mg l^{-1}

Treatments	Days to callus initiation			Percentage of cultures initiating callus growth in 30 days			Callus growth score in 30 days			Callus index		
	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
10 hr photoperiod with ordinary light	10.616	10.582	10.599	88.077	88.616	88.346	3.465	3.166	3.316	305.186	280.558	292.872
Dark	11.916	12.476	12.196	83.164	82.042	82.603	2.537	2.072	2.304	210.987	169.991	190.489
Mean	11.266	11.529	11.397	85.620	85.329	95.474	3.001	2.619	2.810	258.086	225.274	241.690

1. Vellanikkara ecotype 2. Madurai ecotype

Table 14b. Effect of culture environment on callusing in stem cultures of *Tinospora cordifolia*

Basal medium - MS + NAA 2 mg l^{-1}

Treatments	Days to callus initiation			Percentage of cultures initiating callus growth in 30 days			Callus growth score in 30 days			Callus index		
	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
10 hr photoperiod with ordinary light	11.866	11.406	11.636	89.062	88.168	88.615	3.500	3.264	3.382	311.717	287.780	299.748
Dark	13.016	13.872	13.444	80.764	82.874	81.819	2.642	2.761	2.701	213.378	228.815	221.096
Mean	12.441	12.639	12.540	84.913	85.521	85.217	3.071	3.012	3.041	262.547	258.297	260.422

1. Vellanikkara ecotype 2. Madurai ecotype

Vellanikkara ecotype performed better for callus growth score and mean callus index, whereas both the ecotypes showed relatively equal response for percentage of cultures initiating callus and days to callus initiation.

4.6 Indirect organogenesis

4.6.1 Rhizogenesis

Trends in regeneration pattern of roots from calli initiated from leaf and stem segments of experimental ecotypes as affected by effective hormonal combinations are given in Tables 15a and 15b. The results of this study involving various root inducing hormones revealed marked differences in their ability to regenerate roots from leaf and stem calli for parameters tested viz. days to root initiation and percentage of root initiating cultures. Half strength MS medium supplemented with IAA at 2 mg l^{-1}, produced encouraging results initiating roots in 58.372 per cent leaf derived calli and in 53.439 per cent stem derived calli (Plate 3a). NAA at 2 mg l^{-1}, when incorporated to MS medium at half strength resulted in early root induction in leaf (9.98 days) and stem (11.27 days) derived calli.

Next to these treatment, leaf and stem derived calli cultured in MS basal media with IBA at 2 mg l^{-1} registered 40.779 and 39.205 per cent root initiation respectively. Calli cultures incubated with NAA and IAA, each at 4 mg l^{-1}, exhibited lowest percentage of root initiation. Compared to the stem derived calli, leaf derived calli resulted in higher percentage of root initiation (45.368%) within 11.34 days of incubation.

Among the experimental ecotypes, Madurai ecotype registered high percentage of root initiation whereas Vellanikkara ecotype exhibited early root initiation for leaf and stem derived calli cultures. None of the petiole derived calli recorded rhizogenesis with the hormonal combinations tried, in both the ecotypes.

Table 15a. Effect of growth regulators on callus mediated rhizogenesis in leaf cultures of *Tinospora cordifolia*

Media supplements (mg l^{-1})	Days to root initiation			Percentage of cultures initiating roots		
	1	2	Mean	1	2	Mean
MS + NAA 4	10.626	12.654	11.640	33.333	35.612	34.472
MS + IAA 4	12.875	13.108	12.991	37.165	36.445	36.810
MS + IBA 2	11.302	11.986	11.644	39.214	42.345	40.779
½ MS + NAA 2	9.862	10.126	9.994	54.656	58.162	56.409
½ MS + IAA 2	10.662	10.254	10.458	57.128	59.616	58.372
Mean	11.065	11.625	11.345	44.299	46.438	45.368

1. Vellanikkara ecotype, 2 - Madurai ecotype

Table 15b. Effect of growth regulators on callus mediated rhizogenesis in stem cultures of *Tinospora cordifolia*

Media supplements (mg l^{-1})	Days to root initiation			Percentage of cultures initiating roots		
	1	2	Mean	1	2	Mean
MS + NAA 4	12.642	13.781	13.211	36.654	38.282	37.468
MS + IAA 4	13.836	13.512	13.674	33.333	32.016	32.674
MS + IBA 2	12.502	12.498	12.500	38.424	39.986	39.205
½ MS + NAA 2	11.116	11.438	11.277	52.616	50.268	51.442
½ MS + IAA 2	12.012	11.962	11.987	51.114	55.764	53.439
Mean	12.421	12.638	12.529	42.428	43.263	42.845

1. Vellanikkara ecotype, 2 - Madurai ecotype

4.6.2 Shizogenesis

Leaf and stem calli of the experimental ecotypes maintained in the medium supplemented with auxins and cytokinins at varying levels failed to regenerate shoots when incubated under light or in dark (Table 16).

4.6.3 Somatic embryogenesis

The response of experimental ecotypes to somatic embryogenesis is given in Table 17. Among the diverse media employed, embryogenesis was observed in MS medium supplemented with NAA 1 mg l^{-1} and Kinetin 4 mg l^{-1} to which 3.0 per cent sucrose and 1.5 per cent lactose were incorporated (Plate 3b). As presented in Table 17, 41.17 per cent and 37.50 per cent of leaf callus cultures of Vellanikkara and Madurai ecotypes respectively initiated embryoids. As to stem callus cultures, 29.41 per cent and 25.00 per cent cultures initiated embryoids in Vellanikkara and Madurai ecotypes respectively. Mean number of embryoids ranged from 5.16 in leaf cultures of Madurai ecotypes to 6.14 in leaf cultures of Vellanikkara ecotype. Germination of embryoids was possible upon serial subculture in the same media after 4 weeks. Emrbyoids from leaf calli of both the ecotypes germinated better than stem calli derived embryoids. Embryogenesis was not observed in the petiole derived callus of both the ecotypes in any of the media tried.

4.7 Regulation of *in vitro* metabolite production

4.7.1 Standardisation of production media

4.7.1.1 Modifying basal growth medium

4.7.1.1.1 Regulation of growth regulators

a) Preliminary qualitative tests

Results of preliminary tests conducted for detecting the presence of alkaloids in methanol extracts of 4 to 5 week old experimental calli cultured on MS

Plate 3. Indirect organogenesis in *Tinospora cordifolia* Miers.

3 A. Root initiation from stem and leaf calli of *Tinospora cordifolia* Miers.

3 B. Initiation of somatic embryos from leaf calli of *Tinospora cordifolia* Miers.

Table 16 . Effect of growth regulators on callus mediated shizogenesis in leaf and stem cultures of *Tinospora cordifolia*

Basal medium-MS

Hormonal Combinations	Concentration (mgl^{-1})	Days to shoot initiation		Percentage of cultures initiating shoots	
		1	2	1	2
NAA + BA	0.5 + 1.0	-	-	-	-
	1.0 + 2.0	-	-	-	-
	2.0 + 4.0	-	-	-	-
NAA + KIN	0.5 + 1.0	-	-	-	-
	1.0 + 2.0	-	-	-	-
	2.0 + 4.0	-	-	-	-
IAA + BA	0.5 + 1.0	-	-	-	-
	1.0 + 2.0	-	-	-	-
	2.0 + 4.0	-	-	-	-
IAA + KIN	0.5 + 1.0	-	-	-	-
	1.0 + 2.0	-	-	-	-
	2.0 + 4.0	-	-	-	-
NAA + IAA + BA	1.0 + 1.0 + 2.0	-	-	-	-

Table 17. Somatic embryogenesis in *Tinospora cordifolia*

Medium – MS + NAA 1.0 mgl^{-1} + KIN 4.0 mgl^{-1} + 3 % sucrose + 1.5 % lactose

S.No.	Ecotypes	Source of calli	Cultures initiating embryoids (per cent)	Man no. of embryoids initiated	Embryoids germinated (per cent)
1.	Vellanikkara	Leaf	41.17	6.14	25.58
	Vellanikkara	Stem	29.41	3.40	23.52
2.	Madurai	Leaf	37.50	5.16	25.80
	Madurai	Stem	25.00	3.25	23.07

medium incorporated with growth hormones at varying levels are presented in Table 18.

The results clearly indicated that 4 to 5 week old calli of Vellanikkara and Madurai ecotypes subcultured on to MS medium containing NAA or IAA supplemented with BA or Kinetin, at varying levels reacted positively to Dragendorff's reagent with an orange red colour and Mayer's reagents with a white preciptate. When supplemented with auxin alone, the test cultures did not respond positively to any of the above reagents. Cytokinins alone at varying levels also synthesised alkaloids *in vitro* as is evident from Table 18.

b) Developing chromatograms to detect the presence of alkaloids

The solvent system (Butanol:glacial acetic acid:water at 7:1:2), the spotted plates of positively responding samples, when visualised under UV light at 365 nm gave fluorescent yellow colour that corresponds to standard berberine hydrochloride confirming the presence of berberine. The chromatographic pattern of positively responding cultures along with standard berberine hydrochloride is presented in Table 19.

Berberine content of the test samples under the influence of growth regulators as obtained from the standard graph (Fig. 1) are given in Table 20. From the Table, it is inferred that stem cultures of both the ecotypes resulted in maximum berberine yield per gram of callus than leaf and petiole cultures. Among the various growth regulator combinations, NAA 2 mg l^{-1} supplemented with BA or Kin each at 2 mg l^{-1} resulted in highest mean berberine content per gram of callus in both the ecotypes. NAA with BA, each at 2 mg l^{-1} yielded, on an average

Table 18 . Influence of growth regulator combinations on expression of alkaloids in callus cultures of Vellanikkara and Madurai ecotypes of *Tinospora. cordifolia*

Basal medium-MS

Hormonal Combinations	Concentration (mgl^{-1})	Dragendorff's reagent			Mayer's reagent		
		Leaf	Petiole	Stem	Leaf	Petiole	Stem
NAA + BA	1.0 + 1.0	+	+	+	+	+	+
	1.0 + 3.0	+	+	+	+	+	+
	2.0 + 2.0	+	+	+	+	+	+
	2.0 + 3.0	+	+	+	+	+	+
NAA + KIN	1.0 + 1.0	+	+	+	+	+	+
	1.0 + 3.0	+	+	+	+	+	+
	2.0 + 2.0	+	+	+	+	+	+
	2.0 + 3.0	+	+	+	+	+	+
IAA + BA	1.0 +1.0	+	+	+	+	+	+
	1.0 + 3.0	+	+	+	+	+	+
	2.0 + 2.0	+	+	+	+	+	+
	2.0 + 3.0	+	+	+	+	+	+
IAA + KIN	1.0 + 1.0	+	+	+	+	+	+
	1.0 + 3.0	+	+	+	+	+	+
	2.0 + 2.0	+	+	+	+	+	+
	2.0 + 3.0	+	+	+	+	+	+
NAA + IAA + BA	0.5 + 0.5 + 1.0	+	+	+	+	+	+
	1.0 + 1.0 + 1.0	+	+	+	+	+	+
BA	0.5	+	+	+	+	+	+
	1.0	+	+	+	+	+	+
	2.0	+	+	+	+	+	+
KIN	0.5	+	+	+	+	+	+
	1.0	+	+	+	+	+	+
	2.0	+	+	+	+	+	+
NAA	0.5	-	-	-	-	-	-
	1.0	-	-	-	-	-	-
	2.0	-	-	-	-	-	-
IAA	0.5	-	-	-	-	-	-
	1.0	-	-	-	-	-	-
	2.0	-	-	-	-	-	-

Table 19 Pattern of Chromatogram developed by positively responding test calli of *T. cordifolia* screened for the presence of berbrine

Ecotypes	Source of calli	Growth regulators	Solvent system	Rf values	Colour of the spot
Vellanikkara	Leaf	2NAA+2BA	Butanol -glacial acetic acid – Water (7:1:2)	0.522	Light yellow
		2NAA+2BA	Butanol -glacial acetic acid – Water (7:1:2)	0.524	Yellow
Vellanikkara	Petiole	2NAA+2BA	Butanol -glacial acetic acid – Water (7:1:2)	0.525	Yellowish green
		2NAA+2Kin	Butanol -glacial acetic acid – Water (7:1:2)	0.525	Yellow
Vellanikkara	Stem	2NAA+2BA	Butanol -glacial acetic acid – Water (7:1:2)	0.526	Yellow
		2NAA+2Kin	Butanol -glacial acetic acid – Water (7:1:2)	0.523	Light yellow
Vellanikkara		Standard berberine Hcl	Butanol -glacial acetic acid – Water (7:1:2)	0.526	Yellow
Madurai	Leaf	2NAA+2BA	Butanol -glacial acetic acid – Water (7:1:2)	0.540	Yellow
		2NAA+2Kin	Butanol -glacial acetic acid – Water (7:1:2)	0.544	Yellowish green
Madurai	Petiole	2NAA+2BA	Butanol -glacial acetic acid – Water (7:1:2)	0.541	yellow
		2NAA+2Kin	Butanol -glacial acetic acid – Water (7:1:2)	0.540	yellow
Madurai	Stem	2NAA+2BA	Butanol -glacial acetic acid – Water (7:1:2)	0.542	yellow
		2NAA+2Kin	Butanol -glacial acetic acid – Water (7:1:2)	0.544	yellow
Madurai		Standard berberine Hcl	Butanol -glacial acetic acid – Water (7:1:2)	0.542	yellow

Table 20. Influence of growth regulator combinations on berberine production by callus cultures of *Tinospora cordifolia*

Basal Medium - MS

Growth regulators	Concentration (mgl^{-1})	Berberine yield (µg/g of callus)						
		Vellanikkara ecotype			Madurai ecotype			Mean
		leaf	Petiole	stem	Leaf	petiole	stem	
NAA+BA	1.0+1.0	7.497	3.352	7.999	7.418	5.834	8.078	6.696
	1.0+3.0	6.156	3.124	7.086	6.112	3.334	7.626	5.573
	2.0+2.0	7.761	4.91	9.134	7.838	6.547	9.134	7.554
	2.0+3.0	6.765	4.012	7.312	7.018	4.42	7.868	6.232
NAA+KIN	1.0+1.0	6.019	4.963	7.022	6.441	4.012	8.078	6.089
	1.0+3.0	6.812	3.657	6.421	6.812	4.062	7.886	5.808
	2.0+2.0	6.755	5.121	10.058	7.022	4.775	10.401	7.355
	2.0+3.0	7.011	4.124	7.026	7.113	4.126	8.297	6.116
IAA+BA	1.0+1.0	6.086	3.748	7.073	6.781	3.616	7.961	5.877
	1.0+3.0	6.082	3.012	6.617	6.975	4.426	7.662	5.629
	2.0+2.0	6.886	4.963	7.19	7.229	4.461	8.103	6.472
	2.0+3.0	6.962	3.628	7.012	7.321	4.012	7.812	6.124
IAA+KIN	1.0+1.0	6.516	3.458	7.082	3.722	6.675	8.012	5.91
	1.0+3.0	6.562	3.562	6.128	3.826	6.716	7.468	5.71
	2.0+2.0	6.912	4.329	7.497	4.804	7.012	8.401	6.492
	2.0+3.0	6.914	3.826	6.914	4.082	7.064	7.812	6.102
BA	1	4.165	2.168	4.862	4.124	2.268	5.012	3.766
	2	4.662	2.512	5.012	4.802	2.612	5.121	4.12
KIN	1	4.402	2.463	4.462	4.962	2.471	4.966	3.954
	2	4.809	2.801	4.861	5.012	2.703	5.221	4.234
Mean		6.415	3.619	6.84	6.703	3.91	7.545	

7.554 µg of berberine whereas NAA with Kin, each at 2 mg l^{-1} resulted in 7.355 µg of berberine per gram of callus. BA or Kin alone, each at 2 mg l^{-1} resulted in an average of 4.120 µg and 4.234 µg berberine per gram of callus respectively. Hence MS basal medium with NAA at 2 mg l^{-1} supplemented with BA or Kinetin each at 2 mg l^{-1} was selected as basal production medium for *in vitro* berberine production as well as for employing special techniques to enhance the berberine content of *in vitro* cultures.

