IN VITRO CALLUS INDUCTION AND ITS EXPLOITATION IN *Coscinium fenestratum* (Gaertn.) Colebr.

By
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THESIS

Submitted in partial fulfilment of the requirement for the degree of

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DECLARATION

I hereby declare that this thesis entitled **"*In vitro* callus induction and its exploitation in *Coscinium fenestratum* (Gaertn.) Colebr."** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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We, the undersigned members of the Advisory Committee of **Miss.Sindhu, M.,** a candidate for the degree of **Master of Science in Horticulture** with major in Plantation Crops and Spices, agree that the thesis entitled **"*In vitro* callus induction and its exploitation in *Coscinium fenestratum* (Gaertn.) Colebr."** may be submitted by Miss.Sindhu, M., in partial fulfilment of the requirement for the degree.

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Dedicated to dearest

Ma, Appa and brother

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Sindhu, M.

LIST OF ABBREVIATIONS

ABA	- abscissic acid
BA	- benzyl adenine
BAP	- benzyl amino purine
°C	- degree celsius
CCC	- 2 - chloromethyl trimethyl ammonium chloride
cm	- centimeter
2,4-D	- 2,4-dichlorophenoxy acetic acid
2,4,5-T	- 2,4,5-Trichloroethyl phosphonic acid
GA	- gibberellic acid
g	- gram
h	- hour(s)
IAA	- Indole-3-acetic acid
IBA	- Indole-3-butyric acid
2iP	- 2 isopentenyl adenine
KIN	- kinetin, N^6-furfuryl adenine
mg l^{-1}	- milligram(s) per litre
min	- minute(s)
mM	- milli molar
µg	- microgram
µl	- micro litre
µM	- micro molar
MS	- Murashige and Skoog's (1962) medium
½ MS	- Murashige and Skoog's (1962) medium with half the salt concentration
N	- normality
NAA	- naphthalene acetic acid
pH	- hydrogen ion concentration
ppm	- parts per million
psi	- pounds per square inch
rpm	- revolutions per minute
S	- seconds
SH	- Schenk and Hildebrandt medium (1972)
UV	- ultra violet
v/v	- volume in volume
WP	- wettable powder
WPM	- Woody Plant medium (Lloyd and McCown, 1980)
w/v	- weight in volume

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Introduction

INTRODUCTION

Medicinal plants of pharmaceutical significance are on the verge of extinction due to several factors such as their ruthless exploitation, lack of proper conservation of environment, increasing labour costs, economical or technical problems associated with the cultivation of these plants as well as lack of quality planting materials. *Coscinium fenestratum* (Gaertn.) Colebr. is one such endangered plant which has entered the Red Data Book of IUCN (1980). Berberine, an isoquinoline alkaloid having immense medicinal application is usually obtained by extracting the roots and bark of the plants belonging to Ranunculaceae, Menispermaceae and Berberidaceae. *Coscinium fenestratum* (Gaertn.) Colebr, belonging to Menispermaceae is a rich source of berberine. It is a woody climber growing wild in Western Ghats which has become more or less extinct due to over exploitation.

The stem of the plant has long been used in South India and Sri Lanka as a yellow dye and bitter tonic and it has found its way into Europe under the name Tree turmeric. It has often found use as a substitute for *Berberis* spp. and is found useful in debility, fever and certain forms of dyspepsia. It is also said to possess antiseptic properties and hence is used for dressing wounds and ulcers. The stem contains berberine upto 3.5 per cent (Kirtikar and Basu,1975). The tincture and decoction of the stem is an effective antipyretic and antiperiodic. Berberine containing plants (including *Berberis* spp., *Coptis chinensis* and *Hydratis canadiensis*) are used medicinally in all traditional medical systems including Ayurvedic and Chinese dating back to 3000 years. Berberine sulphate is a drug and toxic to *Leishmania tropica* - the organism responsible for oriental sore and has now been tried on a large scale, and proved to be of undoubted value (CSIR, 1975).

The medicinal properties of berberine include : antagonism of the effects of cholera and *Escherichia coli* heat stable enterotoxin, inhibition of intestinal ion secretion, inhibition of smooth muscle contraction, inhibition of ventricular tachyarrhythmias, reduction of inflammation, elevation of platelet count in patients with primary and secondary thrombocytopenia and stimulation of bile secretion and bilirubin discharge. The most common clinical uses of berberine include the treatment of bacterial diarrhoea, intestinal parasites and ocular trachoma infections (Birdsall and Kelly, 1997).

Coscinium fenestratum is a slow grower and hence the wild growth is rapidly removed to obtain the raw material for berberine extraction. The plant as such is highly valued and the entire demand is at present met through collection from wild sources. In *C. fenestratum,* normal propagation is through seeds and the plants take 10 to 12 years for flowering and seed set. Due to indiscriminate collection from wild resources, the plants do not survive upto flowering. No vegetative propagation methods are known to be successful. The availability of planting material is thus a major problem in domestication of this plant.

Now with the enforcement of Intellectual Property Rights and the liberalisation of trade and commerce in the post GATT era, it has become essential that in India we utilise tissue culture techniques for rapid multiplication of this endangered plant and the synthesis of high yield desired compounds *in vitro* which would in turn help to conserve this plant.

The exploration of biosynthetic capabilities of various cell cultures has been systematically investigated in the last three decades by scientists all over the world. Secondary metabolites may be produced in stabler quantity and in shorter period using plant cell cultures than by the plant itself. Industrial processes have been established for production of shikonin and other metabolites that have commercial and pharmaceutical uses. In many cases however, the desired

compounds are not produced, while in others only low quantities could be detected. Enhanced production of target metabolites is essential to lower the production costs.

In this context, a detailed study was undertaken in *Coscinium fenestratum* with the following objectives.

1. To standardise the *in vitro* techniques for callus induction, proliferation and regeneration.
2. To identify and quantify the active principle in *in vitro* cultures.

Review of Literature

REVIEW OF LITERATURE

Higher plants are valuable sources of industrially important natural products which include flavours, fragrances, essential oils, pigments, antimicrobials and drugs. These chemical compounds belong to a metabolic group collectively referred to as secondary metabolites. These substances do not participate in vital metabolic functions of host plant tissues in the same manner as amino acids, nucleic acids or other primary metabolites which are the products of Kreb's cycle. The secondary metabolites produced from primary metabolites includes toxic substances or waste substances which sometimes serve as reserves or just accidental end products eg. alkaloids, steroids, terpenoids, oxygen heterocyclics, glycosides, fats, oils, colouring matter etc. They are produced by plants to ward off predators and to attract pollinators. To some extent secondary substances may also help in combating infectious diseases (Whitaker and Hashimoto, 1986). Many of these metabolites find great application as components in pharmaceutical drugs, pesticides, allelochemicals, plant growth regulators and food and beverages.

Berberine is a yellow coloured alkaloid, used as a drug and produced chiefly from the roots and bark of the South Indian climber *C. fenestratum* and the pharmaceutical industries are exploiting the roots from the natural habitat for processing and export of berberine. Collection of this species without any scientific method of conservation is going on from the hilly tracks of Kerala and Karnataka, where the plant has become more or less extinct because of over exploitation for the production of berberine. Available reports pertaining to these aspects in *in vitro* regeneration of medicinal plants and production of secondary metabolites from berberine yielding species are reviewed here.

2.1 Exploitation of *in vitro* callus for the production of secondary metabolite

The use of tissue culture technique for the biosynthesis of secondary metabolites, offers an avenue to further evaluate and exploit the metabolic potentialities. The cell lines with rates of production of secondary metabolite far in excess of that found in the intact plant have been isolated from *Berberis* for jatrorrhizine (Hinz and Zenk, 1981), *Lithospermum* for shikonin (Fujita *et al.*, 1984), *Coptis* for berberine (Sato and Yamada, 1984) and *Coleus* for rosmarinic acid production (Ulbrich *et al.*, 1985). The cell lines of high biosynthetic potential can be isolated from many cultures but, it is only in a few cases, it has been possible to stabilise these highly productive variants into stable cell lines (Deus-Neumann and Zenk, 1984).

Tabata *et al.* (1975) demonstrated that callus cultures of *Casssia tora* could produce more than 10 times the anthraquinone derivatives (6%) as compared to the crude drug. The production of the red pigment shikonin used both as a dye and a pharmaceutical in cell cultures of *Lithospermum erythrorhizon* on commercial scale is done by Mitsui Petrochemicals (Tabata *et al.*, 1982). The other bioproductions of metabolites of commercial interest are adaptogen-ginsenosides in *Panax ginseng* (Furuya *et al.*, 1984).

2.1.1 Initiation of callus cultures

2.1.1.1 Selection of explants

The age of the tissue may influence whether an explant can be used to initiate a culture or its direct or indirect morphogenetic potential. The youngest and less differentiated tissues are found in plant meristems and the culture of these tissues have been successful in wide range of species (Hughes, 1981). In general, the young tissues have a higher degree of morphogenic competence than older tissues. Rao and Lee (1986) and Lakshmi Sita *et al.* (1986) found that callus could

be induced from young tissues of *Dalbergia latifolia* but not from the mature tissues.

Callus tissues were derived from young flower buds of *Panax notoginseng* on half-strength MS medium supplemented with gibberelic acid and 6-benzyl adenine (Shoyama *et al.*, 1997).

Embryogenic callus tissues were obtained by culturing immature zygotic embryos isolated from immature seeds of *Taxus brevifolia* on Lloyd and Mc Cown medium (MCM) supplemented with 2,4-D and NAA for 4 weeks (Chee, 1996).

In *Lithospermum erythrorhizon,* callus tissues were derived from germinating seeds cultured on semi-solid Murashige and Skoog medium (Oh and Kim, 1994).

Explants taken from the innermost leaves of 2 month old ginger cv. Maran cultured on revised MS medium with 2,4-D induced callus growth (Babu *et al.*, 1992).

Genotype of the explant chosen for propagation is also another important factor in the success of *in vitro* culture. Within a species, some genotypes respond easily, while others fail to respond. Genotype specific effects have been reported in Anthurium (Pierik and Steegman, 1976) and in Geranium (Pillai and Hildebrandt, 1968).

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).

Anjalikumar (1992) reported the use of young leaf discs of *Thevetia purpurea* for production of callus.

The best results of callus growth of *Hyoscyamus muticus* were obtained with petiole explants grown on medium containing 2,4-D and Kinetin (El-Bahr *et al.*, 1997).

2.1.1.2 Surface sterilization

Surface sterilization is done to kill the microbes present on the surfaces of the explant with minimum damage to the plant tissue. Several workers have reported the use of various fungicides in cultures for reducing fungal contamination (Shields *et al.*, 1984). But it was found that most of the systemic fungicides and some antibiotics inhibit growth of the explant cultures. Dodds and Roberts (1985) suggested to avoid the use of antibiotics for sterilization since they or their degradation products metabolised by plant tissue may cause unpredictable results.