4.7.1.1.2 Modification of carbon source

Increasing levels of sucrose in basal medium to 4.5 and 5.0 per cent did not contribute to a favourable influence on the expression of berberine in the test calli as compared to control whereas media supplemented with sucrose (3 per cent) and lactose (1.5 per cent) registered 14.216 µg and 17.318 µg berberine per gram of stem calli and 10.812 µg and 13.625 µg berberine per gram of leaf calli of Vellanikkara and Madurai ecotypes respectively. Calli when subcultured in basal media having 3 per cent lactose gave 10.982 µg berberine per gram of test calli (Table 21).

4.7.1.1.3 Withdrawal of inorganic nutrients

Reducing the nitrogen and phosphorus content of the basal medium to half their original strength exerted beneficial effects on alkaloid expression (Table 22). Phosphorus at half strength resulted in maximum berberine production in Vellanikkara (14.058 µg and 18.126 µg per gram of leaf and stem calli cultures respectively) and Madurai (14.721 µg and 19.624 µg per gram of leaf and stem calli cultures respectively) ecotypes.

Table 21 . Effect of different carbon sources on berberine production in callus cultures of *Tinospora cordifolia*

Basal medium – MS + NAA 2mgl^{-1} + BA 2mgl^{-1}

Carbon Sources	Concentration (%)	Source of Calli	Berberine yield (µg/g of calli)	
			Vellanikkara ecotype	Madurai ecotype
Sucrose	4.0	Leaf	4.016	4.125
Sucrose	4.0	Stem	4.760	5.986
Sucrose	5.0	Leaf	4.362	4.082
Sucrose	5.0	Stem	5.084	6.716
Sucrose + Lactose	2.0+ 1.0	Leaf	10.812	13.625
Sucrose + Lactose	2.0 + 1.0	Stem	14.216	17.318
Lactose	3.0	Leaf	6.286	7.431
Lactose	3.0	Stem	8.086	10.982
Sucrose*	3.0	Leaf	7.761	7.838
Sucrose*	3.0	Stem	9.134	9.134

* control

Table 22. Effect of reducing nitrate and phosphate concentrations on berberine production in callus cultures of *Tinospora cordifolia*

Basal medium-MS

Treatment	Production media (mgl^{-1})	Reduction of inorganic nutrients	Source of calli	Berberine yield (µg/g of callus)	
				Vellanikkara ecotype	Madurai ecotype
1.	2 NAA + 2 BA	½ NO_3	Leaf	13.376	13.160
2.	2 NAA + 2 BA	½ NO_3	Stem	14.126	17.874
3.	2 NAA + 2 KIN	½ NO_3	Leaf	12.762	13.187
4.	2 NAA + 2 KIN	½ NO_3	Stem	12.232	13.876
5.	2 NAA + 2 BA	½ PO_4	Leaf	14.058	14.721
6.	2 NAA + 2 KIN	½ PO_4	Stem	18.126	19.624

4.7.1.1.4 Feeding of precursors

Responses of experimental calli subcultured onto medium with NAA and BA, each at 2 mg l^{-1} concentration, to addition of precursor, phenylalanine as revealed by quantifying the positively responding *in vitro* cultures are presented in Table 23. The observations of the data revealed the favourable influence of phenylalanine on production of berberine in leaf and stem calli of both Vellanikkara and Madurai ecotypes at all levels tested. Phenylalanine at 150 mg l^{-1} produced maximum alkaloid in leaf (17.626 µg/g and 18.012 µg/g of calli of Vellanikkara and Madurai ecotypes respectively) and stem (21.413 µg/g and 20.673 µg/g of calli of Vellanikkara and Madurai ecotypes respectively) derived callus cultures. Stem derived callus cultures synthesised more quantity of the target alkaloid as compared to leaf callus cultures in both the ecotypes studied. Increasing the levels of precursor to 200 mg l^{-1} did not result in enhanced levels of alkaloid in the test cultures.

4.7.1.2 Inducing morphological differentiation

Experimental calli of Vellanikkara and Madurai ecotypes at the stage of differentiation into organoids produced less amount of alkaloid (Table 24).

4.7.1.3 Regulation through cell growth kinetics

Three to eighteen week old calli registered positive for berberine whereas, callus cultures after eighteen weeks did not showed appreciable content of berberine.

Table 23 . Influence of phenyl alanine on berberine synthesis by *in vitro* cultures of *Tinospora cordifolia*

Basal medium – MS

Treatment	Production media (mgl^{-1})	Phenyl alanine concentration (mgl^{-1})	Source of calli	Berberine yield (µg/g of calli)	
				Vellanikkara ecotype	Madurai ecotype
1	2 NAA+2 BA	100	leaf	14.726	15.478
2	2 NAA+2 BA	100	stem	16.013	18.427
3	2 NAA+2 BA	150	leaf	17.626	18.012
4	2 NAA+2 BA	150	stem	21.413	20.673
5	2 NAA+2 BA	200	leaf	14.016	14.986
6	2 NAA+2 BA	200	stem	15.724	18.824
7	2 NAA+2 KIN	100	leaf	15.176	14.086
8	2 NAA+2 KIN	100	stem	15.921	18.472

Table 24 . Berberine synthesis at stage of organoid differentiation of *Tinospora cordifolia*

source of calli	Berberine yield (µg/g of differentiated calli)	
	Vellanikkara ecotype	Madurai ecotype
Leaf	2.268	2.684
Stem	3.024	3.856

4.7.2 Employing special techniques to enhance berberine content in *in vitro* cultures

4.7.2.1 Creating conditions of stress on incubating cultures

4.7.2.1.1 Addition of osmoregulants

Addition of osmoregulants exerted marked increase in berberine production of the cultures (Table 25). The osmoregulants tried, polyethylene glycol at 2 and 3 per cent and mannitol at 1.5 per cent resulted in increased production of berberine in leaf and stem callus cultures of both the ecotypes. On comparing leaf and stem calli cultures, the latter produced maximum berberine (20.826 µg and 20.087 µg per gram of callus) with 3 per cent poly ethylene glycol in Vellanikkara and Madurai ecotypes respectively. Mannitol at 1.5 per cent resulted in 22.728 µg and 20.287 µg per gram of stem derived calli for Vellanikkara and Madurai ecotypes.

4.7.2.1.2 Increasing concentration of agar

Higher level of agar (1.0%) added to the medium P resulted in 14.367 µg and 17.108 µg berberine per gram of leaf and stem derived calli of Vellanikkara ecotype whereas Madurai ecotype resulted 16.323 µg and 17.813 µg per gram of leaf and stem calli cultures respectively (Table 25).

4.7.2.2 Elicitation

Eliciting the production media with autoclaved mycelia of *Pythium aphanidermatum* at levels of 0.5, 1.0 and 1.5 g l^{-1} yielded encouraging results with respect to production of berberine *in vitro,* by leaf and stem calli of experimental ecotypes (Table 26). Addition of 0.5 g l^{-1} fungal mycelia recorded maximum

Table 25 . Effect of stress inducing compounds on berberine production *in vitro*

Basal medium – MS + NAA 2 mgl^{-1}+ BA 2 mgl^{-1}

Sl.No.	Stress inducing compound	source of calli	berberine yield (µg/g of calli)	
			Vellanikkara ecotype	Madurai ecotype
1	Poly ethylene glycol 2%	leaf	16.073	15.923
2	Poly ethylene glycol 2%	stem	19.727	20.601
3	Poly ethylene glycol 3%	leaf	16.823	16.763
4	Poly ethylene glycol 3%	stem	20.826	20.087
5	Mannitol 1.5%	leaf	17.626	18.106
6	Mannitol 1.5%	stem	22.728	20.287
7	Agar 1%	leaf	14.367	16.323
8	Agar 1%	stem	17.108	17.813

Table 26 . Influence of fungal elicitation on berberine production *in vitro*

Basal medium – MS + NAA 2 mgl^{-1}+ BA 2 mgl^{-1}

Sl.No.	Levels of autoclaved mycelia *P.aphanidermatum* (gl^{-1})	source of calli	berberine yield (µg/g of calli)	
			Vellanikkara ecotype	Madurai ecotype
1	0.5	leaf	17.127	19.262
2	0.5	stem	23.176	22.987
3	1.0	leaf	14.326	15.672
4	1.0	stem	16.063	19.017
5	1.5	leaf	13.476	15.639
6	1.5	stem	16.123	18.285

berberine synthesis in stem calli cultures of Vellanikkara (23.176 µg/g) and Madurai (22.987 µg/g) ecotype.

4.7.2.3 Immobilisation

Visual assessment of encapsulated beads as influenced by sodium alginate (Wilson Laboratories) at 3.0, 5.0, 7.0, 9.0 and 11.0 per cent and calcium chloride ($CaCl_2$) at 50, 75 and 100 mM is presented in Table 27.

A 11.0 per cent solution of sodium alginate on complexing with 75 mM calcium chloride solution yielded round, firm beads, ranging from 1.0 to 1.3 cm in diameter (Plate 4). Lower concentrations of sodium alginate yielded fragile beads. Levels of $CaCl_2$ lower than 75 mM, prolonged complexing period, resulting in poor bead quality.

Immobilised calli resulted in increased amount of berberine. Leaf and stem calli when immobilized, produced 15.068 µg and 19.128 µg of berberine per gram of calli in Madurai ecotype and 13.276 µg and 15.068 µg per gram of calli in Vellanikkara ecotype (Table 28).

4.8 Establishing cell suspension cultures

4.8.1 Standardisation of subculturing intervals

Figures 5a and 5b depict the cell count observed in the suspension cultures of the two experimental ecotypes of the species under study over a period of 20 days. The cell count showed a progressive and sustained exponential growth in both the experimental ecotypes up to 16 days and declined thereafter (Plate 5). Based on the above observations, the subculturing intervals were fixed as 16 days

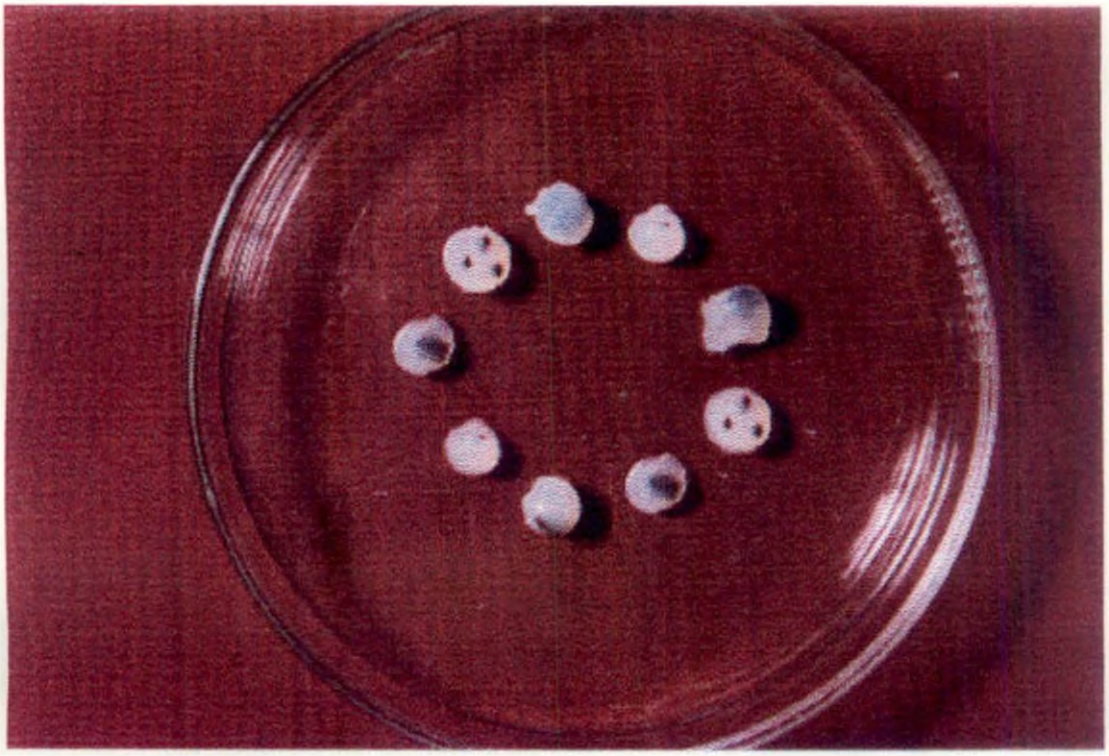


Plate 4. Immobilised calli of *Tinospora cordifolia* Miers.

Plate 5. Suspension cultures derived from leaf calli of *Tinospora cordifolia* Miers.

Table 27 . Standardisation of levels of gel forming chemicals in immobilization of calli of *Tinospora cordifolia*

Calcium chloride (mM)	Sodium alginate (per cent) W/V				
	3.0	5.0	7.0	9.0	11.0
50	-	+	++	++	+++
75	-	+	++	+++	+++++
100	-	+	++	+++	+++

Table 28 . Quantification of berberine in immobilized calli of *Tinospora cordifolia*

Medium – MS + NAA 2 mgl^{-1}+ BA 2 mgl^{-1}

Source of calli	Berberine yield (µg/g of calli)	
	Vellanikkara ecotype	Madurai ecotype
leaf	13.276	15.068
stem	17.624	19.128

Fig.5a. Standardisation of subculturing intervals for suspension cultures of *T.cordifolia.* Production medium:MS+NAA 2 mg/l+BA 2 mg/l

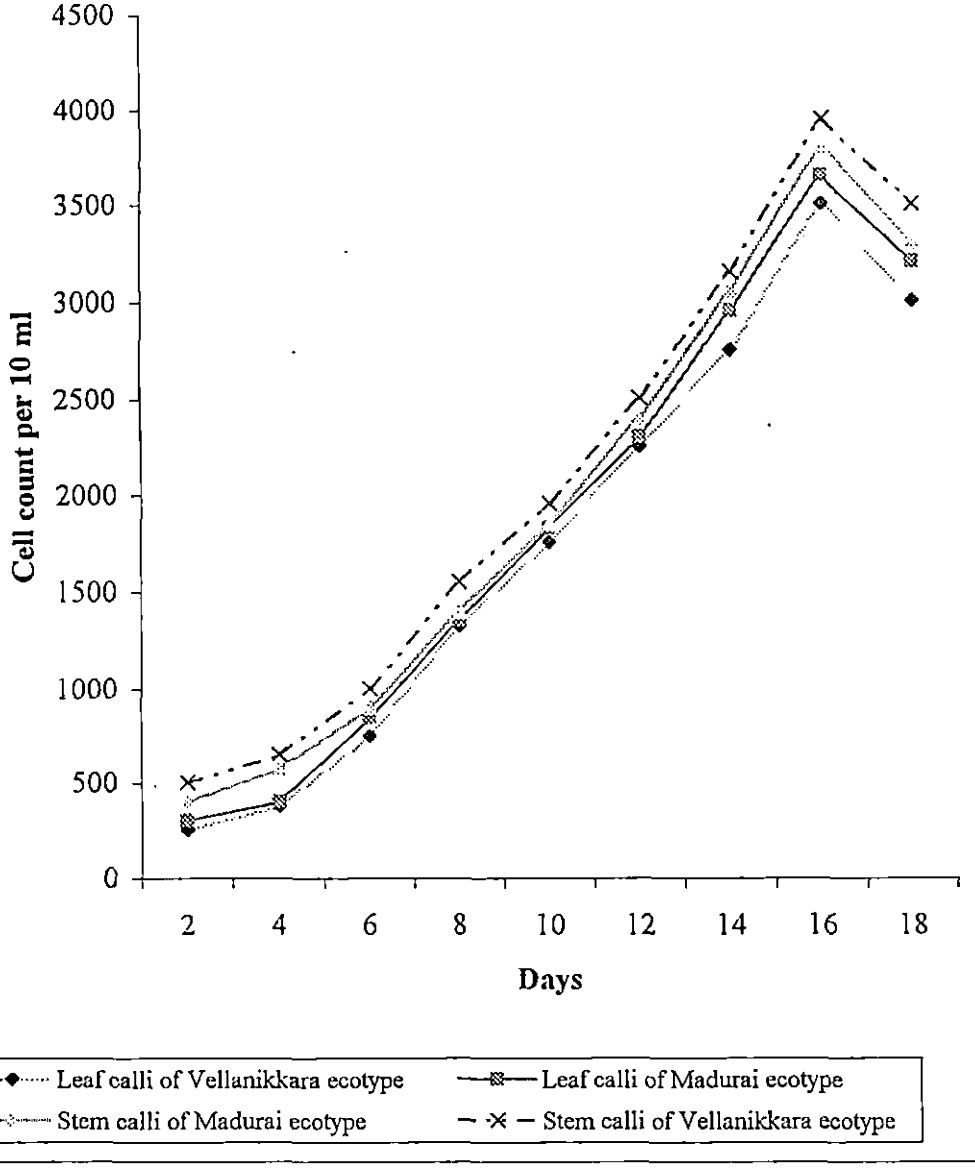
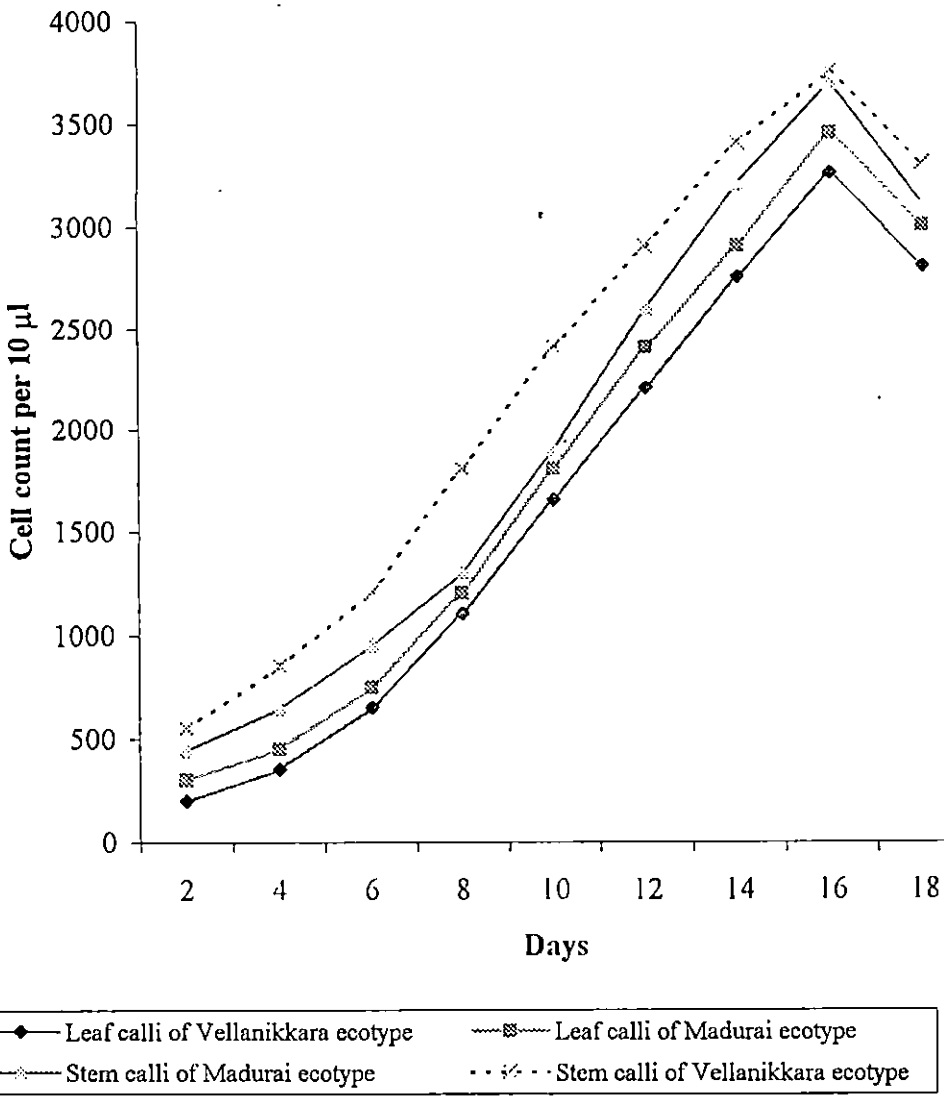


Fig.5b. Standardisation of subculturing intervals for suspension cultures of *T.cordifolia.* Production medium : MS + NAA 2mg/l+KIN 2mg/l.



(Fig. 5a and 5b). Cultures maintained in basal medium enriched with NAA and BA each at 2 mg l^{-1} produced a mean cell count of 3575 and 3850 per 10 µl media for leaf and stem calli respectively (Table 29).

4.8.2 Determination of packed cell volume

The production media (NAA + BA and NAA + Kin, each at 2 mg l^{-1}) gave equal values for mean packed cell volume (0.85 per cent for leaf and 0.95 per cent for stem callus cultures). Madurai ecotype registered maximum cell count while Vellanikkara ecotype registered maximum packed cell volume (Table 29).

4.8.3 Quantification of berberine in suspension cultures

Suspensions established in production medium (MS + NAA and BA, each at 2 mg l^{-1}) produced 4.216 µg and 6.412 µg of berberine per gram of leaf and stem callus respectively in Vellanikkara ecotype whereas leaf and stem calli of Madurai ecotype synthesized 4.562 µg and 6.216 µg of berberine per gram of calli. Suspensions in MS medium incorporated with NAA (2 mg l^{-1}) with Kin (2 mg l^{-1}) synthesized 4.026 µg and 6.012 µg of berberine per gram of leaf and stem cultures in Vellanikkara ecotype while Madurai ecotype produced 4.212 µg and 5.868 µg of berberine per gram of suspensions of leaf and stem derived cultures respectively (Table 29). Suspensions developed from calli of Madurai ecotype resulted in higher berberine than Vellanikkara ecotype (5.214 µg and 5.166 µg per gram of callus). As compared to static culture, suspensions produced less quantity of berberine in the present study (Table 30).

Table 29 . Callus growth and proliferation in liquid suspensions of MS production media at subculturing stage of *Tinospora cordifolia*

Production media (mgl^{-1})	source of calli	cell count (No.of cells per 10 µl)			Packed Cell Voulme(%)			Berberine yield (µg/g of calli)	
		1	2	Mean	1	2	mean	I	2
2 NAA + 2 BA	leaf	3500	3650	3575	0.9	0.8	0.85	4.216	4.562
2 NAA + 2 BA	stem	3900	3800	3850	1.0	0.9	0.95	6.412	6.216
2 NAA + 2 KIN	leaf	3250	3450	3350	0.8	0.9	0.85	4.026	4.212
2 NAA + 2 KIN	stem	3750	3700	3725	1.0	0.9	0.95	6.012	5.868

1 - Vellanikkara ecotype ; 2 - Madurai ecotype

Table 30 . Comparison of static and suspension cultures of *Tinospora cordifolia* for *in vitro* synthesis of berberine

Production media (mgl^{-1})	Source of calli	Berberine yield (µg/g of calli)					
		static cultures			suspension cultures		
		1	2	Mean	1		Mean
MS+2NAA+2BA	leaf	7.761	7.838	7.799	4.216	4.562	4.389
MS+2NAA+2BA	stem	9.134	9.134	9.134	6.412	6.216	6.314
MS+2NAA+2KIN	leaf	6.755	7.022	6.888	4.026	4.212	4.119
MS+2NAA+2KIN	stem	10.058	10.401	10.229	6.012	5.868	5.940
Mean		8.427	8.598		5.166	5.214	

1 - Vellanikkara ecotype , 2 - Madurai ecotype

Table 31 . Berberine content in methonal extracts of ex vitro samples

Extracts	Berberine content (µg/g of sample)	
	Vellanikkara ecotype	Madurai ecotype
Young leaf	-	-
Mature leaf	-	-
Young stem	0.141	0.182
Mature stem	0.262	0.284

4.9 Screening of *ex vitro* sample extracts for berberine by TLC

Thin layer chromatograms of leaf and stem extracts of *ex vitro* plants, both at young and mature stages revealed the presence of berberine only in the stem extracts. The alkaloid was not detected in leaf extracts even under UV detector.

4.9.1 Quantification of berberine in *ex vitro* extracts

Mature stem extract contained comparatively higher quantity of berberine (0.162 µg/g of stem in Vellanikkara ectoype and 0.184 µg/g of stem in Madurai ecotype) as compared to immature stem extracts (Table 31).

Discussion

DISCUSSION

5.1 Standardisation of surface sterilisation of explants

Surface sterilisation, an inevitable procedure for the successful culture and incubation of *in vitro* systems was carried out with the commonly employed sterilant, mercuric chloride at different concentrations for varying lengths of time to standardize the most suitable level. Sterilisation with 0.1 per cent mercuric chloride for eight minutes was found best with respect to percentage of survival, irrespective of source of explants (Table 7). The effectiveness of mercuric chloride as surface sterilant has been observed in various medicinal species. For instance, $HgCl_2$ at 0.1 per cent was most effective in *Piper longum* in which duration of treatment was standardised as 20 min (Bhat *et al.*, 1992). John (1996) also identified sterilisation with 0.1 per cent $HgCl_2$ for 5-10 min as most appropriate to check levels of contamination ensuring better survival in *Holostemma adakodien.* In the present study mercuric chloride (0.1 per cent) treated for eight minutes registered maximum survival of 73.74 per cent in *in vitro* cultures.

Increasing duration of exposure of explants to the sterilant and scaling up the levels of sterilant had adverse effect on the survival of cultures as observed in this study (Table 7). Increasing treatment duration with 0.1 per cent $HgCl_2$ has been reported to result in high mortality in various crops like *Gymnema sylvestre* due to the toxicity of the chemical imposed on the explant (Anu, 1993).

5.2 Influence of season on survival of cultures

Effect of seasonal influence were noticed in this study in the levels of survival of leaf, petiole and stem explants over a period of nine months (Table 8). Contrary to reports wherein percentage of contamination was maximum when the

explants collected during rainy seasons (Sankar, 1998; Sindhu, 1999 and Gholba, 2000), substantial reduction of survival was noticed when the explants were collected during December to March while those collected in June to August registered maximum survival percentage. Reduction in the exudation of phenolic substances by the explants may be the reason for maximum survival of cultures during rainy season in *Tinospora*.

5.3 Standardisation of basal media

Various plant tissue culture media like MS medium, MS medium at half strength and Woody Plant medium were used to initiate callus cultures in this study. Best culture medium was identified based on their efficacy to initiate maximum calli in leaf, petiole and stem cultures (Table 9). MS medium at full strength salt concentration initiated maximum calli in leaf (73.15%), petiole (75.39%) and stem (83.56%) cultures. In MS medium at half strength, only 62.96 per cent leaf explants 63.14 per cent petiole explants and 76.89 per cent stem explants callused.

Highest percentage of culture establishment in full strength MS medium has been reported in *Ginkgo biloba* cultures by Carrier *et al.* (1990). Growth of undifferentiated calli was achieved on MS medium at full strength with varying concentrations of 2,4-D in callus cultures from leaf, stem and root segments of *Solanum mammosum* (Akram *et al.*, 1994). Akram and Yurekhi (1995) observed better callus growth from leaf explants of *Catharanthus roseus* in full strength MS medium supplemented with NAA 2 mg l^{-1} and BA 3 mg l^{-1}. Tailang and Kharya

suspensions of *Glycyrrhiza glabra* from full strength MS medium containing NAA (5.0 mg l^{-1}) and BAP (0.1 mg l^{-1}). Highest percentage of culture establishment in full strength MS medium has been reported in *Gymnema sylvestre* by Gholba (2000).

5.4.1 Effect of auxins on callusing in *Tinospora cordifolia*

Trends in initiation of calli on full strength MS medium supplemented with three auxins NAA, IAA and 2,4-D was examined (Tables 10a, 10b and 10c). MS medium supplemented with NAA at 4 mg l^{-1} recorded highest percentage of callus initiation in leaf (91.43%) and stem (91.01%) cultures. The same auxin at 2 mg l^{-1} recorded maximum callusing in petiole (94.39%) cultures as well. Early callusing was also observed with this auxin at 4 mg l^{-1} for leaf, petiole and stem cultures. IAA at 2 mg l^{-1} registered highest callus growth score in petiole (2.906) and stem (3.113) cultures. For leaf cultures, NAA at 1 mg l^{-1} in combination with IAA at 3 mg l^{-1} resulted in maximum callus growth score and mean callus index value.

Favourable effects of NAA on callusing is observed in various crops like *Papaver bracteatum* (Ilahi and Ghauri, 1994) and *Catharanthus roseus* (Akram and Yurekhi, 1995). Rao *et al.* (1999) obtained maximum percentage of callus initiation in leaf base cultures of *Centella asiatica* in MS medium supplemented with 2 mg l^{-1} NAA. Performance of the auxin 2,4-D with respect to callus induction and proliferation in *Tinospora* was not encouraging, since lowest values were recorded with this auxin for the parameters studied. Contrary to the

above observations, favourable effects of 2,4-D on callus induction was reported in medicinal species like *Plumbago rosea* (Sateeshkumar and Bhavanandan, 1989). Also, John (1996) obtained low callus index values in *Holostemma* on supplementing culture media with 2,4-D.

5.4.2 Comparative response of ecotypes to auxins in inducing and proliferating calli

Vellanikkara and Madurai ecotypes responded equally in leaf and stem cultures for most of the parameters studied whereas for petiole cultures, variation existed between them. Vellanikkara ecotype resulted in high percentage of callusing in leaf (85.618%) and petiole (89.788%) cultures. Madurai ecotype registered high callus growth score (2.478) in petiole cultures.

Sankar (1998) observed superior performance of *Sida acuta* with highest callus index values as compared to the other species tested. Similarly significant variation among species in initiating and proliferating calli was observed in the genus *Ephedra* (O'Dowd *et al.*, 1993). The genotypic variation as indicated by the differential response of the species to growth factors indicate that endogenous requirements of hormones vary inherently.

5.4.3 Comparative performance of explants to callus induction and proliferation

Cultures originating from leaf and stem explants were better performing with reference to the parameters indicating efficient callusing, with the former initiating calli earlier (11.11 days) and latter, registering highest callus growth rate (2.643) and mean callus index value (228.245). Petiole explants registered maximum percentage (88.128) of callus initiation.

Suitability of using leaf segments as explants to induce calli was observed in *Plumbago rosea* (Sateeshkumar and Bhavanandan, 1989). Nair *et al.* (1992) reported the suitability of employing leaf petiole bases as explants to induce calli in *Coscinium fenestratum*. Sankar (1998) reported that efficient callusing was noticed for leaf and stem explants of *Sida* spp. as compared to root explants.

5.5 Effect of cytokinins on callusing

The incorporation of major cytokinins benzyladenine and kinetin at varying levels to basal media did not induce positive response with respect to callusing. Leaf, petiole and stem cultures responded in an inferior manner for all the parameters studied (Tbles 11, 11b and 11c).

Generally, the observation that cytokinins favour cell elongation and shoot bud initiation as compared to callusing holds good in the present study. However, Mercier *et al.* (1992) and Paniego and Giulietti (1994) highlighted favourable effects of cytokinins to promote callus mediated shoot regeneration in *Gomphrena officinalis* and *Artemisia annua* respectively. Anu (1993) reported that *in vitro* cultures reacted unfavourably to kinetin with respect to callus proliferation in *Gymnema sylvestre*. Sindhu (1999) observed that *Coscinium fenestratum* reacted favourably to BA and kinetin wih respect to callusing.

5.6.1 Effect of auxins and cytokinins on callusing

In the present study, IAA and Kinetin, each at 2 mg l^{-1} resulted in high percentage of callusing with maximum callus index values for petiole and stem cultures. NAA and IAA responded favourably for all the parameters of callusing

when supplemented with BA and kinetin than when used singly (Table 12a, 12b and 12c). Leaf cultures performed better than petiole and stem cultures under auxin and cytokinin combinations with respect to percentage of cultures initiating calli with (84.17%) and days to callus initiation (10.48 days). IAA at 1 mg l^{-1} with BA at 2 mg l^{-1} favoured maximum callusing in leaf cultures. Nakagawa *et al.* (1984) observed that NAA and BA induced callus growth in leaf segments cultured on Linsmaier and Skoog medium in *Thalictrum minus* var. hypoteucum. Kin in combination with NAA however reacted unfavourably to callus proliferation in *Gymnema sylvestre* (Anu, 1993).