The most commonly used surface sterilants are sodium hypochlorite (10%) and mercuric chloride (0.01%-0.1%). In *Nardostachys jatamansi*, Mathur (1993) has reported that 2 per cent sodium hypochlorite treatment for 7 min was effective.

Kantharajah and Dodd (1990) have reported in *Passiflora edulis* that seeds could be surface sterilized for 10 min in 10 per cent hypochlorite solution.

A treatment with 1 per cent Labolene for 6-8 min followed by tap water washing and then with 0.1 pr cent mercuric chloride for 15 min in *Adhathoda beddomei* could produce contamination free cultures (Sudha and Seeni, 1994).

Combination of ethyl alcohol and mercuric chloride was effective in *Duboisia myoporoides* (Kukreja *et al.*, 1986) and *Coscinum fenestratum* (Nair *et al.*, 1992).

For surface sterilization of explants taken from the stock plants maintained in the glasshouse, treatment with 0.1 per cent mercuric chloride for 5 min or 10 min was found to be better in *Holostemma annulare* (John, 1996).

In *Trigonella foenum-graecum* for the production of diosgenin by hairy root culture, seeds were surface sterilised with sodium hypochlorite solution (15% wt/vol available chlorine) supplemented with two drops of Triton X-100 for 6 minutes (Merkli *et al.*, 1997).

2.1.1.3 Culture media

Nature of media was also found to be a very important criterion for morphogenesis. Murashige and Skoog (1962) medium is found to be the most popular one and has been successfully reported in *Aristolochia indica, Tylophora indica, Aegle marmelos, Gomphrena officinalis, Valeriana wallichi, Nardostachys jatamansi* and *Piper longum* tissue culture.

For the induction of callus tissues in *Thalictrum minus* var. *hypoleucum* (Ranunculaceae), Linsmaier and Skoog (LS) agar medium supplemented with 2,4-D and BA was most suitable (Nakagawa *et al.*, 1984).

Callus cultures were established from bark, stem and needle tissues of *Taxus brevifolia* (Pacific Yew) on a modified Gamborg's B5 medium supplemented with 0.2 per cent casamino acids (CA), 1 per cent sucrose, 2,4-D one mg l^{-1} (Gibson *et al.*, 1993).

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^{-1} mg l Kinetin (Carrier *et al.*, 1990).

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 callus derived from surface sterilized leaf segments cultured on Linsmaier and Skoog (1965) medium containing NAA and BA for four weeks. Cell suspension cultures were initiated by transferring callus tissues to LS liquid medium supplemented with NAA and BA. These cultures were agitated on a reciprocal shaker at a speed of 100 strokes/min at 25°C and were subcultured every 2-3 weeks (Nakagawa *et al.*, 1984).

In *Coscinium fenestratum,* callus cultures derived from leaf petiole bases on MS medium supplemented with 2,4-D and BA were removed and transferred every 4-5 weeks onto fresh MS medium containing 2,4-D or NAA and BAP. For preparing suspension cultures, callus fragments were transferred to liquid medium of the above composition and subjected to continuous shaking (140 rpm) on a rotary shaker at 28°C in the dark (Nair *et al.*, 1992).

2.1.2.2 Utilising suspension cultures in *in vitro* studies

Cell suspension cultures derived from *Ginkgo biloba* leaves produced ginkgolide B. In cell suspension cultures, the production reached a maximum by the 13^{th} day of subculture and was followed by a sharp decrease (Jeon *et al.*, 1995).

Fujita *et al.* (1981) have found that cell suspension cultures of *Lithospermum erythrorhizon,* which are incapable of synthesising shikonin

derivatives in Linsmaier-Skoog medium, produce these naphthaquinone pigments when grown in White's medium.

Kaul *et al.* (1969) reported that suspension cultures of *Dioscorea deltoidea* could produce upto 1.5 per cent dry weight content of diosgenin. Suspension cultures of *Morinda citrifolia* could be stimulated by appropriate selection methods to produce 20 times more of anthraquinone contents (Zenk *et al.*, 1975).

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).

The production of an antifungal spirostanol saponin (SC-1) was detected in cell suspension cultures of *Solanum chrysotrichum*. The maximum SC-1 production was reached after 20 days using a 2 per cent inoculum and complete MS medium supplemented with 2,4-D, Kinetin and sucrose (Villarreal *et al.*, 1997).

Production of a large quantity of berberine, a water soluble quarternary alkaloid, has been reported from cell suspension cultures of *Thalictrum minus, Thalictrum flavum, Thalictrum rugosum* and *Berberis* species (Sato *et al.*, 1990).

2.1.1.3 Analytical screening techniques for isolating producing lines

Yamada (1985) employed small cell aggregate selection method in cells of *Coptis japonica* and obtained a cell line that produced upto 13.2 per cent berberine.

Hyoscyamus niger cell lines selected by cell aggregate screening method produced increased quantities of hyoscyamine (Hashimoto *et al.*, 1982).

The technique of analytical screening was employed with success by Yamamato *et al.* (1982) in *Euphorbia milli*. Cells developed from original calli was split into several segments. One half of each segment was subcultured and the other used for quantitative analysis. The most refined technique of direct analytical screening was introduced by Ellis (1985) who employed microspectrophotometry wherein the content of a compound in a cell with an absorbance maximum of 300 nm can be monitored.

Cell aggregates obtained from a cell suspension culture of *Thalictrum minus* are grown on small pieces of an agar culture medium and the concentration of berberine which has been released from the cells into the agar piece is assayed by the antibacterial activity against *Bacillus cereus* MT 2026 and subsequent screening of 1000 cell colonies by the agar piece method has resulted in the isolation of four high berberine producing cell lines (Suzuki *et al.*, 1987).

Callus tissues from different explants of *Solanum eleagnifolium* Cav. were cultured on a modified MS medium with 1 mgl^{-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).

2.1.4 Factors regulating synthesis of secondary metabolites *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

In general, sucrose is considered the best carbon source for secondary metabolite production 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.

The optimal yield of Ginkgolide B was obtained in MS medium supplemented with 30 g sucrose in *Ginkgo biloba* L. (Jeon *et al.*, 1995). Stimulation of taxol production at elevated levels of sucrose in nodule cultures of *Taxus* was reported by Ellis *et al.* (1996). In addition to taxol, higher sucrose level also enhanced production of 7-epi-10 acetyl taxol cephalomannine and 7-epi-10-deacetyl cephalomannine so that total content of these taxanes was approximately 39 $\mu g\ g^{-1}$ nodules dry weight.

The better performance of *Ginkgo biloba* callus cultures came from the higher sucrose and nitrogen salts concentration of MS medium (Carrier *et al.*, 1990).

In *Thalictrum rugosum*, cell suspension cultures with 8 per cent sucrose solution as production medium significantly improved berberine productivity and cell biomass compared to MS medium supplemented with 2,4-D and 3 per cent sucrose (Kim *et al.*, 1991c).

2.1.4.1.1.2 Effect of inorganic salts

Cell suspension cultures of *Thalictrum minus* var. *hypoleucum* were found to produce a large amount of berberine (400-800 mg l^{-1}). When the cells were cultured in a modified LS medium containing 20 mM KNO_3 and 40 mM NH_4Cl in place of 20.6 mM NH_4NO_3 as nitrogen source, most of the alkaloid crystallized to form berberine chloride instead of nitrate. Minor alkaloids, thalifendine and magnoflorine were also isolated from the medium and identified (Nakagawa *et al.*, 1984).

Jeon *et al.* (1995) reported that for the maximal production of ginkgolide B alkaloid, cells were cultured in MS medium modified to contain 1.25 mM potassium phosphate with a molar ratio of ammonium to nitrate ions of 1:3.

Yasaki *et al.* (1987) found that shikonin producing *Lithospermum erythrorrhison* cell suspension cultures were inhibited in the LS medium by the presence of NH_4^+ which is contained in the form of NH_4NO_3, whereas it was not inhibited by NO_3^- which is contained in White's medium as the sole nitrogen source.

Setai *et al.* (1988) described a new plant tissue culture procedure, using liquid culture medium which contains either more than 50 mM Na or K ion or more than 10 mM Ca or Mg ion in *Coptis* spp. and *Thalictrum minus* to increase berberine production. Using this procedure metabolites can be easily recovered.

2.1.5.1.1.3 Hormonal regime

The presence of NAA and 2,4-D in the growth medium reduced culture aggregation and repressed secondary metabolism of transgenic cell line of *Catharanthus roseus* G. Don S10 when used for the production of terpenoid indole alkaloids such as ajmalcine and catharanthine. Cultures grown in medium containing 2,4-D showed reduced capacity to supply biosynthetic precursors, which resulted in low levels of accumulation of terpenoid indole alkaloids (Whitmer *et al.*, 1998).

Cell suspension cultures of *Thalictrum minus* var. *hypoleucum* grown in Linsmaier-Skoog medium containing both NAA and BAP are known to produce large amounts of berberine, most of which is released into the medium (Nakagawa *et al.*, 1984).

Production of berberine could be induced by adding BAP to *Thalictrum minus* cells cultured in suspension in a medium containing 2,4-D, early in the growth cycle. In the presence of BAP, the precursor, L-tyrosine was rapidly converted into berberine which was then released into the medium. BAP activated enzymatic reactions subsequent to the formation of the amines in the biosynthesis of berberine (Hara *et al.*, 1993).

Cytokinins are known to stimulate the production of nicotine in callus cultures of *Nicotiana tabacum* (Tabata *et al.*, 1975) and that of phenolic compounds such as anthocyanins (Seitz and Hinderer, 1988), catechins, proanthocyanidins and lignins (Zaprometov, 1988).

Morris (1986) revealed that alkaloid induction in cell cultures of *Catharanthus roseus* is controlled by 2,4-D in medium, its production being increased on reducing levels of 2,4-D. Robins *et al.* (1986) observed that in *Cinchona ledgeriana* suspension cultures, alkaloid production was 50 fold greater at 0.1 mg l^{-1} 2,4-D than at 2 mg l^{-1}.

The incorporation of GA3 to the cell suspension cultures of *Coptis japonica* var. *dissectica* contributed to berberine production by activating primary metabolic steps involved in tyrosine biosynthesis (Hara *et al.*, 1994).

The addition 0.1 µm tyrosine to the cell suspension cultures of *Coptis japonica* var. *dissectica* in LS medium did not increase the production of berberine (Sato *et al.*, 1994).

The addition of IAA to a culture grown in 2,4-D medium or an initial combination of IAA with 2,4-D had no effect on either cell growth or berberine production in *Thalictrum rugosum* cell suspension cultures (Kim *et al.*, 1990).

In cell suspension cultures of *Coptis japonica* on LS medium supplemented with NAA and BA, cells contained large amount of starch. The starch content was decreased and the berberine content increased when gibberellin was added to the medium (Morimoto *et al.*, 1986).

2.1.4.1.1.4 Influence of temperature

The release of benzylisoquinoline 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).

2.1.4.1.1.5 Influence of photoperiod

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.