In *Aristolochia bracteolata,* successful callusing was achieved with NAA and kinetin (Ramashree *et al.*, 1994). Auxins are primarily known to induce cell elongation while cytokinins induce cell division. Both these processes are efficient for growth. So the combined stimulus from auxins as well as cytokinins may have enhanced the rate of growth of the callus (Balandrin and Klocke, 1988). Gokul and Tejawathi (1997) found that in *Cissampelos pareira,* a tropical climber used in Ayurvedic medicine, belonging to Menispermaceae family, stem explants formed callus in MS medium containing NAA and Kinetin. Callus cultures were initiated on MS medium with NAA (2.0 mg l^{-1}) and BA (0.5 mg l^{-1}) in *Coleus forskohlii* (Suryanarayanan and Pai, 1998). Kannan and Jasrai (1998) observed callusing in nodal segments on MS medium with 4.4 µM of BA and 2.6 µM NAA. A drastic inhibition of callusing in *Kaempferia galanga* when kinetin was added to 2,4-D as observed by Joseph (1997) was also noticed in the present study.

5.7 Effect of media supplements on callusing

The phenolic hormone synergist, phloroglucinol, at levels of 100.0 and 125.0 mg l^{-1} and the organic supplement, casein hydrolysate at 100.0, 200.0 and 300.0 mg l^{-1} registered maximum callusing in leaf, petiole and stem cultures (Tables 13a, 13b and 13c). Hunter (1979) observed promotive effects of phloroglucinol in *Cinchona ledgeriana.* He also reported that this phenolic compound can reduce the oxidative browning and suppress contaminants within tissues which adds to its advantage in *in vitro* culture systems. In this investigation, casein hydrolysate at 100 mg l^{-1} showed early induction of calli in leaf, petiole and stem cultures. The same compound at 300 mg l^{-1} registered high callus growth score in petiole cultures (3.413). Casein hydrolysate, considered a complex of twenty amino acids is presumed to be growth promoting in *in vitro* culture system (Adu-Ampomah *et al.*, 1988). Marked reduction in all the parameters studied with respect to callusing was noticed in the study with the incorporation of activated charcoal at two levels viz., 0.25 and 0.5 per cent. Scott and Ellen (1980) stated that, activated charcoal, a known adsorbent, at 0.1 to 5.0 per cent levels, reduces the auxin concentration in the media. This property of activated charcoal of adsorbing growth substances present in culture media accounts for reduced callus growth in media supplemented with this additive.

5.8 Influence of culture environment on callusing

In *Tinospora,* culturing the explants under illumination at 26±1°C proved superior to culturing them under dark (Table 14). Sankar (1998) observed

that incubating leaf and stem explants of *Sida cordifolia* under illumination at 26±1°C was significantly superior to incubating them under dark. However, dark incubation resulted in better callusing in *Kaempferia galanga* cultures (Joseph, 1997). Nair *et al.* (1992) also reported that presence of light inhibited the growth but stimulated berberine synthesis in *Coscinium fenestratum.*

Culturing under dark was found beneficial for callusing with respect to number of days taken for callus initiation and percentage of cultures initiating calli, when leaf segments of *C. fenestratum* were cultured *in vitro*. But maximum callus index values were obtained when cultures were incubated in light (Sindhu, 1999).

5.9 Regeneration of organoids

5.9.1 Callus mediated rhizogenesis

Regeneration of roots from the experimental calli was achieved with varying levels of success in the present study (Tables 15a and 15b). Callus mediated rhizogenesis was promoted by NAA and IAA each at 2 mg l^{-1} for leaf and stem derived calli when cultured in half strength MS medium. The addition of the former resulted in 56.409 per cent and 51.442 per cent rooting respectively in leaf and stem calli cultures whereas the latter induced rooting in 58.372 per cent leaf cultures and 53.439 per cent stem callus cultures. Full strength MS medium supplemented with NAA and IAA each at 4 mg l^{-1} resulted in minimum percentage of rooting. Among the experimental ecotypes, Madurai ecotype registered maximum percentage of rooting than Vellanikkara ecotype. Between the leaf and stem calli cultures, leaf calli recorded high percentage of rooting (45.368%) in

11.345 days of incubation. Sankar (1998) observed maximum percentage of rooting in *Sida* spp. by culturing the explants in MS medium at half strength containing NAA at 1 mg l^{-1}. The auxin, NAA efficiently promoted callus mediated rhizogenesis in *Artemisia* (Brown, 1994). In the present study, auxins supplemented with 2,4-D gave a negative response with respect to root regeneration from leaf and stem cultures whereas Shrivastava and Padhya (1995) observed rooting in leaf calli of *Boerhaevia diffusa* in the presence of 2,4-D.

The cytokinins BA and Kinetin supplemented with auxins also failed to regenerate roots from leaf, stem and petiole derived calli in *Tinospora*. Cytokinins generally recognized as a shoot growth promoter, obviously failed to perform here with a possible reversal of polarity (Mizrahi, 1988). However, Akram *et al.* (1994) observed root regeneration from one year old callus cultures of *Atropa accuminata* in the presence of Kinetin at 1.0 mg l^{-1}. The auxin NAA at 1.0 mg l^{-1} in combination with Kinetin at 0.3 mg l^{-1} resulted root regeneration in 66.51 per cent leaf cultures of *Sida* spp. (Sankar, 1998). The present study confirms to the observation by Sindhu (1999) that addition of BA failed to initiate roots from callus of *Coscinium fenestratum*.

5.9.2 Callus mediated shizogenesis

Various growth regulator treatments, cytokinin (BA and Kinetin) alone and in combination with auxins (NAA and IAA) at varying levels did not result in callus mediated shizogenesis in the present study (Table 16). That the regeneration potential of *in vitro* cultures exhibited marked variation has been a subject of

interest. As interpreted in detail by Mizrahi (1988), the morphogens which are thought to control the differentiation of individual cells and their ordered arrangement into tissues and organs, may not achieve the desired balance in several *in vitro* systems which explain the variation in regenerative potential from culture to culture.

Sankar (1998) reported that root derived calli of *Sida* spp. failed to regenerated shoots. In *Coscinium fenestratum*, Sindhu (1999) could not regenerate shoots from leaf and stem calli cultures.

5.9.3 Somatic embryogenesis

Leaf and stem calli of both Vellanikkara and Madurai ecotype grown on MS medium supplemented with NAA 1 mg l^{-1}, Kinetin 4 mg l^{-1}, sucrose 3.0 per cent and lactose 1.5 per cent differentiated to embryoids. Embryoids germinated when subcultured to the initiating medium. Leaf calli responded better with respect to initiation and germination of embryoids (Table 17). Somatic embryogenesis requires auxin for induction of embryos and a medium devoid of growth regulators for its maturation (Ammirato, 1983). In *Kaempferia galanga* embryogenesis was observed from leaf callus cultures by Vincent *et al.* (1992) in MS medium supplemented with 2,4-D 1.0 mg l^{-1} and BAP 0.5 mg l^{-1}. However, in the present study, 2,4-D did not favour somatic embryogenesis.

Kinetin, the cytokinin observed favouring embryogenesis in this study, also initiated embryoids in *Emilia sonchifolia* L. wherein nodular calli produced somatic embryoids (Shanthamma *et al.*, 1991). In *Sida cordifolia*, Sankar (1998)

observed somatic embryogenesis from leaf callus cultured on half MS medium supplemented with NAA and Kinetin, each at 1.0 mg l^{-1} to which 1.0 per cent sucrose and 2.0 per cent maltose were incorporated. Callus cultures of *Coscinium fenestratum* did not yield somatic embryos (Sindhu, 1999).

5.10 Regulation of *in vitro* metabolite production

5.10.1 Standardization of production medium

5.10.1.1 Modifying basal growth medium

5.10.1.1.1 Regulation of growth factor combinations

Among the growth factor combinations attempted in the study, the auxin NAA and IAA, each, in combination with BA or Kinetin each at 2 mg l^{-1} proved beneficial for the synthesis of berberine. Calli produced from stem segments cultured on full strength MS medium registered maximum amount of berberine as compared to leaf segments (Table 20). Cytokinins are known to stimulate the production of secondary metabolites in *in vitro* systems. In the present study also, the cytokinins BA and Kinetin, each at 2 mg l^{-1}, registered higher production of berberine.

Favourable influence of NAA and Kinetin in terms of product synthesis was also observed by Sakamoto *et al.* (1993) in *Aralia cordata* callus cultures. *In vitro* synthesis of tabernosine in *Vinca herbaceae* cell cultures occurred with NAA and Kinetin as reported by Zagorska *et al.* (1993). Ginkgolide B production in cultured cells derived from *Ginkgo biloba* leaves reached optimum levels with NAA 1.0 mg l^{-1} and Kinetin 0.3 mg l^{-1} (Jeon *et al.*, 1995). In general, the

observation by Rhodes *et al.* (1986) that 2,4-D is less good for product synthesis than IAA, with NAA tending to have an intermediate effect holds good in this study. The favourable influence of IAA in combination with BA on product synthesis was observed in *Coscinium* by Sindhu (1999) wherein IAA 2 mg l^{-1} and BA 1 mg l^{-1} proved beneficial for the synthesis of berberine.

5.10.2 Modification of carbon source

Increasing the sucrose concentration to 4.0 and 5.0 per cent did not exert a beneficial influence on berberine synthesis (Plate 6), in the present study, as also observed by Sankar (1998) for ephedrine synthesis in *Sida* spp. But Ellis *et al.* (1996) observed stimulation of taxol content by increasing the sucrose concentration to 8.0 per cent in *Taxus* sp.

Substituting sucrose with lactose totally or in proportions of 2:1 brought about noticeable increase in berberine synthesis in the present study (Table 21). Substitution of sucrose with maltose totally in culture systems resulted favourably with respect to product synthesis as in *Artemisia annua* (Rao and Narasu, 1997) and *Digitalis lanata* (Kreis and Reinhard, 1986). Asaka *et al.* (1994) reported that ginseng embryoids produced maximum amount of saponins when raised in media containing 30 g sucrose and 30 g glucose per litre.

5.10.3 Withdrawal of inorganic nutrients

Maintaining phosphate levels below optimum is often employed as a strategy to confer a growth limiting environment to *in vitro* systems that stimulate product synthesis (Sakamoto *et al.*, 1993). In the present study, reduction of

phosphate of basal MS medium to half strength increased berberine production (Plate 6). The berberine yield obtained when phosphate was reduced to half strength was 18.126 µg/g of callus. Reducing nitrate to half the original concentration also resulted in increased berberine synthesis by leaf and stem cultures (Table 22) though the increase was less as compared to that obtained by reducing phosphate concentration.

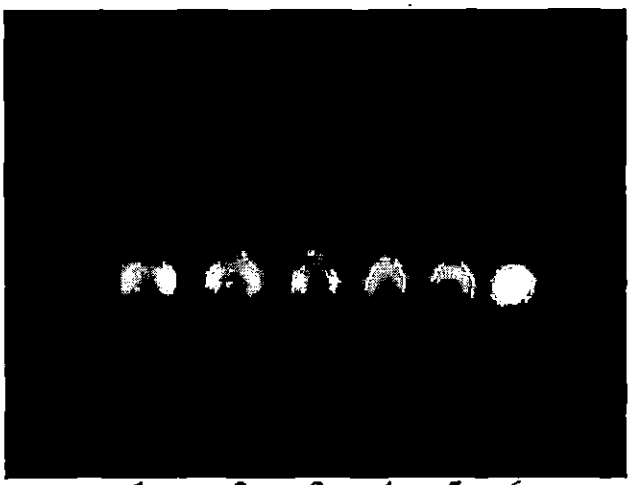
Typical examples of beneficial effect of phosphate withdrawal include accumulation of *cinnamoyl putrescence* in *Nicotiana* (Schiel *et al.*, 1984). Capsaicin synthesis in cell cultures of *Capsicum annum* increased by elimination of nitrate from basal medium (Ravishankar *et al.*, 1988). Increase in phosphate levels is also observed to have a positive influence on product synthesis, as observed for saponin accumulation in cell cultures of *Panax ginseng* (Zhong and Zhu, 1995). When ammonium phosphate was reduced to quarter strength, the berberine yield obtained was 10.079 µg/g callus in *Coscinium fenestratum* (Sindhu, 1999).

5.10.4 Supply of precursors

Phenyl alanine, the amino acid precursor of the target alkaloid berberine at levels of 100 mg l^{-1}, 150 mg l^{-1} and 200 mg l^{-1} had a desirable influence on the biosynthetic capability of leaf and stem calli of *Tinospora cordifolia* which responded positively when subjected to appropriate tests employed to screen berberine (Table 23). Phenyl alanine at 150 mg l^{-1} registered maximum yield of berberine (21.413 µg/g callus). These findings correlate well with many published

Plate 6. Thin layer chromatogram of methanol extract of *in vitro* cultures of *Tinospora cordifolia* Miers.

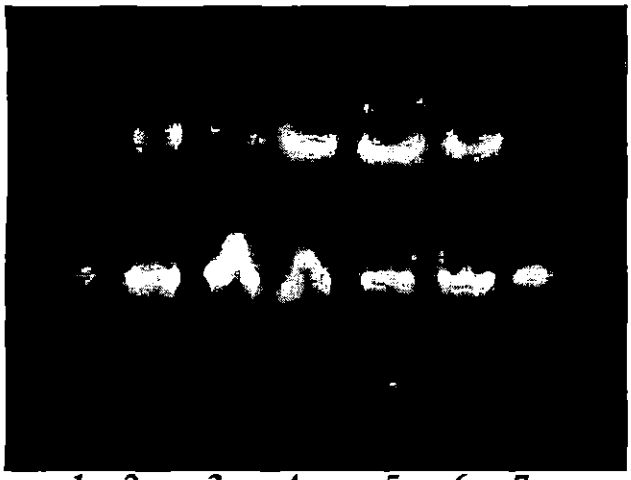
A



B

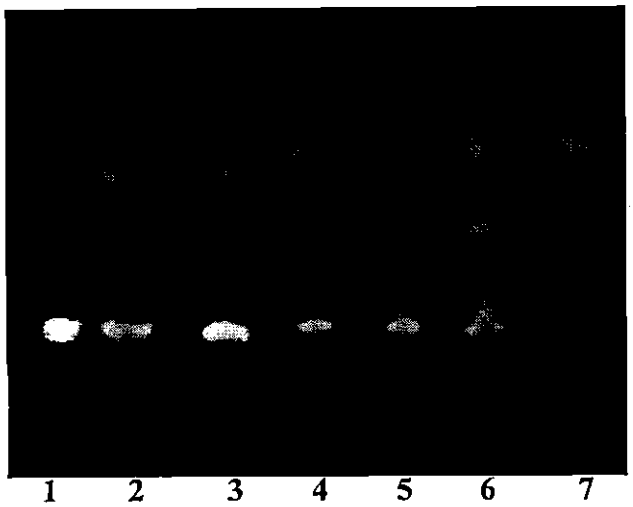
C

D



E

F



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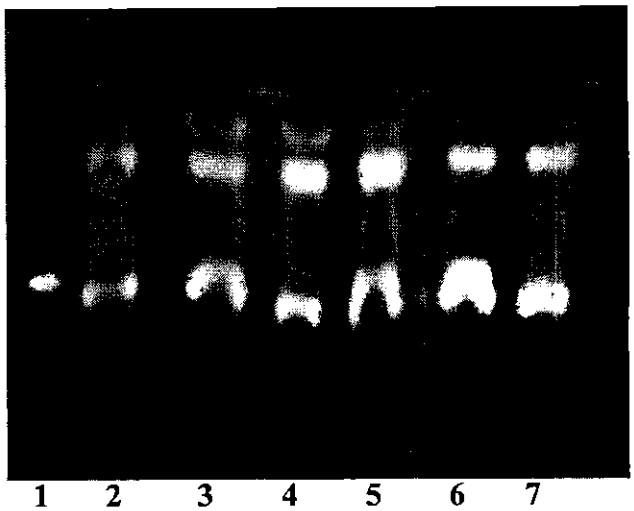

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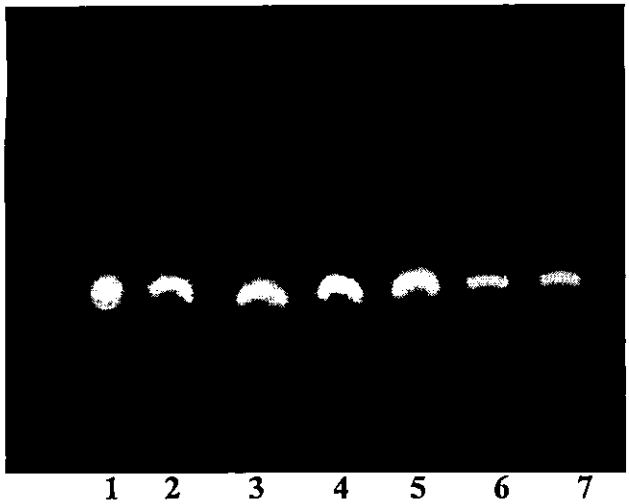

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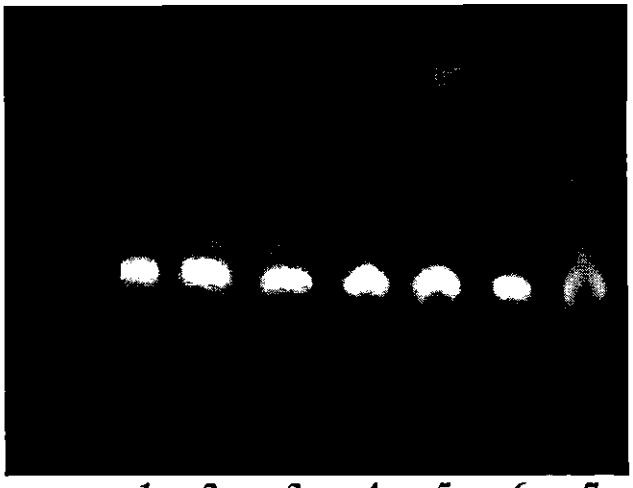

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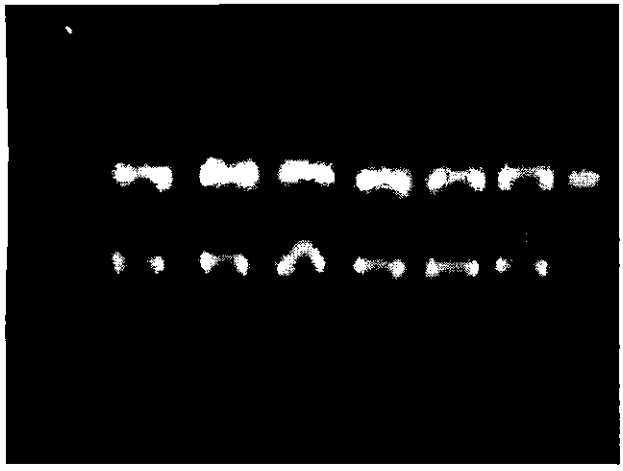
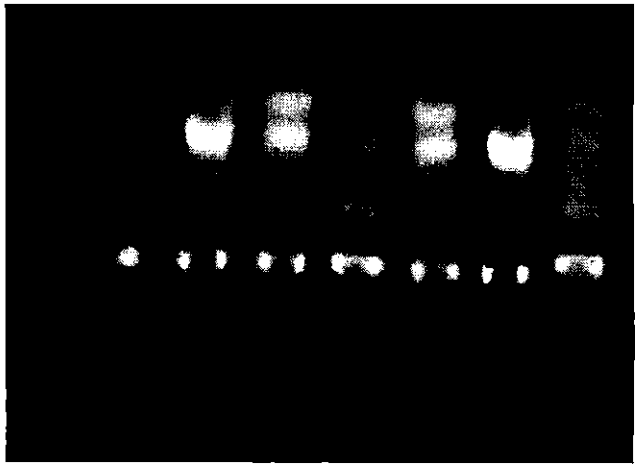


K



L

reports as detailed herewith. Feeding of precursors is employed with spectacular results in various medicinal plants. Deus and Zenk (1982) observed that the indole alkaloid precursor, tryptophan, stimulated alkaloid production in one cell line of *Catharanthus roseus* nearly three fold. In *Capsicum frutescens,* more than one precursor effectively contributed to product synthesis, where both phenyl alanine and isocapric acid stimulated capsaicin synthesis (Lindsey and Yeomann, 1984). In *Capsicum annum*, addition of D-limonene and L-ascorbic acid increased the production of capsaicin (Veeresham *et al.*, 1992).

Sankar (1998) reported that between phenyl alanine and methionine, phenyl alanine at 50.0 and 100.0 mg l^{-1} induced synthesis of ephedrine in *Sida* spp. whereas methionine was not effective. In the present study, when the levels of the prescursor was increased to 200 mg l^{-1} berberine content did not register an increase revealing that there is an optimum level of the precursor which the culture can tolerate. The unsuitability of employing high precursor levels to enhance product release is reported in *Cinchona pubescens* suspension cultures as well by Robins *et al.* (1987).

5.10.5 Inducing morphological differentiation

Experimental calli of both the ecotypes at the stage of differentiation into roots or embryoids did not react favourably to synthesis of berberine as observed in this study was also inferred by Sankar (1998). This observation is in contrast with many published reports as in *Boerhaavia diffusa* (Shrivastava and Padhya, 1995) and *Solanum platanifolium* (Jaggi and Singh, 2001) where calli at differentiation stage synthesized the respective secondary products.

5.10.6 Regulation through cell growth kinetics

In the present investigation, three to eighteen week old calli tested positive for berberine synthesis whereas productivity diminished as age of calli advanced beyond eighteen weeks. At 13 weeks after callus initiation the calli synthesized maximum berberine in this study. Sankar (1998) observed ephedrine synthesis in 4 to 5 week old calli of *Sida* spp., which diminished thereafter.

In this context, regulation of secondary product synthesis, subjected to cell growth kinetics of *in vitro* system becomes relevant. While certain products like sinapyl alcohol in *Nicotiana tabacum* are synthesized only by actively growing cultures (Anderson *et al.,* 1981) certain others like triphlolides in *Tripterygium wilfordii* are synthesized only as growth slows down (Misawa *et al.*, 1985). The former are termed as growth related products and latter, non-growth related ones. Berberine obviously being in the former category, does not express in aged *in vitro* systems, the cells having lost their biosynthetic potential.

5.10.7 Regulation of photoperiod

Synthesis of target alkaloid was not observed in dark on incubating the calli in medium P (MS supplied with NAA and BA or Kin each at 2 mg l^{-1}). Importance of light for the optimal expression of certain metabolic pathways in cultured cells has been demonstrated in many secondary products like flavanoids (Hahlbrock and Griseback, 1979) and cardenolides (Ohlsson *et al.*, 1983). Interestingly, though plant cell cultures are fully photosynthetically competent and rarely exposed to phtotosynthetically significant light intensities, the behaviour of cultures can be influenced by photoperiodicity (Seibert and Kadkade, 1980). In the present study also light favourably influenced product synthesis in *in vitro* cultures.

Conversely, instances where light has an inhibitory influence on product synthesis are reported as in the formation of ephedrine in *Sida* spp. (Sankar, 1998).

5.11 Employing special techniques to upgrade the content of berberine

5.11.1 Creating conditions of stress

5.11.1.1 Addition of osmo regulants

The osmo regulant polyethylene glycol at 2 and 3 per cent elicited a positive response in leaf and stem calli of *Tinospora cordifolia* on biosynthesis of berberine (Table 25). Polyethylene glycol at 2 per cent elicited a positive response in leaf calli of *Sida cordifolia* on biosynthesis of ephedrine (Sankar, 1998). Change in osmotic pressure modifies the properties of cell membrane and hence influences the uptake and exchange of chemicals by the cell wall (Rudge and Morrison, 1986). In the present study, osmotic pressure is increased by adding mannitol to the culture medium. Mannitol at varying levels responded positively for berberine synthesis in the present study, an observation also reported in *Panax notoginseng* for saponin production by Zhang *et al.* (1995) and in *Nicotiana* by Gangopadhyay *et al.* (1997).

However, Sindhu (1999) reported that none of the osmoregulants, added to the basal growth medium such as polyethylene glycol, mannitol and sorbitol at 1.5 to 3 per cent could sustain callus growth and berberine synthesis in *Coscinium fenestratum*.

5.11.1.2 Increasing concentration of agar

The acidic polysaccharide component of agar reportedly has beneficial influence on synthesis of certain products (Cejka, 1985). Berberine biosynthesis was favourably influenced upon, with increase in levels of agar from 0.75 to 1.0 per cent (Table 25).

5.11.2 Elicitation

Synthesis of berberine in leaf and stem calli of *T. cordifolia* was positively influenced on incorporating autoclaved mycelia of *Pythium aphanidermatum* at levels of 0.5 g l^{-1}, 1.0 g l^{-1} and 1.5 g l^{-1} (Table 26). Among the various levels tried, fungal mycelia at 0.5 g l^{-1} exerted maximum berberine synthesis by leaf calli cultures (19.262 µg) of madurai ecotype and stem calli cultures (23.176 µg) of Vellanikkara ecotype respectively. These findings correlate well with many published reports as detailed herewith. Anthraquinone levels in *Cinchona ledgeriana* suspensions doubled following treatment with autoclaved mycelia of *Phytophthora cinnamomi* (Wijnsma *et al.*, 1985). Vazquez-Flota *et al.* (1994) observed increase in sanguinarene levels in *in vitro* cultures of *Papaver somniferum* following addition of *Botrytis* preparations.

The elicitor, *Pythium aphanidermatum* used to advantage in this study was employed with success in *Catharanthus roseus* cell suspensions by Moreno *et al.* (1996). They attributed the success to changes in enzyme activities which channeled intermediates through the desired metabolic pathway. In the present study also enhanced enzyme activity responsible for channeling intermediaries through secondary metabolic pathway might have contributed to the positive effect of elicitor. Another possible explanation on the positive effect of autoclaved mycelia of *Pythium* is the presence of polyglacturonic acid, as one of the mycelial cell wall components which hydrolyse the cell walls of *in vitro* cultures, releasing cell wall bound enzymes for secondary pathway as suggested by Rijhwani and

Shanks (1996) in *Catharanthus roseus*. Chitosan, another component of fungal cell wall also contribute to increased levels of secondary products. An example cited is the biosynthesis of carvone in dill (Doernenburg *et al.*, 1990).

5.11.3 Immobilization studies

Standardization studies on levels of the gel forming agent sodium alginate and gel forming chemical calcium chloride identified a 11.0 per cent solution of former and $CaCl_2$ at 75 mM as the ideal complexing combination (Table 27). During immobilization, cells within a gel bead are closely subjected to physical and chemical environment different from those in the periphery and such heterogeneity results in increased molecular conversation between constituent cells resulting in better expression of secondary metabolite pathways (Lindsey and Yeoman, 1983).

The immobilized cell cultures synthesized increased level of berberine (15.068 µg and 19.128 µg per gram of calli) in the present study (Table 28). This is in agreement with the observation made by Subramani *et al.* (1989) wherein immobilized cells of *Solanum xanthocarpum* synthesized 47.7 µg of solasodine per 100 ml medium. Similarly, O'Dowd *et al.* (1993) and Sankar (1998) observed absence of ephedrine synthesis in immobilized cell cultures of *Ephedra gerardiana* and *Sida* spp. respectively.

5.12.1 Establishing suspension cultures

In the present investigation, cell growth as indicated by serial cell counts reached the maximum at 16 days after initiation of suspensions (Fig. 5a

and 5b) and declined thereafter steadily fixing subculture interval at 16 days. This is in conformity to the theory that in suspensions, cells undergo lag, exponential, stationary and declining phases and subculturing is done at the end of exponential phase since cell growth declines thereafter (Gamborg and Shyluk, 1981). Sankar (1998) standardised the subculturing interval for *Sida* spp. as 17 days for ephedrine production. In *Solanum xanthocarpum* suspension cultures, the cells attained maximum fresh weight in 12 days (Subramani, 1989). Leaf and stem derived suspensions showed marked difference in cell counts, in both Vellanikkara and Madurai ecotypes. Madurai ecotype registered maximum cell count (3650 cells/10 µl) whereas Vellanikkara ecotype registered maximum packed cell volume (0.92%). Genetic differences in the efficiency to derive suspensions from static cultures has been reported by Sankar (1998) who observed that among the various species, *Sida cordifolia* resulted in maximum cell count in cell suspensions.

5.12.2 Quantification of berberine from suspension cultures

Berberine synthesis in shake cultures did not exceed that in static cultures of *T. cordifolia*, in the present study (Table 30). O'Dowd *et al.* (1993) and Sankar (1998) observed ephedrine only in traces in cell suspensions of *Ephedra* spp. and *Sida* spp. respectively. However, Sindhu (1999) observed highest berberine recovery from *Coscinium fenestratum in vitro* in half strength MS liquid medium. Among the explants tried leaf cultures of Madurai ecotype registered maximum berberine whereas in Vellanikkara ecotype, stem cultures registered the highest amount of alkaloid.

5.13 Estimation of berberine in *ex vitro* sample extracts

Among the leaf and stem extracts, berberine was noticed only in stem extracts of the plant. Chopra *et al.* (1956) reported that, *Tinospora* is one of the indigenous sources of berberine containing small amounts of alkaloid. From *Tinospora malabarica*, Bowen and Motawe (1985) isolated three alkaloids from the stem methanol extract by flash column chromatography and were further purified by TLC. Chatterjee and Pakrashi (1991) reported the presence of the alkaloid berberine, in the stem of *T. cordifolia.*

Expression of secondary products in plants is dependent on the stage of maturity. Mizrahi (1988) emphasized the necessity of harvesting medicinal crop species at optimum harvest stages, in the context of synthesis of secondary compounds at certain stages of maturity at specialized cells. In the present study, mature stem extracts resulted in higher amounts of berberine (0.262 µg and 0.284 µg) than immature stem extracts (0.141 µg and 0.182 µg in Vellanikkara and Madurai ecotypes respectively). Sindhu (1999) obtained maximum berberine from tender leaf (90.321 µg/25 g leaves) as compared to stem (0.250 µg/25 g stem) in *Coscinium fenestratum.* Similarly, Sankar (1998) registered maximum content of ephedrine from leaf (0.0089%) and stem (0.0080%) extracts, harvested at 7 months after planting of *Sida cordifolia.*

5.14 Comparative yield of berberine from *in vitro* and *ex vitro* sources

In vitro derived calli and suspensions yielded higher amounts of berberine than *ex vitro* samples. Highest berberine yield was obtained when the

fungal elicitor, *P. aphanidermatum* was added at 0.5 g l^{-1} to the production medium. The recovery was 23.176 µg and 22.987 µg per gram of stem derived calli of Vellanikkara and Madurai ecotypes respectively.

In *ex vitro* samples, mature stem extracts have comparatively higher amount of berberine (0.162 µg and 0.184 µg per gram of stem in Vellanikkara and Madurai ecotypes respectively) than the immature stem and leaf extracts. This is in agreement with the observations made by Sankar (1998) and Sindhu (1999) wherein they observed higher quantity of alkaloid production by *in vitro* cultures of *Sida* spp. for ephedrine and *Coscinium fenestratum* for berberine respectively.

SUMMARY

The present investigation entitled "Utilization of *in vitro* cultures of *Tinospora cordifolia* Miers. (chittamrithu) for berberine" was undertaken at the Plant Tissue Culture and Biochemistry Laboratories, College of Horticulture, Vellanikkara, Thrissur, during the period from 1999 to 2001. The study was aimed to standardise the *in vitro* techniques for initiation and proliferation of static and suspension cultures of *Tinospora cordifolia*, to screen *in vitro* cultures for synthesis of berberine and quantify it and to enhance the product synthesis in *in vitro* cultures. The results of the investigation are summarised below.

Leaf, petiole and stem segments of mature plants of the experimental ecotypes were used as explants. Surface sterilization with mercuric chloride at 0.1 per cent for 8 min. was most effective in reducing microbial contamination in all the explant registering 73.74 per cent survival.

Better establishment of cultures and the least rate of contamination was obtained during the period from June to August.

MS medium at full strength was standardised as the best basal culture medium for initiating callus cultures in *Tinospora cordifolia* where 73.15 per cent leaf cultures, 75.39 per cent petiole cultures and 83.56 per cent stem cultures callused.