In *Coscinium fenestratum*, light induced the blackening of callus cultures and retarded their growth in callus and suspension cultures. However light increased the berberine content to 3.5 per cent of dry cell weight (2.15% in the dark) (Nair *et al.*, 1992).

In *Thalictrum rugosum* cell suspension cultures, cell growth and production of berberine were assayed for 10 subcultures. 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. Light helped to maintain the cells in the viable state. Berberine production was high in light grown cultures (216.5 mg l^{-1}) than in dark culture (159.0 mg l^{-1}).

2.1.4.1.1.7 Levels of oxygen and carbon dioxide

Breuling *et al.* (1985) reported that oxygen tensions were beneficial for berberine production by cells of *Berberis wilsonae*.

Cultured cells of *Thalictrum minus* transferred from culture flasks to a bubble column bioreactor, produced little berberine and turned dark brown, even when supplied with sufficient oxygen. The physiological damage caused by forced

aeration could be prevented by adding 2 per cent CO_2 to the air in the bioreactor (Kobayashi *et al.*, 1991).

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 CO_2 and ethylene to the airlift system, the berberine content was increased two fold in *Thalictrum rugosum* cell suspension in an improved airlift bioreactor (Kim *et al.*, 1991b).

2.1.4.1.1.8 Addition of precursors

Vanadyl sulphate (10-100 mg l^{-1}) when added to cell suspension cultures of *Catharanthus roseus* stimulated increased intracellular accumulation of catharanthine and ajmalcine (Smith *et al.*, 1987).

The 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).

Addition of various concentrations of (0.5-2.0 mm) acetylsalicyclic acid (ASA) to tumour lines of *Catharanthus roseus* cultivated *in vitro* and requiring 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).

To enhance berberine production in established plant cell cultures of *Thalictrum minus* subsp. *saxatile*, Smolko and Peretti (1994) found that addition of 10^{-9} M pectate lyase was beneficial which also simulated secretion of the alkaloid into the medium.

In *Thalictrum rugosum* cell suspension cultures on MS medium with 2,4-D, maximum enhancement in berberine production was achieved by adding cupric sulfate (200-500 μm) on 6th day of culture (Kim *et al.*, 1991c).

Berberine production was significantly enhanced (over 400%) by addition of spermidine in *Thalictrum minus* cell suspension cultures grown in LS medium supplemented with NAA and BA. Spermidine caused an increase in ethylene generation, which was closely associated with berberine biosynthesis (Hara *et al.*, 1991), whereas other polyamines such as cadaverine, putrescine and spermine were ineffective.

Maximum production of tropane alkaloids (3.97%) in *Hyoscyamus muticus* callus cultures was obtained by supplementing the medium with phenylalanine, while maximum growth was obtained using isoleucine (El-Bahr *et al.*, 1997).

Tailang and Kharya (1996) reported that when callus cells derived from stem, leaf and root segments of *Glycyrrhiza glabra* were exposed to *maleic hydrazide* at lower to moderate (100-500 ppm) concentration for four hours, glycyrrhetinic acid production was favoured.

2.1.4.1.1.9 Effect of osmotic stress in secondary metabolite production

A stimulating effect of osmotic stress caused by mannitol on alkaloid production in cultured *Hyoscyamus muticus, Atropa belladonna, Datura stramonium* cells is reported. The reduced growth in cells caused by mannitol has been attributed to the rapid accumulation of alkaloids in response to osmotic stress (Saker *et al.*, 1997).

2.1.4.1.1.10 Separation and purification of alkaloids under *in vitro* conditions

Berberine alkaloid containing plant tissues or cells are treated with 2-4C aliphatic alcohol to give an extract which is then treated with a chloride ion and or nitric acid ion containing salt exchange agent to deposit the alkaloid as an ion and or salt. The salt is separated from solution (Setai *et al.*, 1987).

The callus produced from the leaf of *Coptis japonica* var. *dissecta* on modified liquid LS medium was collected by filtration, dried and berberine type alkaloids were extracted. The alkaloids were detected by HPLC. The berberine type alkaloid content in the callus was 11.1 per cent. The content in callus cultured in normal LS medium was 8.0 per cent (Setai *et al.*, 1989)

Callus derived from the leaf of *Berberis julianae* on B_5 medium supplemented with 2,4-D and kinetin was transferred into its liquid medium. After culturing in the liquid medium for 20 days, cells were collected through filtering. Fresh cells were extracted with HCl solution. After adding NaCl to the HCl extract, orange crystals were obtained and recrystallised from ethanol with a yield of 1 per cent (dry weight of cells). The orange crystal was identified with jatrorrhizine by HPLC, UV, IR and MS (Sheng, 1986).

Cell suspension cultures of callus tissues, obtained from *Thalictrum minus* cultures on LS agar medium supplemented with NAA and BA in the dark and subcultured at 4 week intervals were produced by transferring callus to LS liquid medium containing NAA and BA. Yellow crystals formed in the liquid medium after 18 days of culture were collected. The medium was filtered and the filtrate was passed through a column of Amberlite XAD-2 to obtain berberine nitrate and berberine chloride respectively (Nakagawa *et al.*, 1984). Most of the berberine produced was released from the cells into the medium.

The methanol extract of the callus tissue of *Nandina domestica* was chromatographed over Sephadex LH-20. A new compound along with 11 protoberberine type alkaloids including berberine, palmatine and jatrorrhizene were identified by NMR, MS and UV spectral data (Ikuta and Itokawa, 1982).

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

The first report of isolation of alkaloid from the family Menispermaceae by Bowen and Motawe was from *Tinosporia malabarica* in 1985. Three alkaloids were isolated from the stem methanol extract by flash column chromatography and were further purified by TLC.

The dried powdered leaves of *Stephania venosa* (Menispermaceae) were defatted with petroleum ether and extracted with cold methanol. The extracts were concentrated to a syrup which gave known alkaloids on further analysis and identified by comparison with authentic samples (Pharadai *et al.*, 1985).

An unknown yellow base, designated menisporphine, was isolated from the rhizome of *Menispermum dauricum* (Kunitomo *et al.*, 1983).

Isoquinoline alkaloids were isolated from aerial parts of *Berberis wilsoneae*. A mixture of tertiary alkaloids was obtained from the extract after alkalization and extraction with ether. Berberine chloride was obtained from the aqueous layer after the separation of tertiary alkaloids and after addition of HCl and extraction with ether (Hrochova and Kostalova, 1985).

Isolation of berberine iodide from *Berberis oblonga* was done by concentrating the extracts of young shoots, diluting the concentration extract with distilled water, filtering, adding ammonia and extracting tertiary alkaloids with ether. The mother liquor was then acidified and berberine iodide was precipitated by addition of aqueous potassium iodide solution (ASMI, 1984).

Alkaloids, such as palmatine, isotetrandrine, aromoline, jatrorrhizine, berberine, berberine chloride, columbamine, oxycanthine and magnoflorine were isolated from the root bark of *Berberis cartagena* (Basher *et al.*, 1996).

2.2 *In vitro* regeneration

2.2.1 Enhanced release of axillary buds

Micropropagation by axillary bud proliferation has proved to be the most reliable method for large scale production of many crop plants (Satyakala *et al.*, 1995).

Multiple shoots were generated from *Woodfordia fruiticosa* nodal segments when cultured in Schenk and Hildebrandt medium supplemented with BA 0.2 mg l^{-1} for 4-5 weeks (Krishnan and Seeni, 1994). Formation of shoots were reported in single node segments of *Aegle marmelos* when cultured in MS medium supplemented with BA and IAA (Ajithkumar and Seeni, 1998).

Shoot bud induction and elongation was obtained in *Piper colubrinum* using root, internode, leaf and petiole explants derived from *in vitro* plantlets in ½ MS medium supplemented with different concentrations of BA (Kelkar and Krishnamurthy, 1998).

Multiple shoots were induced from seedling and axillary buds of mature plants of *Dendrocalamus strictus* on MS medium supplemented with BA and Kinetin (Ravikumar *et al.*, 1998).

Propagation of *Trichopus zeylanicus* was achieved by culturing shoot tips of 2 month old seedlings by Krishnan *et al.* (1995) and that of young, purple shoots observed to show maximum establishment and growth by Seema and Keshavachandran (1998).

Similar results were obtained in medicinal plants like *Chlorophytum borivilianum* (Aparna *et al.*, 1998), *Piper longum* (Banerjee *et al.*, 1998), *Phyllanthus kozhikodianus* (Asha, 1998), *Curculigo orchioides* (Rekha and Reddy, 1998), *Aristolochia indica* (Kavitha and Raju, 1995), *Adhathoda beddomei* (Sudha and Seeni, 1994), *Janakia arayalpathra* (Gangaprasad *et al.*, 1998) and *Mentha piperita* (Faure *et al.*, 1998).

2.2.2 Organogenesis

Plant regeneration through direct or indirect organogenesis has been reported in many medicinal plants.

Adventitious shoots were obtained from callus when cultured on ½ MS medium supplemented with kinetin and silver thiosulphate in *Panax notoginseng* (Lem *et al.*, 1997).

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).

Similar reports on organogenesis were obtained in a number of medicinal plants like *Gloriosa superba* (Somani *et al.*, 1989), *Chrysanthemum morifolium* (Bhattacharya *et al.*, 1990), *Tylophora indica* (Mhatre *et al.*, 1984; Keshavachandran *et al.*, 1996), *Papaver somniferum* (Jaiswal and Narayan, 1985) and *Mentha piperita* (Cellarova *et al.*, 1984).

2.2.3 Somatic embryogenesis

Somatic embryogenesis has the greatest potential for producing large number of plantlets, and is a phenomenon in which the production of embryos takes

place from somatic cells of explants directly or indirectly by induction on callus formed by explants (Purohit *et al.* 1993).

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). Regenerated somatic embryos were capable of developing into plantlets.

Secondary somatic embryogenesis and shoot organogenesis was obtained from primary somatic embryos in *Papaver somniferum* (Ovecka *et al.*, 1997).

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 media, the plantlets were regenerated from somatic embryos.

Similarly somatic embryogenesis were reported in *Panax ginseng* (Choi *et al.*, 1998), *Azadirachta indica* (Hwang *et al.*, 1997), *Tylophora indica* (Mhatre *et al.*, 1984), *Rauwolfia caffra* (Upadhyay *et al.*, 1992), *Aconitum* spp. (Giri *et al.*, 1993), *Atropa belladonna* (Thomas and Street, 1970) and *Digitalis* spp. (Kuberski *et al.*, 1984).

Materials and Methods

MATERIALS AND METHODS

The study entitled "*In vitro* callus induction and its exploitation in *Coscinium fenestratum* (Gaertn.) Colebr." was carried out at the Plant Tissue Culture and the Biochemistry Laboratories of the College of Horticulture, Kerala Agricultural University, Vellanikkara, Thrissur from July 1997 to February 1999. The details regarding the experimental materials and methodology adopted for conducting various aspects of the study are presented in this chapter.