MS medium enriched with NAA at 4.0 mg l^{-1} recorded highest percentage of callus initiating cultures registering callusing in 91.43 per cent leaf cultures and 91.01 per cent stem cultures. The same auxin at 2.0 mg l^{-1} registered maximum callusing in petiole cultures (94.39 per cent).

Stem and petiole explants registered maximum callus index values of 228.145 and 214.125 respectively whereas leaf explants were superior for early induction of callus (11.11 days) under the influence of various auxins.

Both Vellanikkara and Madurai ecotypes registered values on par with each other for mean callus index in all the cultures.

On supplementing cytokinins to auxin enriched media, most favourable and consistent response was obtained with kinetin at 2 mg l^{-1}. This cytokinin when supplemented to media containing IAA 2 mg l^{-1} initiated calli in 96.06 per cent petiole cultures and 98.37 per cent stem cultures.

Early induction of callus in leaf (9.89 days) and petiole (10.83 days) cultures was observed on incubating them in medium containing BA 4 mg l^{-1} or kinetin 3 mg l^{-1} supplemented with NAA 2 mg l^{-1}.

The cytokinins employed had almost no favourable influence on the response of 2,4-D to induce calli.

Under the influence of auxin and cytokinin combinations, leaf and petiole callus cultures of Madurai eecotype registered maximum callus growth score and mean callus index values. Both the ecotypes responded equally for percentage of culture initiation.

Media additives, phloroglucinol at 100 mg and 125 mg l^{-1} and casein hydrolysate at 100, 200 and 300 mg l^{-1} registered favourable influence on callusing.

Drastic inhibition of callusing resulted when activated charcoal was supplemented to culture medium at 0.25 per cent and 0.50 per cent levels.

Incubating the cultures under illumination at 26 ± 1°C was superior to incubation in dark.

Cultures maintained in MS medium at half strength supplemented with IAA at 2 mg l^{-1} initiated roots in 58.37 per cent leaf derived calli and 53.44 per cent stem derived calli. MS medium at half strength with NAA or IBA, each at 2 mg l^{-1}, also registered root initiation in leaf and stem derived calli.

Leaf derived calli registered higher percentage of root regeneration than stem derived calli. Callus cultures derived from Madurai ecotype performed better with respect to root regeneration as compared to Vellanikkara ecotype.

None of the treatments resulted in shoot regeneration from callus cultures.

MS medium supplemented with NAA 1 mg l^{-1} and kinetin 4 mg l^{-1} to which 3.0 per cent sucrose and 1.5 per cent lactose was incorporated was successful in initiating embryoids in 41.17 per cent and 37.50 per cent leaf cultures of Vellanikkara and Madurai ecotypes respectively.

Callus cultures maintained in MS medium with cytokinins, alone or in combination with auxins responded positively for the presence of alkaloid.

Basal media containing NAA in combination with BA, or Kin, each at 2 mg l^{-1} was identified as production media for *in vitro* synthesis of berberine from *Tinospora cordifolia* resulted 7.554 µg and 7.355 µg berberine per gram of callus respectively. *In vitro* cultures of both the experimental ecotypes failed to synthesize the alkaloid under the influence of auxins alone.

Increasing levels of sucrose in basal medium to 4.0 and 5.0 per cent did not attribute a favourable influence on the expression of berberine whereas sucrose (3%) along with lactose (1.5%) registered 17.318 µg berberine per gram of the test calli, substitution of sucrose with lactose at 3% resulted in the synthesis of 10.982 µg berberine per gram of test calli.

Reducing the phosphorus level in basal media to half strength produced encouraging response registering an increased level of berberine synthesis in *in vitro* cultures (18.126 µg and 19.624 µg per gram of stem derived calli of Vellanikkara and Madurai ecotypes respectively).

Favourable influence of the precursor phenyl alanine on the expression of alkaloid in leaf and stem calli was evident at levels of 100.0 mg l^{-1}, 150.0 mg l^{-1} and 200.0 mg l^{-1}.

Experimental calli at the stage of differentiation into organoids synthesised less amount of berberine(3.024 µg and 3.856 µg per gram of differentiated calli of Vellanikkara and Madurai ecotypes respectively).

Response of experimental calli at varying stages of growth over a period ranging from 2 week to eight months with respect to synthesis of berberine revealed that 3 week to 4½ month old calli were suitable for production of berberine.

The osmoregulants, poly ethylene glycol at 2.0 and 3.0 per cent and mannitol at 1.5 per cent registered positive influence on berberine production.

Incorporation of autoclaved mycelia of *Pythium apanidermatum* at levels of 0.5, 1.0 and 1.5 mg l^{-1} resulted in maximum synthesis of berberine by leaf and stem cultures of *Tinospora*.

Visual assessment of immobilized beads of experimental calli revealed the suitability of employing 11.0 per cent solution of sodium alginate and 75 mM calcium chloride solution for entrapping experimental calli. Immobilised calli of leaf and stem explants produced 15.068 µg and 19.128 µg of berberine per gram of calli.

Based on the cell count of liquid suspensions, sub culturing interval was fixed at 16 days.

As compared to static cultures, suspensions synthesized lesser amount of berberine in both the ecotypes (6.412 µg and 6.216 µg per gram of stem derived calli of Vellanikkara and Madurai ecotypes respectively).

Berberine was detected only in stem extracts of *ex vitro* samples of both the ecotypes. When quantified, the content of the target alkaloid in *in vitro* systems was higher as compared to that from *in vivo* stem samples.

The highest berberine yield (23.176 µg/g of callus) was obtained from stem cultures maintained in production media supplemented with autoclaved mycelia of *Pythium aphanidermatum* at 0.5 g l^{-1}.

Madurai ecotype performed better with respect to berberine synthesis with a mean value of 17.565 µg of berberine per gram of callus whereas Vellanikkara ecotype synthesized 16.051 µg of berberine per gram of callus under various treatments.

References

REFERENCES

Agarwal, S., Kumar, A., Gupta, M.M., Verma, R.K., Singh, D.V. and Kumar, S. 2001. Production of bilobalide in cultures of clone GBC^{-1} of *Ginkgo biloba*. *J. med. arom. Pl. Sci.* **22**(4A) & **23**(1A): 194-196

Adu-Ampomah, Y., Novak, F.J., Afza, R. and Pereapallos, M. 1988. Initiation and growth of somatic embryos in cocoa (*Theobroma cacao* L.). *Café, Cacao, The* **32**(3): 187-200

Akram, M., Afridi, K. and Hameed, S. 1994. Direct and via callus root regeneration in *Atropa accuminata* Royle. *Hamdard Medicus* **37**(1): 113-116

Akram, E. and Yurekhi, A.K. 1995. Effect of different nutrient media and explant sources on callus induction of *Catharanthus roseus* L.G. Don Plants. *Turkish J. Bot.* **19**(6): 569-572

Ambasta, S.P. 1986. *The Useful Plants of India*. CSIR, New Delhi, 639p.

Ammirato, P.V. 1983. Embryogenesis. *Handbook of Plant Cell Cultures* Vol.I. *Techniques for Propagation and Breeding* (eds. Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y.), Macmillan, New York, pp.82-123

Anderson, R.A., Kemp, T.R. and Vaughn, T.H. 1981. Coniferyl alcohol, sinapyl alcohol and scopoletin in tobacco callus tissue during growth of a subculture. *Physiol. Plant* **53**: 89

Anis, K.V., Kuttan, G. and Kurran, R. 2000. Antitumour and anticarcinogenic activity of isoquinoline alkaloids berberine and coralyine. *Abstracts of Symposia on Cancer Biology*, held at Amala Cancer Hospital and Research Centre, Thrissur, Kerala, India, 5-6 January, p.123

Anjalikumar, A. 1992. Somatic embryogenesis and high frequency plantlet regeneration in callus cultures of *Thevetia purpurea*. *Pl. Cell Tiss. Org. Cult.* **31**: 47-50

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

Anu, K.I. 1993. Standardisation of *in vitro* propagation technique in *Gymnema sylvestre* Br. M.Sc.(Hort.) thesis, Kerala Agricultural University, Thrissur, Kerala, India, pp.54-78

Asaka, I., Li, I., Hirotani, M., Asada, Y., Yoshikawa, T. and Furaya, Y. 1994. Mass production of ginseng (*Panax ginseng*) embryoids on media containing high concentrations of sugar. *Planta Med.* **60**(2): 146-148

Ayabe, S., Ida, K. and Furaya, T. 1986. Induction of stress metabolites in immobilized *Glycyrrhiza echnata* cultured cells – echination production. *Pl. Cell Rep.* **5**(3): 186-189

Balandrin, M.F. and Klocke, J.A. 1988. Medicinal Aromatic and Industrial Materials from Plants. *Biotechnology in Agriculture and Forestry.* Vol.4. *Medicinal and Aromatic Plants.* I (ed. Bajaj, Y.P.S.), Springer Verlag, London, pp.3-30

Banerjee, N.S., Philip, S., Manoj, S., Soniya, E.V., Thomas, G. and Das, M.R. 1998. Genetic biodiversity and *in vitro* regeneration in Long pepper (*Piper longum* L.) – a case study. *Abstracts of Int. conf. on conservation of Tropical species, communities and Ecosystems.* Thiruvananthapuram, Kerala, India, 11-13 December, p.111

Baquar, S.R. and Tasnif, M. 1984. *Medicinal Plants of Southern West Pakistan.* Periodical Expert book agency, New Delhi, p.2

Basher, K.K.Z., Koshar, M., Telezhametskaya, M.V. and Khamidav, I. 1996. Alkaloids of *Berberis cartagena* – chemistry of natural compounds. *Pl. Cell Rep.* **15**(1): 88

Bhalsing, S.R. and Maheswari, V.L. 1998. Plant tissue culture – a potential source of medicinal compounds. *J. Sci. Ind. Res.* **57**: 703-708

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

Bhattacharya, P., Dey, S., Das, N. and Bhattacharya, C.B. 1990. Rapid mass propagation of *Chrysanthemum morifolium* by callus derived from stem and leaf explants. *Pl. Cell Rep.* **9**:439-442

Bhojwani, S.S. and Razdan, M.K. 1983. *Plant Tissue Culture. Theory and Practice.* Elsevier Publications, Amsterdam, The Netherlands, 312p.

Bowen, I.H. and Motawe, H.M. 1985. Isolation and identification of Kokkusaginine from *Tinospora malabarica. Planta Med.* **51**: 529

Brown, G.D. 1994. Secondary metabolism in tissue cultures of *Artemisia annua.* *J. nat. Prod.* **57**: 975

Carrier, J.D., Consentino, G., Neufeld, R., Rho, D., Weber, M. and Archambault, J. 1990. Nutritional and hormonal requirements of *Ginkgo biloba* embryo derived callus and suspension cultures. *Pl. Cell Rep.* **9**: 635-638

Cejka, A. 1985. Preparation of media. *Biotechnology* Vol. 2. *Fundamentals of Biochemical Engineering* (eds. Rehm, H.J., Reed, J. and Brauer, H.), VCH, Weinheim, pp.629-641

Chatterjee, A. and Pakrashi, S.C. 1991. *The Treatise on Indian Medicinal Plants* Vol.I. CSIR, New Delhi, p.136

*Choi, H.J., Dale, M.C., Heinstein, P.F. and Okos, M.R. 1996. Continuous production of taxol using an immobilized plant cell reactor. *Abstract Papers of 211th National Meeting of American Chemical Society*, New Orleans, USA, 25-28 October, p.1

*Choi, Y.E., Yang, D.C., Yoon, E.S. and Choi, K.T. 1998. Plant regeneration via adventitious bud formation from cotyledon explants of *Panax ginseng*. *Pl. Cell Tiss. Org. Cult.* 52 : 177-181

Chopra, R.N., Nayar, S.L. and Chopra, I.C. 1956. *Glossary of Indian Medicinal Plants*. CSIR, New Delhi, p.244

*Ciddi, V., Srinivasan, V. and Shuler, M.L. 1995. Elicitation of *Taxus* sp. cell cultures for production of Taxol. *Biotech. Lett.* **17**(12): 1343-1346

Dattagupta, S. and Datta, P.C. 1984. A search for alkaloids in callus cultures of black *Datura* by changing tissue environment. *Bangladesh J. Sci. Ind. Res.* **19**(1-4): 30-36

*Deus, B. and Zenk, M.H. 1982. Exploitation of plant cells for the production of natural compounds. *Biotech. Bioeng.* **24**: 1965

Devadoss, P., Hsu, A.F. and Chen, P.K. 1984. Morphine alkaloids in tissue cultures of *Papaver somniferum*. *In vitro* **20**(3): 279

Dodds, J.A. and Roberts, L.W. 1982. *Experiments in Plant Tissue Culture*. Cambridge University Press, London, p.28

Doernenburg, H., Werrmann, U. and Knorr, D. 1990. Improvement of secondary metabolite production by precursor and elicitor addition to cell cultures of dill. *Food Biotech.* 4(1): 477

Dougall, D.K. 1980. Nutrition and metabolism. *Plant Tissue Culture as a source of Biochemicals* (ed. Staba, J.E.), CRC Press, Florida, pp.21-57

El-Bahr, M.K., Ghanem, S.A., El-Missery, M.M. and El-Nasr, M.M.S. 1997. Production of tropane alkaloids in tissue cultures of *Hyoscyamus muticus. Fitoterapia* **68**(5): 423-428

Ellis, B.E. 1985. Destabilisation of production and growth characteristics to cloning and rosmarinic acid producing cultures of *Anchusa officinalis. J. Cell Biochem.* **96**: 253

Ellis, D.D., Zelden, E.L., Brodhagen, M. and McCown, B.H. 1996. Taxol production in nodule cultures of *Taxus. J. nat. Prod.* **59**(3): 246-250

Endo, T. and Yamada, Y. 1985. Alkaloid production in cultured roots of three species of *Dublosia* – scopolamine and hyocyamine production. *Phytochem.* **24**(6): 1233-1236

Farnsworth, N.R. 1984. Value of drugs obtained from higher plants. *The Role of Medicinal Plants in Drug Development* (eds. Krogsgaard-Larsen, P., Chstensen, S.B. and Kobod, H.), Munksgaard, Copenhagen, pp.17-30

Fridborg, G. and Eriksson, T. 1975. Effects of activated charcoal on growth and morphogenesis in cell cultures. *Physiol. Plant* **34**: 306-308

Fujita, T. 1988. Shikonin : production by plant (*Lithospermum erythrorhizon*) cell cultures. *Biotechnology in Agriculture and Forestry,* Vol.4. *Medicinal and Aromatic Plants* I (ed. Bajaj, Y.P.S.), Springer-Verlag, Berlin, pp.226-235

* Fukui, H., Nakagawa, K., Tsuda, S., and Tabata, M. 1982. Production of isoquinoline alkaloids by cell suspension cultures of *Coptis japonica. Plant Tissue Culture,* Japanese Association for Plant Tissue Culture, Tokyo, Japan, pp. 313-314

Funk, C., Gugler, K. and Brodelius, P. 1987. Increased secondary product formation in plant cell suspension cultures after treatment with a yeast carbohydrate preparation (elicitor). *Phytochem.* **26**(2): 401-405

Gamborg, O.L. and Shyluk, J.P. 1981. Nutrition, media and characteristics of cell and tissue cultures. *Plant Tissue Culture: Methods and Applications in Agriculture* (ed. Thrope, T.A.), Academic Press, New York, pp.21-24

Gangopadhyay, G., Basu, A.S., Mukherjee, B.R. and Gupta, S. 1997. Effects of salt and osmotic shocks on unadapted and adapted callus lines of tobacco. *Pl. Cell Tiss. Org. Cult.* **49**: 45-52

Garnier; F., Depierreux, C., Petit-Paly, G., Hamdi, S., Chenieux, J.C. and Rideau, M. 1996. Induction of the accumulation of tryptamine and phenols by endogenous elicitors in cell suspension cultures of periwinkle. *J. Pl. Physiol.* **148**(6): 701-706

Gholba, D.N. 2000. *In vitro* callus induction in Gurmar (*Gymnema sylvestre* R.Br.) for secondary metabolite synthesis. M.Sc. (Hort.) thesis, Kerala Agricultural University, Thrissur, India, pp.148-151

Gill, R. and Saxena, K.P. 1993. Somatic embryogenesis in *N. tabacum* L. induction by thidiazuron of direct embryo differentiation from cultured leaf discs. *Pl. Cell Rep.* **12**: 154-159

Giri, A., Ahuja, P.S. and Ajayakumar, P.V. 1993. Somatic embryogenesis and plant regeneration from callus cultures of *Aconitum heterophyllum* Wall. *Pl. Cell Tiss. Org. Cult.* **32**(2): 213-218

Gokul, H., Sowmya, S. and Tejavathi, D.H. 1998. Effect of stress on alkaloid production in the cultures of *Cissampelos pareira* L. *Abstracts of National Seminar on Plant Biotechnology for sustainable Hill Agriculture*, Defence Agricultural Research Laboratory, U.P., May 6-8th, p.10

Gokul, H. and Tejawati, D.H. 1997. *In vitro* culture and production of alkaloids in *Cissampelos pareira* L. *Souvenir and Abstracts of National Symposium on Emerging Trends in Plant Tissue Culture and Molecular Biology,* Hyderabad, 29-31 January, p.65

Gontier, E., Sangwan, B.S. and Barbolin, L.N. 1994. Effect of calcium alginate immobilization on growth and tropane alkaloids of a stable suspension cell line of *Datura innoxia* Mill. *Pl. Cell Rep.* **13**(9): 533-536

Gupta, P.K. 1995. *Elements of Biotechnology.* Rastogi and company, Meerut, p.267

Hahlbrock, K. and Griseback, J. 1979. Enzymic controls in the biosynthesis of lignin and flavanoids. *A. Rev. Pl. Physiol.* **30**: 105-130

Hara, M., Morimoto, T. and Fujita, Y. 1987. Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhzom. Pl. Cell Rep.* **6**: 8-11

Hara, M., Kobayashi, Y., Fukui, H. and Tabata, M. 1991. Enhancement of berberine production by spermidine in *Thalictrum minus* cell suspension cultures. *Pl. Cell Rep.* **10**(10): 494-497

Hara, Y., Laugel, T., Morimoto, T. and Yamada, Y. 1994. Effect of gibberellic acid on berberine and tyrosine accumulation in *Coptis japonica. Phytochem.* **36**(3):643-646

Harbone, J.B. 1973. *Phytochemical Methods.* Chapman and Hill Ltd. London, 271p.