3.1 Materials

3.1.1 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 Glasswares

Borosilicate glassware of Corning/Borosil brand were used for the study. The glasswares were cleaned initially by soaking in potassium dichromate solution for 12 hrs followed by thoroughly washing with jets of tap water in order to remove completely all traces of dichromate solution. They were then soaked in detergent solution (Teepol 0.1%), thoroughly washed with tap water and rinsed twice with double 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.

3.1.3 Culture media

3.1.3.1 Composition of media

Murashige and Skoog (MS) medium (1962), Schenk and Hildebrandt (SH) medium (1972) and Woody Plant medium (WPM) (Lloyd and McCown, 1980) were used for the study. The nutrient media included inorganic salts, vitamins, growth regulators, organic supplements and a carbon source. The composition of different basal media tried are given in Appendix I.

3.1.3.2 Preparation of medium

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

To prepare one litre of medium, the required quantity of each stock solution was added. A known quantity of double distilled water was added and the required quantity of sucrose was weighed, added as solids and dissolved fully. The pH of the solution was adjusted using an electronic pH meter between 5.5 and 5.8 as required, using 0.1N NaOH and 0.1N HCl. The volume was made up to one litre. Agar (0.7% w/v) was added to the medium and melted by keeping the solution in microwave oven for 10 min. The medium was transferred to test tubes (15 cm x 25 cm) at the rate of 15 ml each or to 100 ml Erlenmeyer flasks at the rate of 25 ml each. The test tubes or flasks were plugged with non-absorbent cotton and autoclaved at 121°C and 1.06 kg cm^{-2} for 20 min (Dodds and Roberts, 1982). The medium was allowed to cool to room temperature and stored in the storage room until use.

3.1.4 Growth regulators

Auxins (2,4-D, NAA, IBA, IAA) and cytokinins (BA, 2iP, Kin) were incorporated in the media for callus induction and regeneration. Stress inducers

such as ABA, mannitol and spermidine were added to the subcultured media for the synthesis of secondary metabolites.

3.1.5 Carbon source

Sucrose, maltose and lactose were used as the source of carbon for the study and was added to the medium at the rate of 3, 4, 5, 6, 7 and 8 per cent, to MS media, MS media at 1/2 strength and 1/4 strength, at various stages, for studying the quantity of alkaloid produced.

3.1.6 Transfer area and aseptic manipulation

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

3.1.7 Culture room

The cultures were incubated at 26±2°C in an airconditioned culture room with 16 hrs photoperiod (1000 lux) from fluorescent tubes. Humidity in the culture room varied between 60 to 80 per cent according to the climate prevailing. Cultures were also incubated under dark to study the effect of light and dark conditions on culture establishment, growth, callus development and regeneration.

3.1.8 Source of explant

The explants were taken from a vine of *Coscinium fenestratum* growing in the medicinal plant garden attached to the Department of Plantation Crops and Spices, Vellanikkara. A portion of the vine of *Coscinium fenestratum* is presented in Plate 1. In order to control the pest and diseases it was sprayed with a systemic fungicide, Bavistin 50 WP at 0.1 per cent concentration and a botanical insecticide, neem kernal extract, at 0.1 per cent concentration before the onset of monsoon at

Plate 1. A portion of the vine of field grown *Coscinium fenestratum* .

Plate 1

fortnightly intervals. Leaf segments, nodal segments, internodal segments and immature fruits were used as explants for the study.

3.2 Methods

3.2.1 Preparation of explant

Mature, semi mature and tender shoots, immature light green leaves and immature fruits were used as explants. They were washed with tap water 2-3 times followed by distilled water and subjected to surface sterilization treatments.

3.2.2 Standardisation of surface sterilization

The explants were subjected to various surface sterilants at different concentrations and durations as given below.

Treatment No.	Sterilant	Concentration (%)	Duration (min)
1	$HgCl_2$	0.05	2.5
2	Alcohol wipe + $HgCl_2$	70.00 0.05	2.5
3	$HgCl_2$	0.05	5.0
4	Alcohol wipe + $HgCl_2$	70.00	5.0
5	$HgCl_2$	0.05	7.5
6	Alcohol wipe + $HgCl_2$	70.00 0.05	7.5
7	$HgCl_2$	0.10	2.0
8	$HgCl_2$	0.10	3.0

Treatment No.	Sterilant	Concentration (%)	Duration (min)
9	$HgCl_2$	0.10	4.0
10	$HgCl_2$	0.10	5.0
11	$HgCl_2$	0.10	6.0
12	$HgCl_2$	0.10	7.0
13	$HgCl_2$	0.10	10.0
14	Alcohol wipe + $HgCl_2$	70.00 0.10	5.0
15	Alcohol wipe + $HgCl_2$	70.00 0.10	6.0
16	Alcohol wipe + $HgCl_2$	70.00 0.10	7.0
17	Cetrimide + $HgCl_2$	0.03 0.10	5.0 5.0
18	Cetrimide + $HgCl_2$	0.05 0.10	5.0 5.0
19	Control	-	-

After the treatments, explants were washed free of the sterilants with sterile water, drained over blotting paper and inoculated on to the media prepared under the hood of laminar air flow cabinet. The observations on percentage of cultures contaminated, dried and survival percentage were recorded, three weeks after incubation.

3.2.2.1 Effect of season on culture establishment

The explants were collected from July to January and inoculated after surface sterilization using 0.1 per cent mercuric chloride. 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.

3.3 Routes of micropropagation attempted

In order to standardise the *in vitro* exploitation of *Coscinium*, different *in vitro* techniques such as induction of callus and exploitation of induced calli through organogenesis/somatic embryogenesis were tried. *In vitro* callus induction from immature fruit was also attempted.

3.3.1 Effect of auxins on callus induction

Leaf segments, nodal and internodal segments were cultured in MS basal medium supplemented with auxins such as 2,4,5-T, 2,4-D, NAA, IBA, IAA and cytokinins such as BA and Kin each at 0.5, 1 and 2.5 mg l^{-1} to study their relative effects on callus induction and growth. Observations were recorded on percentage of cultures initialing calli and the period taken for initiation. Numerical scores (1-4) were given for assessing callus growth rate. Callus index (CI) was calculated as

$$CI = P \times G$$

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 subcultured to the very same medium at 4-5

weeks interval for further proliferation. The best combinations identified in the MS basal medium were repeated with ½ MS, ¼ MS, SH and WPM media.

3.3.2 Effect of cytokinins on callusing

Major cytokinins benzyl adenine (BA), kinetin (Kin) and 2-isopentenyl adenine (2iP) at variable concentrations, each at 0.5, 1 and 2.5 mg l^{-1} were incorporated singly and in combination with or without auxins (2,4-D, NAA, IAA) to the basal media. Response of leaf and stem explants to varying combinations 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 earlier in 3.3.1. The best among them were subsequently subcultured.

3.3.3 Effect of other media additives on callusing

Media additives like phloroglucinol (100 and 125 mg l^{-1}) and activated charcoal (0.5 and 0.25 mg l^{-1}) were supplemented to basal media (½ MS) containing IAA (2 mg l^{-1}) + BA (1 mg l^{-1}) and IAA (2 mg l^{-1}) + 2,4-D (1 mg l^{-1}) to assess their relative effects on callusing of nodal and internodal 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 were observed by incubating the cultures in dark and under illumination. MS and ½ MS medium were employed for culturing leaf and stem explants.

3.4 Induction of regeneration

Calli initiated from ½ MS medium supplemented with IAA and BA were subcultured on to media with different growth regulator combinations as detailed below in order to induce regeneration.

3.4.1 Effect of auxins and auxin synergist

Uniform bits of calli of 3 months age initiated from leaf segments were subcultured on to media (solid and liquid) supplemented with the following auxin combinations and the auxin synergist, phloroglucinol.

Auxins and auxin synergist	Concentration ($mg\ l^{-1}$)
IAA	0.25, 5.00, 10.00
IBA	0.25, 5.00, 10.00
NAA	0.25, 5.00, 10.00
IAA+IBA	0.50+0.50, 1.00+0.50
IAA+NAA	0.50+0.50, 0.50+1.00
IBA+NAA	0.50+0.50, 1.00+5.00
IBA+Phloroglucinol	1.00+50.00
IAA+Phloroglucinol	0.50+100.00
Basal medium (1/2 MS)	-

Cultures were incubated in light. Response of calli were recorded at fortnightly intervals.

3.4.2 Effect of cytokinins on callus regeneration

Various levels of cytokinins (as given below) were incorporated to ½ MS medium (liquid and solid) to evaluate their effects singly and in combination with respect to regeneration from callus. Cultures were maintained in light. Response of calli were recorded at fortnightly intervals.

Cytokinins	Concentration (mgl^{-1})
Kinetin	0.25, 5.00, 10.00
BA	0.25, 5.00, 10.00
2iP	0.25, 5.00, 10.00
Kinetin + BA	0.25 + 0.50
BA + Kinetin	0.50 + 1.00
Kinetin + 2iP	1.00 + 0.50
Basal medium (1/2 MS)	-

3.4.3 Effect of growth regulators, osmoregulants and carbon sources

Calli maintained on ½ MS medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1} were subcultured to media incorporated with various auxins and cytokinins as detailed below.

Growth regulators incorporated for inducing regeneration.

Growth regulators	Concentration ($mg\ l^{-1}$)
2,4-D	0.25, 0.5.00
2,4-D + BA	0.25 + 0.25
BA	0.25, 0.50, 2.50, 5.00
Kinetin	0.50, 2.50, 5.00
Kinetin + BA	1.00 + 1.00
CCC	0.10, 0.20
Basal medium (½ MS)	-

Osmoregulants added for inducing regeneration by replacing sucrose

Osmoregulants	Concentration (%)
Sorbitol	1.50, 2.00, 3.00
D-Mannitol	1.50, 2.00, 3.00
PEG	1.50, 2.00, 3.00
Basal medium (½ MS)	-

Varying sources of carbon as given below were employed to assess their relative efficacy to induce regeneration. Cultures were maintained in light and dark. Observations were recorded after 2 weeks.

Carbohydrate sources employed for initiating regeneration in *Coscinium*

Source of CHO	Concentration (%)
Maltose	3.00
Lactose	3.00
Glucose	3.00
Sucrose	5.00
Sucrose	8.00
Maltose + Sucrose	1.50+ 1.50
Lactose + Sucrose	1.50 + 1.50
Glucose + Sucrose	2.00 + 1.00
Basal medium (1/2 MS)	-

3.5 Screening calli for alkaloid, berberine

Leaf induced calli obtained from solid and liquid media were screened for berberine content.

3.5.1 Preparation of callus extract from solid medium

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

3.5.2 Tests for detection of alkaloids

3.5.2.1 Qualitative 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).

1) Test employing Dragendorff's reagent
2) Test employing Mayer's reagent

1) Test employing Dragendorff's reagent

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

2) Test employing Mayer's reagent

Two ml of the test solution was made solvent free by evaporation 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.

3.5.3 Detection of alkaloids by chromatography

The methodology followed was as per Harbone (1973).