* Hashimoto, T., Sato, F., Mino, M. and Yamada, Y. 1982. Production of tropane alkaloids from cultured solanaceae cells. *Plant Tissue Culture*, Japanese Association for Plant Tissue Culture, Tokyo, Japan, pp. 305-306.

Hayashi, H., Fukui, H. and Tabata, M. 1988. Examination of triterpenoids produced by callus and cell suspension cultures of *Glycirrhiza glabra. Pl. Cell Rep.* 7: 508-511

Hernandez, G.G. and Vargas, L.M. 1997. Effect of acetylsalicylic acid on secondary metabolism of *Catharanthus roseus* tumor suspension cultures. *Pl. Cell Rep.* **16**: 287-290

Hilton, M.G. and Rhodes, M.J.C. 1993. Factors affecting the growth and hyoscyamine production during batch cultures of transformed roots of *Datura stramonium. Planta Med.* **59**(4): 340-344

Hunter, C.S. 1979. *In vitro* culture of *Cinchona ledgeriana* L. *J. hort. Sci.* **54**(2): 111-114

Hwang, W.W., Kim, W.J. and Sagawa, Y. 1997. Induction of somatic embryogenesis in *Azadirachta indica. Pl. Cell Tiss. Org. Cult.* **50**(2): 91-95

Ilahi, I. and Ghauri, E.G. 1994. Regeneration in cultures of *Papaver bracteatum* as influenced by growth hormones and temperatures. *Pl. Cell Tiss. Org. Cult.* **38**(1): 81-83

Jaggi, K.R. and Singh, J. 2001. Solasodine production in cultures of *Solanum platanifolium. J. med. arom. Pl. Sci.* **22**(4A) & **23**(1A): 197-200

Jain, S.K. 1968. *Medicinal Plants*. National Book Trust, India, 201p.

Jaiswal, V.S. and Narayan, P. 1985. Plant regeneration from hypocotyls callus of *Solanum torrum* Swartz. *J. Pl. Physiol.* **119**: 381-383

Jasrai, Y.T., Yadava, N. and Mehta, A.R. 1993. Somatic embryogenesis from leaf induced cell cultures of *Plantago ovata* fors K. *J. Herbs Spices med. Pl.* 1(4): 11-16

Jeon, M.H., Sung, S.H., Hah, H. and Kim, Y.C. 1995. Ginkgolide-B production in cultured cells from *Ginkgo biloba* L. leaves. *Pl. Cell Rep.* **14**(8): 501-504

John, S.A. 1996. Standardisation of *in vitro* techniques for rapid multiplication of *Holostemma annulare* K. Schum. M.Sc.(Hort.) thesis, Kerala Agricultural University, Thrissur, India, pp.42-48

Joseph, M. 1997. Indirect organogenesis and embryogenesis in *Kaempferia galanga* L. M.Sc.(Hort.) thesis, Kerala Agricultural University, Thrissur, India, pp.64-67

Kalpana, H.S., Prakash, T.G., Prasad, B.N. and Suresh Sondur, S. 1996. Colchicine production from *in vitro* callus cultures of *Gloriosa suporba. Souvenir and Abstracts of National Symposium on Horticultural Biotechnology*, IIHR, Bangalore, 28-30 October, p.106

Kannan, V.R. and Jasrai, Y.T. 1998. Micropropagation of medicinal plant – *Vitex negundo. J. med. arom. Pl. Sci.* **20**: 693-696

Keshavachandran, R., Manual, J. and Savitha, A.R. 1997. Regeneration of plantlets through organogenesis in *Tylophora indica* (Burm. F.) Merill. Abstracts of *National Symposium on Emerging trends in Plant Tissue Culture and Molecular Biology,* Hyderabad, 29-31 January, p.37

*Kim, D.I., Pedersen, H. and Chin, C.K. 1988. Effect of light on berberine production in cell suspension cultures of *Thalictrum rugosum. Biotech. Lett.* **10**(1): 709-712

*Kim, D.I., Pedersen, H. and Chin, C.K. 1991a. Cultivation of bioreactor stimulatory effect of Co_2 and ethylene in alkaloids production. *Biotech. Bioeng.* **38**(4): 331-339

*Kim, D.I., Pedersen, H. and Chin, C.K. 1991b. Stimulation of berberine production in *Thalictrum rugosum* suspension cultures in response to addition of cupric sulphate. *Biotech. Lett.* **13**(3): 213-216

Kreis, W. and Reinhard, E. 1986. Highly efficient 12-beta hydroxylation of digitoxin in digitalis lanata cell suspensions using a two staged culture method - deacetyl lanatoside production. *Planta Med.* **52**(4): 418-419

Kuberski, C., Scherbner, H., Stepup, C., Diettrich, B. and Luckner, M. 1984. Embryogenesis and cardemolide formation in tissue cultures of *Digitalis lanata. Phytochem.* **23**: 1407-1412

Kulkarni, V.M. and Rao, P.S. 1999. *In vitro* propagation of sweet flag (*Acorus calamus,* Araceae). *J. med. arom. Pl. Sci.* **21**: 325-330

Kumari, N. and Saradhi, P.P. 1992. Regeneration of plants from callus cultures of *Origanum vulgare* L. *Pl. Cell Rep.* **11**: 476-479

Kunitomo, J., Satoh, M. and Sheng, T. 1983. Structure and synthesis of menisporphine, a new type of isoquinoline alkaloid of *Menispermum dauricum* DC. *Tetrahedron* **39**(20): 3261-3265

Lem, H., Lee, H. and Eriksson, T. 1997. Regeneration of *Panax notoginseng* by organogenesis and nuclear DNA analysis of regenerants. *Pl. Cell Tiss. Org. Cult.* **49**: 179-187

Lindsey, K. and Yeomann, M.M. 1983. The relationship between growth rate, differentiation and alkaloid accumulation in cell culture. *J. exp. Bot.* **34**(145): 1055-1065

*Lindsey, K. and Yeomann, M.M. 1984. The synthetic potential of immobilized cells of *Capsicum frutescens* Mill cv. *annuum. Planta* **162**: 495

*Linsmaier, E.F. and Skoog, F. 1965. Organic growth factor requirements of tobacco tissue culture. *Physiol. Pl.* **18**: 100-127

Majerus, F. and Parcilleux, A. 1988. Alkaloid accumulation in calcium alginate entrapped cells in *catharanthus roseus* using a limiting growth medium, enhanced tryptamine, ajmalicine and serpentine production. *Pl. cell Rep.* **5**(4): 302-305

Maurya, R., Wazir, V., Tyagi, A. and Kapil, R.S. 1995. Clerodane diterpenoids from *Tinospora cordifolia. Phytochem.* **38**(3): 659-661

Mercier, H., Vieira, C.J. and Figneirado-Ribeiro, R.C.L. 1992. Tissue culture and Plant Propagation of *Gompherena officinalis* – a Brazilian Medicinal Plant. *Pl. Cell Tiss. Org. Cult.* **28**: 249

Merkli, A., Christen, P. and Kapetanidis, I. 1997. Production of diosgenin by hairy root cultures of *Trigonella foenum-graecum* L. *Pl. Cell Rep.* **16**: 632-636

Mhatre, M.M., Bapat, V.A. and Rao, P.S. 1984. Plant regeneration in protoplast culture of *Tylophore indica. J. Pl. Physiol.* **115**: 231-235

Miketova, P., Schram, K.H., Whitney, J.L., Voicic, S. and Volk, K.J. 1998. Mass spectrometry of selected components of Biological interest in green tea extracts. *J. nat. Prod.* **61**: 461-467

Misawa, M., Hayashi, M. and Takayama, S. 1985. Accumulation of antineoplastic agents by plant tissue cultures. *Primary and Secondary Metabolism of Plant Cell Cultures* (eds. Neumann, K.H., Basz, W.J. and Reinhard, E.), Springer-Verlag, Berlin, pp.235-242

Mizrahi, A. 1988. *Biotechnology in Agriculture.* Alan R. Liss, INC, New York, 261p.

*Moreno, P.R.H., Poulsen, C., Van-der-Heijden, R. and Verpoorte, R. 1996. Effects of elicitation on different metabolic pathways in *Catharanthus roseus* (L.) G. Don cell suspension cultures. *Enzyme. microb. Technol.* **18**(2): 99-107

*Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* **15**: 473-497

Nair, A.J., Sudhakaran, P.R., Rao, J.M. and Ramakrishna, S.V. 1992. Berberine synthesis by callus and cell suspensions of *Coscinium fenestratum. Pl. Cell Tiss. Org. Cult.* **29**: 7-10

Nakagawa, K., Konagal, A. and Tabata, M. 1984. Release and crystallisation of berberine in liquid medium of *Thalictrum minus. Pl. Cell Rep.* 3(6): 254-257

*Nakazima, H., Sonomoto, K., Sato, F. and Fukui, S. 1985. Entrapment of *Lavendula vera* cells and production of pigments of entrapped cells. *J. Biotech.* **2**: 107-117

Narayanaswamy, S. 1997. Regeneration of plants from tissue cultures. *Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture* (eds. Reinert, J. and Bajaj, Y.P.S.), Springer-Verlag, Berlin, pp.179-204

Nef-Campa, C., Trouslot, M.P., Trouslot, P. and Chrestin, H. 1994. Long term effect of *Pythium* elicitor treatment on the growth and alkaloid production of *Catharanthus roseus* Cell suspensions. *Planta Med.* **60**(2): 149-152

Nigra, H.M., Caso, O.H. and Giulietti, A.M. 1987. Production of solasodine by calli from different parts of *Solanum eleagnifolium* cav. Plants. *Pl. Cell Rep.* **6**: 135-137

O'Dowd, N.A., Mc Cauley, P.G., Richardson, D.H.S. and Wilson, G. 1993. Callus production, suspension culture and *in vitro* alkaloid yields of *Ephedra. Pl. Cell Tiss. Org. Cult.* **34**: 149-155

Ohlsson, A.B., Bjork, L. and Gatenbeck, S. 1983. Effect of light on cardenolide production of pigments by entrapped cells. *J. Biotech.* **2**: 107-117

Paniego, N.B. and Giulietti, A.M. 1994. *Artemisia annua* L. dedifferentiated and differentiated cultures. *Pl. Cell Tiss. Org. Cult.* **36**(2): 163-168

Paniego, N.B., Rodriguez-Talou, J. and Giulietti, A.M. 1995. Artimisin production by transformed cultures of *Artemisia annua. Pharm. Wld. Sci.* **17**: 6-10

Panse, V.G. and Sukhatme, P.V. 1978. *Statistical Methods for Agricultural Workers*. ICAR, New Delhi, 341p.

Pasqua, G., Monacelli, B., Cuteri, A., Finocchiaro, O., Botta, B., Vitali, A., Monache-G-delle and Delle-Monache, G. 1991. Cell suspension cultures of *Maclura Pornifers* : Optimization of growth and metabolite production. *J. Pl. Physiol.* 139(2): 249-251

Purohit, M., Pande, D., Haseeb Mughal, M. and Sreevastava, P.G. 1997. Regenerating cultures of *Ammi majus*. A potential source of high Xanthotoxin. *Souvenir and Abstracts of National Symposium on Emerging Trends in Plant Tissue Culture and Molecular Biology*, Osmania University, Hyderabad, India, 29-31 January, p.62

Rajkumar, M., Veerasham, C. and Kokate, C.K. 1997. Production of azadirachtin in callus cultures of *Azadirachta indica. Souvenir and Abstracts of National Symposium on Emerging trends in Plant Tissue Culture and Molecular Biology,* Osmania University, Hyderabad, India, 29-31 January, p.66

Ramamurthy, G. and Reddy, K.G. 1997. Optimization of colchicine production in *Gloriosa superba* Linn. cultures. *Souvenir and Abstracts of National Symposium on Emerging Trends in Plant Tissue Culture and Molecular Biology,* Osmania University, Hyderabad, India, 29-31 January, p.67

Ramashree, A.Q., Hariharana, M. and Unnikrishnan, K. 1994. Micropropagation and callus induction of *Aristolochia bracteolata* Lcm-a medicinal plant. *Phytomorph.* 44(3-4): 247-252

Rao, P.K., Rao, S.S. and Sadanandam, M. 1999. Tissue culture studies of *Centella asiatica. Indian J. Pharm. Sci.* 23: 392-393

Rao, K.V. and Narasu, M.L. 1997. Factors affecting *in vitro* production of artemisine. *Souvenir and Abstracts of National Symposium on Emerging Trends in Plant Tissue Culture and Molecular Biology*, Osmania University, Hyderabad, India, 29-31 January, p.66

Ravishankar, G.A., Sarma, K.S., Venketaramanan, V. and Kadyan, A.K. 1988. Effect of nutritional stress on capsaicin production in immobilized cell cultures of *Capsicum annuum. Curr. Sci.* 57(7): 381-382

Raychaudhuri, S.R. 1998. Development of somatic embryos of *Plantago ovata in vitro. Abstracts of National Conference on Recent trends in Spices and Medicinal Plant Research,* Calcutta, India, 26-29 April, p.13

Rhodes, M.J.C., Payne, J. and Robins, R.J. 1986. Cell suspension cultures of *Cinchona ledgeriana* - The effect of a range of auxins and cytokinins on the production of Quinoline alkaloids. *Planta Med.* 52(3): 226

Rijhwani, S.K. and Shanks, J.V. 1996. Effect of fungal wall components on indole alkaloid synthesis by *Catharanthus roseus* hairy root cultures. *Abstract papers of 211th National meeting of American Chemical Society,* New Orleans, USA, 25-28 October, p.59

Robins, R.J., Hanley, A.B., Richard, S.R. and Rhodes, M.J.C. 1987. Uncharacteristic alkaloid synthesis by suspension cultures of *Cinchona pubescens* fed with L-tryptophan. *Pl. Cell Tiss. Org. Cult.* **9**: 49-50