3.5.3.1 Preparation of gel plate

Thin layer chromotography 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 silica gel layer. The plates were allowed to set for 10 minutes at room temperature and then placed in hot air oven maintained at 120-150°C for an hour to dry and activate the same and stored.

3.5.3.2 Application of sample

Methanol extracts of test calli along with the standard berberine were used. Five microlitres of the sample was applied with a capillary tube on a pre-coated silica plate at 2 cm from the base at 2 cm intervals, along with the standard berberine hydrochloride.

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

Solvent systems employed for developing chromatograms of *in vitro* and *ex vitro* extract from Coscinium are as follows.

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

3.5.4 Detection of berberine

For the detection of berberine, Dragendorff's reagent was sprayed on chromatographic plates 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.

$$\text{RF value} = \frac{\text{Distance a compound moved in chromatography}}{\text{Distance the solvent travelled}}$$

3.6 Screening of berberine from liquid medium

3.6.1 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 XAD 16 x 1 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 neutral Amberlite

XAD 20 g was used to set up a compact column of 16 x 1 cm.

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.

3.6.2 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 collected fractions were spotted in precoated 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 presence of berberine.

3.7 Screening of *ex vitro* samples for berberine

3.7.1 Preparation of extracts

Samples of bark and stem (30 g each), mature and tender leaves (25 g each) were ground separately to fine pulp by using a mixer grinder. Hot 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.

3.7.2 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).

3.8 Optimisation of *in vitro* metabolite production

3.8.1 Regulation of growth regulators

Basal growth medium (½ MS) was supplemented with varying combinations of growth regulators as given below. Experimental calli were cultured in the above media and analysed for berberine after 3 to 5 months to identify the best growth regulator combination for alkaloid production, which was identified as production medium (medium P).

Growth regulators incorporated for inducing synthesis of berberine in *in vitro* cultures of *C. fenestratum*

Growth regulators	Concentration (mg l^{-1})
NAA + BA	2.00 + 1.00
BA	0.25, 2.50, 5.00
Kinetin	0.25, 2.50, 5.00
IAA + 2,4-D	2.00 + 1.00
2,4-D + BA	0.25 + 1.00
	0.50 + 1.00
	0.75 + 1.00
IAA + BA	0.25 + 1.00
	0.50 + 1.00
	0.75 + 1.00
	1.00 + 1.00
	2.00 + 1.00
2,4-D	0.25, 2.50, 5.00
IAA	1.00, 2.00
NAA	1.00, 2.00
2,4-D	1.00, 2.00
IAA + Kinetin	0.25 + 1.00
	0.50 + 1.00
	0.75 + 1.00
Basal medium (½ MS)	-

3.8.2 Addition of osmoregulants

Osmoregulants like sorbitol and mannitol at 1, 2 and 5 per cent and polyethylene glycol at 0.2 and 2 per cent were added singly to ½ strength MS solid medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1} (medium P) to which 3 month old leaf calli were subcultured for subsequent screening.

3.8.3 Increasing concentration of agar

Concentration of the gelling agent agar was increased to 1, 1.5, 2 and 5 per cent and added to production medium for subculturing the experimental calli.

3.8.4 Addition of stress inducers

Stress inducers like ABA (0.25, 0.5 and 1 mg l^{-1}) and spermidine (0.25, 0.5 and 2.5 mg l^{-1}) were added singly to the production medium to which 3 months old leaf calli were subcultured for subsequent screening of berberine.

3.8.5 Influence of age of calli on berberine production

Calli initiated from leaf segments subcultured on production medium were screened for synthesis of berberine at varying stages of growth from 4 to 12 months at monthly intervals. Observations were made on the influence of age of calli on the expression of the secondary metabolite.

3.9 Optimisation of liquid medium for *in vitro* metabolite production

MS medium at half strength without agar supplemented with IAA 2 mg l^{-1} + BA 1 mg l^{-1} and NAA 2 mg l^{-1} + BA 1 mg l^{-1} singly was incorporated to jam bottles @ 30 ml. The bottles were covered with plastic lid and autoclaved as described in 3.1.3.

Friable leaf callus of 4 months to 1 year age were inoculated in 30 ml each of the callus growth medium. Bottles were incubated at 108 rpm at 26±2°C with a 16 hour photoperiod.

3.9.1 Modification of carbon source

Chemical composition of basal growth medium (1/2 MS) enriched with best hormone combination (IAA + BA, 2 and 1 mg l^{-1} respectively) was modified by increasing the levels of sucrose to 4, 5 and 8 per cent. The carbon source sucrose was replaced by maltose, lactose and galactose each at 3, 5 and 8 per cent respectively to subculture experimental calli for subsequent screening of berberine.

3.9.2 Addition of stress inducer to the liquid medium

Addition of spermidine at 10 µg, 30 µg, 50 µg, 60 µg and 100 µg to the production medium was done for studying the effect of spermidine on the synthesis of berberine.

3.9.3 Withdrawal of inorganic nutrients

Nitrate and phosphate ion supply of ½ MS basal liquid medium was reduced to 75, 50 and 25 per cent of the original concentration and calli grown on them for 4 weeks and screened for the presence of berberine.

3.10 Quantification of berberine

3.10.1 Estimation of Lambda max

Lambda max of berberine was identified using UV spectrophotometer. Natural and standard berberine hydrochloride were used for this purpose. The extracts were prepared from *ex vitro* plant as described in 3.7.1. Since the extract was collected from the plant, it could contain some other compounds (phenols and

other alkaloids) other than berberine which are treated as impurities for the study. To remove these, purification by column chromatography followed by thin layer chromatography was carried out for extracts collected from different parts of the plant viz., stem and leaves. Silica Gel G (20 x 15 cm) column was saturated with solvent mixture (butanol : acetic acid : water - 7 : 2 : 1). The concentrated 2 ml sample was poured on top of the column without disturbing the top layer. The flow rate was 3 ml/ 2 minute.

The fractions were concentrated under air flow for 5 days and TLC was performed against standard berberine hydrochloride. The alkaloid spots which gave yellow colour identical to that of standard berberine at 365 nm were noted. The spots were scraped and dissolved in 4.5ml methanol and centrifuged at 1500 rpm for 18 minutes. The supernatant liquid was collected and read in the UV spectrophotometer at 365 nm. The same method was adopted for extracts from leaves also. The wavelength was 228 nm for the extract obtained from stem and leaf samples.

The standard berberine at 0.001 per cent concentration gave 4 peaks at 228 nm, 264 nm, 348 nm and 429 nm. The fractions 12 to 35 gave a λ max from 213 nm to 229 nm. Fractions 33, 36, 37 and 41 gave two peaks at 228 nm and 282 nm. The same procedure was adopted for leaf extract also.

Adsorption chromatography was also carried out for leaf and stem extracts. Cation exchanger, Amberlite XAD-200 was used for this purpose and the procedure was the same as described for liquid media (Nakagawa *et al.*, 1984).

TLC was performed against standard berberine for detection of berberine. The spots gave Rf value identical to that of standard berberine. The corresponding fractions were concentrated under airflow for one day and scanned in the UV spectrophotometer. The fraction gave two peaks at 228 nm and 269 nm.

228 nm peak was common for all samples (stem, leaf and standard berberine hydrochloride). The other 3 peaks (264 nm, 348nm and 429nm) of standard berberine hydrochloride were not recorded in the samples (leaf and stem). So the peak at 228 nm was selected as the lambda max of marker berberine in the present study.

The absorbance of 2, 4, 6, 8, 10, 12 and 14 µg standard berberine hydrochloride was found out at 228 nm. Methanol was used as the solvent as in the case of *ex vitro* and *in vitro* extracts. The different concentrations of the standard were read in the UV spectrophotometer 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).

3.10.2 Estimation of berberine from *in vitro* and *ex vitro* sources

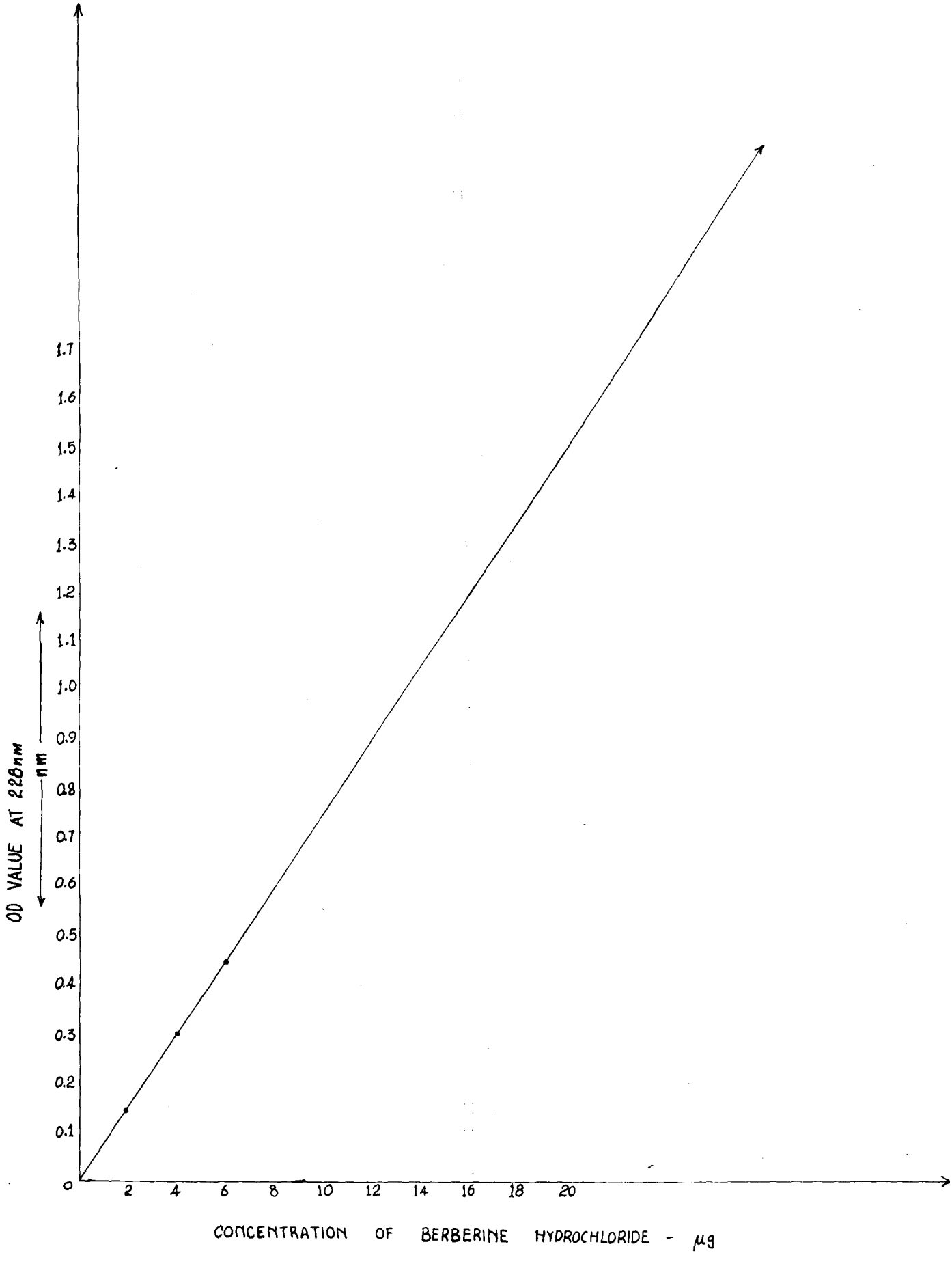
The methanol extracts of calli, suspension cultures, stem and leaves that gave positive test for the presence of berberine were subjected to adsorption chromatography (ion exchange chromatography) as described earlier.

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 airflow for 2 days and dissolved in 2 ml of methanol. From this 10 µl to 100 µl samples were taken and made up to 2.5 ml each with methanol.

The samples were read in the UV spectrophotometer at 228 nm against a reagent blank (methanol). The absorbance of berberine from different extracts were recorded. The standard curve was prepared by standard berberine hydrochloride procured from Sigma Chemicals, UK. The standard graph (Fig.1) was used for estimating the concentration of berberine from samples of various treatments.

FIG. 1

STANDARD GRAPH OF BERBERINE, OD AT 228nm



Results

RESULTS

The results of the studies on "*In vitro* callus induction and its exploitation in *Coscinium fenestratum* (Gaertn.) Colebr." carried out during 1997-1998 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

4.1.1 Surface sterilisation of leaf and fruit explants

The results of the experiment for surface sterilisation of explants taken from the climber growing in the field are presented in Tables 1a, 1b and 1c.

Among the different treatments tried, the least contamination rate was observed in cultures receiving treatments T_{17}, T_{18} and T_{11} (Table 1a) and T_{12} and T_{13} (Table 1c). However, treatments T_{17} ($HgCl_2$ 0.1% for 5 minutes preceded by 0.03% Cetrimide immersion for 5 minutes) and T_{18} ($HgCl_2$ 0.1% for 5 minutes preceded by Cetrimide 0.05% immersion for 3 minutes) resulted in better growth rates (89.77% and 93.12% respectively).

Treatments T_{10}, T_{11} and T_{12} also registered less contamination (19.64%, 19% and 18.77% respectively) but comparatively lesser growth rates (78.36%, 63.1% and 60% respectively). Treatments involving longer durations and higher concentrations of $HgCl_2$ treatments were not promising. They resulted in higher percentage of bleached cultures and subsequent drying and the percentage of establishment varied from zero per cent to 49 per cent.

Table 1a. Surface sterilisation of the leaf and fruit explants

Treatment No.	Sterilant	Concentration (%)	Duration (min.)	Contamination (%)	Fungal (%)	Bacterial (%)	Survival (%)	Cultures bleached (%)	Cultures showing growth (%)
1	$HgCl_2$	0.05	2.50	100.00	77.77	22.23	-	-	-
2	alcohol wipe + $HgCl_2$	70.00 0.05	2.50	90.00	88.00	2.25	10.00	-	-
3	$HgCl_2$	0.05	5.00	89.00	67.23	21.77	11.00	-	-
4	alcohol wipe + $HgCl_2$	70.00 0.05	5.00	87.00	26.47	24.53	49.00	-	-
5	$HgCl_2$	0.05	7.50	51.00	26.47	24.53	49.00	-	-
6	alcohol wipe + $HgCl_2$	70.00 0.05	7.50	51.00	28.12	22.88	49.00	-	-
7	$HgCl_2$	0.10	2.00	62.50	36.70	23.30	37.50	-	-
8	$HgCl_2$	0.10	3.00	51.10	34.10	25.00	40.90	-	-
9	$HgCl_2$	0.10	4.00	31.25	27.00	4.25	62.50	-	-
10	$HgCl_2$	0.10	5.00	19.64	10.21	9.43	80.36	-	48.36
11	$HgCl_2$	0.10	6.00	19.00	10.11	8.89	81.00	10.00	63.10
12	$HgCl_2$	0.10	7.00	18.77	10.21	8.56	78.23	13.00	60.00
13	$HgCl_2$	0.10	10.00	0.00	0.00	0.00	0.00	100.00	0.00
14	alcohol wipe + $HgCl_2$	70.00 0.10	3.00	53.71	21.12	32.59	26.19	20.00	0.00
15	alcohol wipe + $HgCl_2$	70.00 0.10	5.00	13.77	10.13	3.64	53.23	33.00	-
16	alcohol wipe + $HgCl_2$	70.00 0.10	7.00	0.00	0.00	0.00	28.00	72.00	0.00
17	Cetrimide + $HgCl_2$	0.03 0.10	5.00 5.00	12.19	7.31	4.88	87.81	-	89.97
18	Cetrimide + $HgCl_2$	0.05 0.10	5.00 5.00	13.83	6.74	7.09	86.13	-	93.12
19	Control	-	-	100.00	81.00	19.00	-	-	-

4.1.2 Surface sterilisation of explants during the rainy season (May, June, July)

Combination of a fungicide (Emisan) and mercuric chloride were tried for sterilisation of explants for the rainy season. Treatment T_4 (Cetrimide 0.05% for 5 minutes, Emisan 0.1% for 5minutes, and 0.1% $HgCl_2$ for 5 minutes) was the best and gave 87 per cent establishment of cultures with a growth rate of 82 per cent (Table 1b).

4.1.3 Surface sterilisation of shoot buds, nodal and internodal segments

Surface sterilisation treatments tried for the leaves were also repeated for the above explants and the contamination rate was cent percent. Another constraint was the exudation of phenols from the cut ends. Hence pre-treatment with antioxidants were needed. So Emisan, mercuric chloride and antioxidant combinations were tried for sterilisation of semimature and tender shoots, the results of which are presented in Table 1c.

The treatments T_{11}, T_{12} and T_{13} showed the least contamination rate (11.41%, 11% and 17% respectively). But only the latter two treatments showed better growth rate (13% and 15%) respectively. T_{12} (Cetrimide 0.1% for 5 min, Emisan 0.1% for 5 min, citric acid 0.1% + ascorbic acid 0.1% for 10 min and mercuric chloride 0.1% for 3 min) was the best among the treatments.

4.1.4 Seasonal variation in the rate of establishment of explants

The data on seasonal influence on culture establishment and sustained growth of explants are presented in Table 2 and Fig.2. When the explants were sterilised with 0.1 per cent mercuric chloride for 5 min at monthly intervals, better establishment of foliar explants were obtained during the period from October to January, maximum in November (96.1%) followed by October (94.4%) and

Table 1b. Surface sterilisation of the explants taken from leaf in rainy season

Treatment No.	Sterilant	Concentration (%)	Duration (min.)	Contamination (%)	Fungal (%)	Bacterial (%)	Survival (%)	Cultures bleached	Cultures showing growth
1	Emisan + $HgCl_2$	0.10 0.10	5.00 3.00	34.00	21.00	13.00	66.00	-	64.00
2	Emisan + $HgCl_2$	0.10 0.10	5.00 5.00	21.00	17.00	4.00	79.00	-	79.00
3	Emisan + $HgCl_2$	0.10 0.10	7.00 5.00	17.00	14.00	3.00	62.00	21.00	42.00
4	Cetrimide + Emisan $HgCl_2$	0.05 0.10 0.10	5.00 5.00 5.00	43.00	15.00	8.00	87.00	-	82.00
5	Control	-	-	100.00	79.00	21.00	-	-	-

Table 1c. Surface sterilisation of the explants taken from stem

Treatment No.	Sterilant	Concentration (%)	Duration (min.)	Contamination (%)	Fungal (%)	Bacterial (%)	Survival (%)	Cultures bleached (%)	Cultures showing growth (%)
1	Emisan +	0.10	5.00	11.00	7.00	4.00	16.00	60.00	2.00
	$HgCl_2$	0.10	5.00						
2	Emisan +	0.10	7.00	10.30	7.00	13.00	66.00	-	6.00
	$HgCl_2$	0.10	5.00						
3	Emisan +	0.10	10.00	-	-	-	5.00	95.00	-
	$HgCl_2$	0.10	10.00						
4	Emisan +	0.10	10.00	-	-	-	3.00	98.00	-
	$HgCl_2$	0.10	10.00						
5	$HgCl_2$	0.10	10.00	-	-	-	5.00	95.00	-
6	$HgCl_2$	0.10	7.50	10.30	10.30	-	9.00	80.00	-
7	$HgCl_2$	0.10	5.00	15.32	13.18	1.14	20.00	78.00	
8	$HgCl_2$	0.10	2.50	81.32	58.71	22.61	-	-	-
9	Cetrimide +	0.10	5.00						
	Emisan +	0.30	5.00	33.18	21.51	1.67	57.00	10.00	-
	$HgCl_2$	0.10	5.00						
10	Cetrimide +	0.10	5.00						
	Emisan +	0.30	5.00	25.00	20.48	4.52	43.00	32.00	-
	$HgCl_2$	0.10	5.00						
11	Cetrimide +	0.10	5.00	15.44	11.41	5.03	84.56	82.00	-
	$HgCl_2$	0.10	5.00						
12	Cetrimide +	0.10	5.00						
	Emisan +	0.10	5.00						
	$HgCl_2$ +	0.10	5.00	11.00	7.00	4.00	70.00	19.00	13.00
	Citric acid +	0.10	10.00						
	Ascorbic acid	0.10	10.00						
13	Cetrimide +	0.10	5.00						
	Emisan +	0.10	5.00						
	$HgCl_2$ +	0.10	3.00	17.00	14.00	6.00	83.00	-	18.00
	Citric acid +	0.10	10.00						
	Ascorbic acid	0.10	10.00						
14	Control	-	-	100.00	80.00	20.00	-	-	-

Table 2. Effect of season of collection of explants* on *in vitro* culture establishment in *Coscinium fenestratum*