Rudge, K. and Morrison, P. 1986. The effect of osmotic stress on growth and alkaloid accumulation in *Catharanthus roseus. Secondary Metabolism in Plant Cell Cultures* (eds. Morrisson, P., Scragg, A.H. and Fowler, M.W.), Cambridge University Press, London, pp.75-81

Sakamoto, K., Iida, K., Asada, Y. and Furaya, T. 1993. Effects of nutrients on anthocyanin production in cultured cells of *Aralia cordata. Phytochem.* **33**(2): 1357-1360

Saker, M.M., Rady, M.R. and Ghanem, S.A. 1997. Elicitation of tropane alkaloids in suspension cultures of *Hyoscyamus, Datura* and *Atropa* by osmotic stress. *Fitoterapia* **68**(4): 338-342

Sankar, M.A. 1998. *In vivo* and *in vitro* screening of *Sida* spp. for ephedrine content. Ph.D.(Hort.) thesis, Kerala Agricultural University, Thrissur, India, pp.198-206

Sankar, M.A., Nair, S.G. and Augustine, A. 2001. Ephedrine synthesis in *in vitro* cultures of *Sida* species through precursor feeding. *J. med. arom. Pl. Sci.* **22**(4A) & **23**(1A): 248-251

Sasse, F., Knoblock, K.H. and Berlin, J. 1983. Induction of secondary metabolism in cell suspension cultures of *Catharanthus roseus, Nicotiana tabacum* and *Peganum harmala. Pl. Cell Tiss. Org. Cult.* **3**: 343-344

Sateeshkumar, K. and Bhavanandan, K. 1989. Regeneration of plants from leaf callus of *Plumbago rosea* Linn. *Indian J. exp. Biol.* **27**(4): 368-369

Sato, F. and Yamada, Y. 1984. High berberine producing cultures of *Coptis japonica* cells. *Phytochem.* **23**(2): 281-285

Sato, F., Kobayashi, Y., Fukui, H. and Tabata, M. 1990. Specific differences in tolerance to exogenous berberine among plant cell cultures. *Pl. Cell Rep.* **9**: 133-136

Schiel, O., Jarchow-Redecker, K., Piehl, G.W. and Berlin, J. 1984. Increased formation of cinnamoyl putrescence by feed batch fermentation of cell suspension cultures of *Nicotiana tabacum*. *Pl. Cell Rep.* **3**: 18

*Schlatmann, J.C., Moreno, P.R.H., Seller, M., Vinke, J.H., ten-Hoopen, H.J.G., Verpoorte, R. and Heizmen, J.J. 1997. Two stage batch process for the production of ajmalicine by *Catharanthus roseus*, the link between growth and production stage. *Biotech. Bioeng.* **47**(1): 53-59

Scott, J.N. and Ellen, G.S. 1980. Stability of IAA and IBA in nutrient medium to several tissue culture procedures. *HortScience* **25**(7): 800-802

Seibert, M. and Kadkade, P.G. 1980. Environmental Factors: A. Light. *Plant Tissue Culture as Source of Biochemicals* (ed. Staba, E.J.). CRC Press, Boca Raton, pp.123-142

Shanthamma, C., Sreenath, K. and Rao, B.S. 1991. Somatic embryogenesis and secondary metabolite production in *Emilia sonchifolia* (L.) Dc. *In vitro* **27**(3): 149

Shrivastava, N. and Padhya, M.A. 1995. 'Punarnavine' profile in the regenerated roots of *Boerhaavia diffusa* L. from leaf segments. *Curr. Sci.* **68**(6): 653-656

Siddique, A.A. and Zafar, R. 1995. *Tinospora cordifolia* Miers. A review. *Hamdard Medicus* **38**(3): 85-90

Sindhu, M. 1999. *In vitro* callus induction and its exploitation in *Coscinium fenestratum* (Gaertn.) Colebr. M.Sc.(Hort.) thesis, Kerala Agricultural University, Thrissur, India, pp.126-130

Sindhu, M., Kesavachandran, R., Nybe, E.V. and Augustin, A. 2000. Isolation and estimation of berberine from *in vitro* and *ex vitro Coscinium* plant samples. *Proceedings of National Symposium on Medicinal Plant and Industrial Biotechnology,* Cochin University of Science and Technology, Cochin, India, 1-2 December, p.25

Singh, V., Wadhuwani, A.M. and Johri, B.M. 1983. *Dictionary of Economic Plants in India.* Indian Council of Agricultural Research, New Delhi, India, p.231

Sivarajan, V.V. and Balachandran, I. 1994. *Ayurvedic Drugs and their Plant Sources*. Oxford and IBH, New Delhi, India, p.39

Smith, J.I., Smart, N.J., Misawa, M., Kurz, W.G.W., Tallevi, S.G. and Dicosmo, F. 1987. Increased accumulations of indole alkaloids by small cell lines of *Catharanthus roseus* in response to addition of vanadyl sulphate. *Pl. Cell Rep.* **6**: 142-145

Sohni, Y.R. and Bhatt, R.M. 1996. Activity of a crude extract formulation in experimental hepatic liver amoebiasis and in immunomodulation studies. *J. Ethnopharmacol.* **54**(2-3): 119-124

Subramani, J., Bhatt, P.N. and Mehta, A.R. 1989. Alkaloid production by the immobilized cells of *Solanum xanthocarpum. Curr. Sci.* **58**(9): 511-513

Sudha, C.G. and Seeni, S. 1994. *In vitro* multiplication and field establishment of *Adhatoda beddomei* C.B. Clarke, a rare medicinal plant. *Pl. Cell Rep.* **13**: 203-207

Suryanarayanan, M. and Pai, J.S. 1998. Studies in micro propagation of *Coleus forskohlii. J. med. arom. Pl. Sci.* **20**: 379-382

Tabata, M., Mizukami, H., Hiraoka, N. and Keneshima, M. 1974. Pigment formation in callus cultures of *Lithospermum erythrorhizon. Phytochem.* **13**: 927

Tailang, M. and Kharya, M.D. 1998. Effect of some growth hormones and sodium azide on biomass yield and glycyrrhetinic acid content in callus and cell suspension cultures of *Glycyrrhiza glabra. J. med. arom. Pl. Sci.* **20**: 36-41

Toivonen, L., Leakso, S. and Rosenqvisit, H. 1992. The effect of temperature on hairy root cultures of *Catharanthus roseus*. Growth, indole alkaloid accumulation and memberane liquid composition. *Pl. Cell Rep.* **11**(8): 395-399

Ulbrich, B., Wiesner, W. and Arens, H. 1985. Large scale Production of Rosmarinic acid from plant cell culture of *Coleus blumei*. Primary *and secondary metabolism of plant cell cultures* (eds. Neumann, K.H., Barz, W. and Reinhard, E.), Springer Verlag, Berlin, pp.293-303

Upadhyay, N., Makoveyenchuk, A.Y., Nikolaeva, L.A. and Batygina, T.B. 1992. Organogenesis and somatic embryogenesis in leaf callus cultures of *Rauwolfia caffra* Sond. *J. Pl. Physiol.* **140**(2): 218-222

Vander, P. Eijkelboom, C. and Hagendoorn, M.J. 1995. Relation between primary and secondary metabolism in plant cell suspensions of *Morinda citrifolia. Pl. Cell Tiss. Org. Cult.* 43(2): 111-116

Vazquez-Flota, F., Moreno-Valenzuela, O., Miranda-Ham, M.C. and Lyolla-Vargas, V.M. 1994. Catharanthine and ajmalicine synthesis in *Catharanthus roseus* hairy root cultures. *Pl. Cell Tiss. Org. Cult.* **38**(2-3): 273-279

Veeresham, V., Kokate, C.K., Ramesh, B. and Venkateswarlu, V. 1992. Influence of precursor supplementation on bioproduction of capsaicin in static culture of *Capsicum annum. Indian J. Pharm. Sci.* **54**(5): 178-182

Vincent, K.A., Bejoy, M., Hariharan, M. and Mathew, K.M. 1991. Plantlet regeneration from callus cultures of *Kaempferia galanga* L. – a medicinal plant. *J. Pl. Physiol.* **34**(4): 396-400

Vincent, K.A., Hariharan, M. and Mathew, K.M. 1992. Embryogenesis and plantlet formation in tissue culture of *Kaempferia galanga* L. - a medicinal plant. *Phytomorph.* **42**(3,4): 253-256

Wijnsma, R., Van Weerden, I.N. and Srendson, A.B. 1985. Anthraquinones as phytoalexins in cell and tissue cultures of *Cinchona* spp. *Pl. Cell Rep.* 4: 241

Wijnsma, R., Verpoorte, R., Harkes, P.A.A. and Svendsen, A.B. 1986. The influence of initial sucrose and nitrate concentrations on the growth of *Cinchona ledgeriana* cell suspension cultures and the production of alkaloids and anthraquinones. *Pl. Cell Tiss. Org. Cult.* **7**(1): 21-29

Yamada, Y. and Sato, F. 1981. Production of berberine in cultured cells of *Coptis japonica. Phytochem.* **20**(3): 545-547

Yamamoto, H., Suzuki, Y., Suga, H., Fukui, H. and Tabata, M. 1987. Participation of an active transport system in berberine secreting cutlured cells of *Thalictrum minus. Pl. Cell Rep.* **6**: 356-359

Yazaki, K., Fukki, H., Kikuma, M. and Tabata, M. 1987. Regulation of shikonin production by glutamine in *Lithospermum erythrorhizon* cultures. *Pl. Cell Rep.* **6**: 131-134

Zagorska, N., Pyuskyuliev, B. and Ballova, B. 1993. Callus cultures of *Vinca herbaceae* and their secondary production . *In vitro* **29**(3): 165-166

Zenk, M.H., El-Shagi, H. and Schutte, U. 1975. Anthraquinone production by cell suspension cultures of *Morinda citrifolia. Planta Med.* **41**(1): 79-81

*Zhang, Y.H., Zhong, J.J. and Yu, J.T. 1995. Effect of osmotic pressure on cell growth and production of ginseng saponin and polysaccharide in suspension cultures of *Panax notoginseng. Biotech. Lett.* **17**(12): 1347-1350

*Zhang, Y.H., Zhong, J.J. and Yu, J.T. 1996. Effect of nitrogen source on cell growth and production of ginseng saponin and polysaccharide in suspension cultures of *Panax notoginseng*. *Biotech. Prog.* **12**(4): 567-571

*Zhong, J.J. and Zhu, Q.X. 1995. Effect of initial phosphate concentration on cell growth and ginsenoside saponin production by suspended cultures of *Panax notoginseng*. *Appl. Biochem. Biotech.* **55**(3): 241-247

* Originals not seen

Appendix

APPENDIX-I

Composition of plant tissue culture basal media used in the study

Constituents	Media (concentrations in mg l^{-1})		
	MS	SH	Wood Plant medium
NH_4NO_3	1650	-	400
KNO_3	1900	2500	-
$CaCl_2.2H_2O$	440	200	96
$CaCl_2$	-	400	-
$MgSO_4.7H_2O$	370	400	370
KH_2PO_4	170	-	170
$(NH_4)_2SO_4$	-	300	-
$Ca(NO_3)_2.4H_2O$	-	-	556
Na_2SO_4	-	200	-
$Na_2H_2PO_4H_2O$	-	300	-
KCl	-	65	-
Kl	0.83	0.75	-
H_3BO_3	6.2	5	6.2
$MnSO_4.4H_2O$	22.3	10.0	-
$MnSO_4.H_2O$	-	-	22.3
$ZnSO_4.7H_2O$	8.6	1.0	8.6
$NaMoO_4.2H_2O$	0.25	-	-
MoO_3	-	0.001	-
$CuSO_4.5H_2O$	0.025	0.01	0.25
$CoCl.6H_2O$	0.025	-	-
$Fe_2(SO_4)_3$	-	2.5	-
$FeSO_4.7H_2O$	27.8	-	27.8
$Na_2EDTA.2H_2O$	37.3	-	37.3
Organic			
Sucrose	30,000	20,000	20,000
Inositol	100	-	100
Nicotinic acid	0.5	0.05	0.5
Pyridoxine HCl	0.5	0.01	0.5
Thiamine HCl	0.1	0.01	1
Glycine	2	3	2

UTILISATION OF *IN VITRO* CULTURES OF *Tinospora cordifolia* Miers. (CHITTAMRITHU) FOR BERBERINE

By

M. KALIMUTHU

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

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DEPARTMENT OF PLANTATION CROPS AND SPICES
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ABSTRACT

The present investigation on "Utilisation of *in vitro* cultures of *Tinospora cordifolia* Miers. (Chittamrithu) for berberine" was carried out in the Plant Tissue Culture and Biochemistry Laboratories, College of Horticulture, Vellanikkara, Thrissur during the period 1999-2001. The study was undertaken with the objective to standardise the *in vitro* techniques for initiation and proliferation of static and suspension cultures of *T. cordifolia* and to screen the *in vitro* cultures for synthesis of berberine and quantify it. It was also envisaged to enhance the level of product synthesis in *in vitro* cultures.

Leaf, petiole and stem derived callus cultures of Vellanikkara and Madurai ecotypes were established *in vitro*. Surface sterilisation with mercuric chloride ($HgCl_2$) at 0.1 per cent for 8 min was most effective in all the explants. MS medium at full strength supplemented with NAA at 4 mg l^{-1} was observed ideal for initiation and proliferation of calli. Kinetin at 3 mg l^{-1} and BA at 4 mg l^{-1} enhanced the callus inducing property of NAA. Both the ecotypes responded equally for most of the parameters observed, with respect to callusing. The auxin synergist, phloroglucinol at levels of 100.0 mg l^{-1} and 125 mg l^{-1} and casein hyrdolysate at 100, 200 and 300 mg l^{-1} registered favourable influence on callusing. Incubating leaf and stem cultures under illuminated conditions at 26±1°C was significantly superior to incubation in dark.

Successful regeneration of roots from leaf and stem calli of the experimental ecotypes was achieved on MS medium at half strength supplemented

with NAA or IAA each at 2 mg l^{-1}. Calli derived from Madurai ecotype performed better with respect to root regeneration. None of the treatments tried resulted in a positive response with respect to shoot initiation from callus cultures of both the ecotypes. Substituting sucrose with lactose in proportions of 2:1 in MS medium at full strength fortified with NAA at 1 mg l^{-1} and Kin at 4 mg l^{-1} initiated embryoids in both the ecotypes.

MS media at full strength supplemented with NAA and BA or NAA and Kin each at 2 mg l^{-1}, was standardised as the production medium, which recorded maximum berberine synthesis. Butanol-glacial acetic acid-water at 7:1:2 was identified as the appropriate solvent system for detecting the alkaloid with Dragendorff's reagent as the localizing spray.

Substituting sucrose with lactose maintaining a proportion of 2:1 and reducing the phosphorus level in basal medium to half the original strength resulted in increased levels of berberine synthesis. The precursor phenyl alanine at 100, 150 and 200 mg l^{-1} elicited synthesis of berberine. Addition of osmoregulants, polyethylene glycol at 2.0 and 3.0 per cent and mannitol at 1.5 per cent exerted a favourable influence on synthesis of berberine in *Tinospora*.

Incorporation of autoclaved mycelia of *Pythium aphanidermatum* at 0.5, 1.0 and 1.5 g l^{-1} and immobilisation of calli with sodium alginate-calcium chloride complex revealed a positive influence on synthesis of berberine.

Liquid suspensions of Vellanikkara and Madurai ecotypes registered 0.92 and 0.87 per cent of packed cell volume. Based on critical cell density, the

liquid suspension were subcultured at 16 days interval. As compared to static cultures, suspensions synthesized lesser quantity of berberine.

Berberine was detected only in stem extracts of *ex vitro* plants. When compared to *ex vitro* samples, *in vitro* cultures yielded higher quantities of berberine.

The highest berberine yield (23.176 µg/g of callus) was obtained from stem cultures maintained in solid MS media supplemented with NAA 2 mg l^{-1} + BA 2 mg l^{-1} and autoclaved mycelia of *P. aphanidermatum* at 0.5 g l^{-1}.

Madurai ecotype performed better with respect to berberine synthesis with a mean value of 17.565 µg of berberine/gram of callus whereas Vellanikkara ecotype synthesized 16.051 µg/g of callus under positively responding treatments.