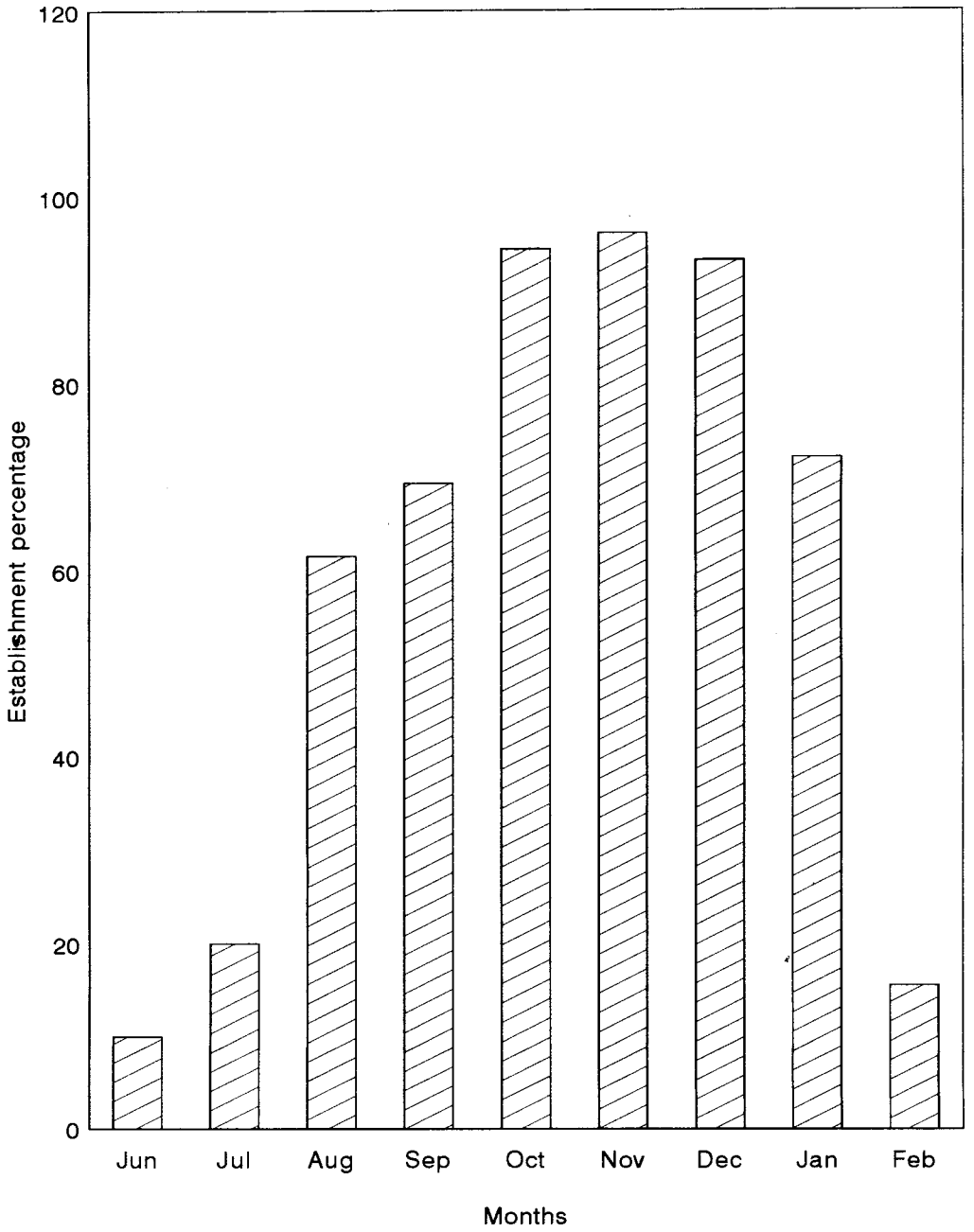
Treatment	Treatment month**	Establishment percentage***
1	June	10.00
2	July	20.00
3	August	61.50
4	September	69.30
5	October	94.40
6	November	96.10
7	December	93.20
8	January	72.10
9	February	15.50

* Explants include nodal segments, shoot tips and leaf segments

** Experiments done in the year 1997-1998

*** Observations recorded three weeks after inoculation

Fig.2. Effect of season on *in vitro* establishment of explants of *C. fenestratum*



December (93.2%), while the rate of contamination was higher during the other months of the year.

4.2 Induction and proliferation of calli

4.2.1 Effect of auxins on induction and proliferation of calli

The response of leaf, stem and petiole cultures of *Coscinium fenestratum* to auxin combinations in initiation and proliferation of callus in MS medium are presented in Table 3.

From the results it could be inferred that the 5 auxins used in the study, namely 2,4-D, 2,4,5-T, IAA, IBA and NAA at varying levels had significant influence on the leaf and leaf petiole cultures with respect to days taken for callus induction, percentage of cultures initiating calli, callus growth score and mean callus index values. Percentage of cultures initiating calli varied significantly in leaf bit cultures under the influence of various auxins and photoperiod. Maximum calli, 71 and 42 percentage respectively were initiated in treatment T_{86} and T_{87} with callus indices of 71 and 42 respectively in the dark in MS basal medium (Table 3).

Culturing in dark reduced the number of days taken for callus initiation than under light (1200 lux). In treatment T_{86}, the leaf segments had taken only 16 days in the dark than 19 days under continuous illumination for callus initiation. Thus it can be seen from Table 3 that culturing in dark is congenial for callus initiation in *Coscinium.*

4.2.2 Effect of cytokinins on callusing

As presented in Table 4, incorporation of cytokinins such as benzyladenine (BA), kinetin and 2- isopentenyl adenine (2iP) at varying levels to basal media did not have any significant effect on callusing except BA at 1 mg l^{-1}

Table 3. Effect of auxins on callus induction under light and dark in solid MS media

Treatment No.	Treatment concentration MS media ($mg\ l^{-1}$)	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
1	2,4-D	*Stem	3.28	34.00	24	24	3.28	34.00	Brown	Brown
	0.25	**Petiole	10.00	14.10	22	25	10.00	14.10	Brown	Brown
		***L+P	0.00	0.00	-	-	0.00	0.00	-	-
		****Leaf seg	35.00	37.00	26	24	35.00	37.00	Brown	Brown
2	2,4-D	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	1	Petiole	20.00	18.00	21	21	20.00	18.00	Brown	Brown
		L+P	30.00	30.00	23	25	30.00	30.00	Brown	Brown
		Leaf seg	26.34	18.70	26	25	26.34	18.70	Brown	Brown
3	2,4-D	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Petiole	10.21	17.23	22	24	10.21	17.23	Brown	Brown
		L+P	21.30	20.52	24	26	21.30	20.52	Brown	Brown
		Leaf seg	25.00	25.41	26	24	25.00	25.41	Brown	Brown
4	2,4-D	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	25.00	27.00	26	25	25.00	27.00	Brown	Brown
5	2,4,5-T	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	1	Petiole	12.13	5.38	23	25	12.13	5.38	Brown	Brown
		L+P	27.38	28.10	21	22	27.38	28.10	Brown	Brown
		Leaf seg	30.24	30.6	26	25	30.24	30.60	Brown	Brown
6	2,4,5-T	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	30.80	27.4	26	23	30.80	27.12	Brown	Brown
		Leaf seg	20.00	25.23	25	25	20.00	25.23	Brown	Brown
7	IAA	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Petiole	19.00	13.00	21	20	19.00	13.00	Brown	Brown
		L+P	60.00	22.00	27	25	60.00	22.00	Brown	Brown
		Leaf seg	63.00	61.00	26	24	63.00	61.00	Brown	Brown

Contd.

Table 3. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
8	IAA	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Petiole	20.00	12.00	22	21	20.00	12.00	Brown	Brown
		L+P	21.00	31.00	25	24	21.00	31.00	Brown	Brown
		Leaf seg	38.26	45.00	26	25	38.26	45.00	Brown	Brown
9	IAA	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Petiole	11.00	10.00	21	20	11.00	10.00	Brown	Brown
		L+P	53.00	21.00	26	25	53.00	21.00	Brown	Brown
		Leaf seg	78.00	79.00	27	26	78.00	79.00	Brown	Brown
10	NAA	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Petiole	10.00	12.00	27	27	10.00	12.00	Brown	Brown
		L+P	12.80	15.00	26	25	12.80	15.00	Brown	Brown
		Leaf seg	23.00	25.00	28	25	23.00	25.00	Brown	Brown
11	NAA	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	1	Petiole	1.00	2.00	29	27	1.00	2.00	Brown	Brown
		L+P	12.00	15.00	26	25	12.00	15.00	Brown	Brown
		Leaf seg	35.00	35.00	27	26	35.00	35.00	Brown	Brown
12	NAA	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Petiole	20.00	25.00	27	26	20.00	25.00	Brown	Brown
		L+P	78.00	80.00	26	26	78.00	80.00	Brown	Brown
		Leaf seg	61.00	59.00	26	26	61.00	59.00	Brown	Brown
13	IBA	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Petiole	18.00	21.00	29	28	18.00	21.00	Brown	Brown
		L+P	17.00	37.00	25	24	17.00	37.00	Brown	Brown
		Leaf seg	21.00	29.00	25	24	21.00	29.00	Brown	Brown
14	IBA	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	1	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	11.28	0.00	-	-	11.28	0.00	Brown	-

Contd.

Table 3. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
15	IBA	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Petiole	3.17	0.00	25	-	3.17	0.00	Brown	Brown
		L+P	5.20	0.00	30	-	5.20	0.00	Brown	Brown
		Leaf seg	12.00	0.00	26	-	12.00	0.00	Brown	Brown
16	NAA	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Petiole	12.00	14.00	19	21	12.00	14.00	Brown	Brown
		L+P	17.00	21.00	23	20	17.00	21.00	Brown	Brown
		Leaf seg	33.00	40.00	20	19	33.00	40.00	Brown	Brown
17	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	11.00	20.00	29	21	11.00	20.00	Brown	Brown
	0.25+1	L+P	17.00	31.00	21	20	17.00	31.00	Brown	Brown
		Leaf seg	10.00	60.00	23	18	10.00	60.00	Brown	Brown
18	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	14.00	15.00	21	18	14.00	15.00	Brown	Brown
	0.25+2.5	L+P	31.00	29.00	22	20	31.00	29.00	Brown	Brown
		Leaf seg	41.00	70.00	24	19	41.00	70.00	Brown	Brown
19	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	2.50	25	24	0.00	2.50	Brown	Brown
	1+0.25	L+P	12.00	15.00	22	22	12.00	15.00	Brown	Brown
		Leaf seg	10.00	10.00	20	19	10.00	10.00	Brown	Brown
20	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	3.00	0.00	21	22	3.00	0.00	Brown	Brown
	1+1	L+P	6.60	6.60	25	21	6.60	6.60	Brown	Brown
		Leaf seg	17.00	36.00	27	25	17.00	36.00	Brown	Brown
21	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	10.00	11.00	27	24	10.00	11.00	Brown	Brown
	1+2.5	L+P	21.30	22.00	25	21	21.30	22.00	Brown	Brown
		Leaf seg	50.00	66.00	26	20	50.00	66.00	Brown	Brown

Contd.

Table 3. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
22	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	9.00	7.00	21	19	9.00	7.00	Brown	Brown
	2.5+0.25	L+P	27.00	29.00	24	23	27.00	29.00	Brown	Brown
		Leaf seg	30.00	33.00	25	20	30.00	33.00	Brown	Brown
23	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	9.00	11.00	24	20	9.00	11.00	Brown	Brown
	2.5+1	L+P	17.00	21.00	22	19	17.00	21.00	Brown	Brown
		Leaf seg	10.00	60.00	20	18	10.00	60.00	Brown	Brown
24	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	4.00	8.00	19	20	4.00	8.00	Brown	Brown
	2.5+2.5	L+P	12.00	15.00	24	21	12.00	15.00	Brown	Brown
		Leaf seg	33.00	40.00	25	21	33.00	40.00	Brown	Brown
25	2,4-D +	Stem	0.00	10.00	-	-	0.00	10.00	-	Brown
	2,4,5-T	Petiole	0.00	2.40	25	21	0.00	2.40	Brown	Brown
	0.25+0.25	L+P	5.00	7.00	28	20	5.00	7.00	Brown	Brown
		Leaf seg	10.00	33.00	27	19	10.00	33.00	Brown	Brown
26	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	
	0.25+1	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
27	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25+2.5	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	5.00	10.00	21	19	5.00	10.00	Brown	Brown
28	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+0.25	L+P	8.30	9.00	28	20	8.30	9.00	Brown	Brown
		Leaf seg	5.00	10.00	27	21	5.00	10.00	Brown	Brown

Contd.

Table 3. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
29	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+1	L+P	12.00	23.00	26	25	12.00	23.00	Brown	Brown
		Leaf seg	15.00	10.00	25	23	15.00	10.00	Brown	Brown
30	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	10.00	13.30	21	20	10.00	13.30	Brown	Brown
	1+2.5	L+P	11.00	14.10	26	24	11.00	14.10	Brown	Brown
		Leaf seg	13.00	33.00	25	21	13.00	33.00	Brown	Brown
31	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	10.00	14.00	19	18	10.00	14.00	Brown	Brown
	2.5+0.25	L+P	12.00	15.00	23	17	12.00	15.00	Brown	Brown
		Leaf seg	10.00	33.30	25	21	10.00	33.30	Brown	Brown
32	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	10.00	13.00	25	21	10.00	13.00	Brown	Brown
	2.5+1	L+P	15.00	18.00	23	22	15.00	18.00	Brown	Brown
		Leaf seg	0.00	33.00	26	22	0.00	33.00	Brown	Brown
33	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	10.00	13.00	26	24	10.00	13.00	Brown	Brown
	2.5+2.5	L+P	0.00	0.00	-	-	0.00	0.00	Brown	Brown
		Leaf seg	0.00	4.00	23	20	0.00	4.00	Brown	Brown
34	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	21.00	22.00	21	23	21.00	22.00	Brown	Brown
	0.25+0.25	L+P	20.00	18.00	25	24	20.00	18.00	Brown	Brown
		Leaf seg	31.00	29.00	26	26	31.00	29.00	Brown	Brown
35	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	18.00	20.00	21	24	18.00	20.00	Brown	Brown
	0.25+0.1	L+P	23.00	25.00	24	26	23.00	25.00	Brown	Brown
		Leaf seg	34.00	35.00	26	27	34.00	35.00	Brown	Brown

Contd.

Table 3. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
36	2,4,5-T + NAA 0.25+2.5	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	19.00	21.00	23	22	19.00	21.00	Brown	Brown
		L+P	21.00	23.00	24	23	21.00	23.00	Brown	Brown
		Leaf seg	25.00	28.00	22	21	25.00	28.00	Brown	Brown
37	2,4,5-T + NAA 1+0.25	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	31.00	33.00	22	21	31.00	33.00	Brown	Brown
		L+P	45.00	48.00	21	22	45.00	48.00	Brown	Brown
		Leaf seg	78.00	80.00	26	26	78.00	80.00	Brown	Brown
38	2,4,5-T + NAA 1+1	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	28.00	30.00	22	23	28.00	30.00	Brown	Brown
		L+P	29.00	31.00	24	25	29.00	31.00	Brown	Brown
		Leaf seg	31.00	3.00	26	27	31.00	3.00	Brown	Brown
39	2,4,5-T + NAA 1+2.5	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	23.00	25.00	23	24	23.00	25.00	Brown	Brown
		L+P	28.00	30.00	24	25	28.00	30.00	Brown	Brown
		Leaf seg	29.00	32.00	25	25	29.00	32.00	Brown	Brown
40	2,4,5-T + NAA 2.5+0.25	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	27.00	30.00	23	24	27.00	30.00	Brown	Brown
		L+P	29.00	31.00	24	25	29.00	31.00	Brown	Brown
		Leaf seg	31.00	33.00	26	27	31.00	33.00	Brown	Brown
41	2,4,5-T + NAA 2.5+1	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	28.00	30.00	23	24	28.00	30.00	Brown	Brown
		L+P	29.00	32.00	25	25	29.00	32.00	Brown	Brown
		Leaf seg	37.00	39.00	26	27	37.00	39.00	Brown	Brown
42	2,4,5-T + NAA 2.5+2.5	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	28.00	29.00	24	25	28.00	29.00	Brown	Brown
		L+P	31.00	32.00	25	26	31.00	32.00	Brown	Brown
		Leaf seg	37.00	39.00	26	27	37.00	39.00	Brown	Brown

Contd.

Table 3. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
43	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	25.00	11.00	24	25	25.00	11.00	Brown	Brown
	0.25+0.25	L+P	37.00	21.00	25	26	37.00	21.00	Brown	Brown
		Leaf seg	48.00	30.00	26	27	48.00	30.00	Brown	Brown
44	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	10.00	11.00	25	24	10.00	11.00	Brown	Brown
	0.25+0.5	L+P	11.00	15.00	26	25	11.00	15.00	Brown	Brown
		Leaf seg	9.00	10.00	27	26	9.00	10.00	Brown	Brown
45	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	3.00	5.00	22	24	3.00	5.00	Brown	Brown
	0.25+2.5	L+P	5.00	8.00	24	23	5.00	8.00	Brown	Brown
		Leaf seg	8.00	10.00	26	25	8.00	10.00	Brown	Brown
46	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	0.00	8.00	-	23	0.00	8.00	-	Brown
	1+0.25	L+P	0.00	9.00	-	18	0.00	9.00	-	Brown
		Leaf seg	0.00	20.00	-	25	0.00	20.00	-	Brown
47	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	1.00	2.00	23	24	1.00	2.00	Brown	Brown
	1+1	L+P	5.00	7.00	24	20	5.00	7.00	Brown	Brown
		Leaf seg	10.00	11.00	25	26	10.00	11.00	Brown	Brown
48	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+2.5	L+P	0.00	9.00	-	23	0.00	9.00	Brown	Brown
		Leaf seg	0.00	8.00	-	24	0.00	8.00	Brown	Brown
49	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	0.00	2.00	-	21	0.00	2.00	-	Brown
	2.5+0.25	L+P	0.00	8.00	-	22	0.00	8.00	-	Brown
		Leaf seg	0.00	15.00	-	25	0.00	15.00	-	Brown

Contd.

Table 3. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
50	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	0.00	3.00	-	21	0.00	3.00	-	Brown
	2.5+1	L+P	0.00	8.00	-	18	0.00	8.00	-	Brown
		Leaf seg	0.00	10.00	-	26	0.00	10.00	-	Brown
51	IBA +	Stem	0.00	0.00	-	-	0.0	0.00	-	-
	2,4-D	Petiole	0.00	2.00	-	21	0.00	2.00	-	Brown
	2.5+2.5	L+P	0.00	9.00	-	18	0.00	9.00	-	Brown
		Leaf seg	0.00	11.00	-	23	0.00	11.00	-	Brown
52	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	8.00	-	21	0.00	8.00	-	Brown
	0.25+0.25	L+P	0.00	10.00	20	23	0.00	10.00	-	Brown
		Leaf seg	0.00	9.00	17	18	0.00	9.00	-	Brown
53	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	Brown
	0.25+1	L+P	0.00	5.00	-	27	0.00	5.00	-	Brown
		Leaf seg	0.00	7.00	-	21	0.00	7.00	-	Brown
54	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25+2.5	L+P	0.00	5.00	-	21	0.00	5.00	-	Brown
		Leaf seg	5.00	13.30	23	20	5.00	13.30	Brown	Brown
55	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+0.25	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
56	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	5.00	7.50	19	21	5.00	7.50	Brown	Brown
	1+1	L+P	0.00	13.00	-	23	0.00	13.00	-	Brown
		Leaf seg	17.00	28.00	27	21	17.00	28.00	Brown	Brown

Contd.

Table 3. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
57	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+2.5	L+P	5.00	10.00	22	25	5.00	10.00	Brown	Brown
		Leaf seg	10.00	21.30	27	21	10.00	21.30	Brown	Brown
58	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	Brown	Brown
	2.5+0.25	L+P	0.00	8.00	-	25	0.00	8.00	-	Brown
		Leaf seg	7.00	16.00	21	27	7.00	16.00	Brown	Brown
59	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5+1	L+P	0.00	8.00	-	21	0.00	8.00	-	Brown
		Leaf seg	7.00	16.00	22	26	7.00	16.00	Brown	Brown
60	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5+2.5	L+P	0.00	5.00	-	25	0.00	5.00	-	Brown
		Leaf seg	11.00	22.00	23	27	11.00	22.00	Brown	Brown
61	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	IAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25+0.25	L+P	0.00	5.00	-	-	0.00	5.00	-	Brown
		Leaf seg	11.00	24.00	25	22	11.00	24.00	Brown	Brown
62	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	IAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25+1	L+P	0.00	5.00	-	-	0.00	5.00	-	Brown
		Leaf seg	21.00	25.00	23	25	21.00	25.00	Brown	Brown
63	IBA +	Stem	0.00	0.00	-	-	0.00	-	-	-
	IAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25+2.5	L+P	0.00	5.00	-	-	0.00	5.00	-	Brown
		Leaf seg	23.00	24.00	27	21	23.00	24.00	Brown	Brown

Contd.

Table 3. Continued

Treat-ment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
64	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	IAA	Petiole	17.00	10.00	21	25	17.00	10.00	Brown	Brown
	1+0.25	L+P	10.00	21.00	23	22	10.00	21.00	Brown	Brown
		Leaf seg	21.00	25.00	24	25	21.00	25.00	Brown	Brown
65	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	IAA	Petiole	33.00	5.00	22	25	33.00	5.00	Brown	Brown
	1+1	L+P	21.00	25.00	27	23	21.00	25.00	Brown	Brown
		Leaf seg	37.00	41.00	24	22	37.00	41.00	Brown	Brown
66	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	IAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+2.5	L+P	28.00	29.00	21	24	28.00	29.00	Brown	Brown
		Leaf seg	27.00	33.00	25	27	27.00	33.00	Brown	Brown
67	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	IAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5+0.25	L+P	27.00	29.00	29	25	27.00	29.00	Brown	Brown
		Leaf seg	29.00	32.00	27	21	29.00	32.00	Brown	Brown
68	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	IAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5+1	L+P	27.00	28.00	25	21	27.00	28.00	Brown	Brown
		Leaf seg	28.00	32.00	28	24	28.00	32.00	Brown	Brown
69	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	IAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5+2.5	L+P	27.00	21.00	20	25	27.00	21.00	Brown	Brown
		Leaf seg	30.00	33.00	21	23	30.00	33.00	Brown	Brown
70	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25+0.25	L+P	13.00	24.00	25	26	13.00	24.00	Brown	Brown
		Leaf seg	27.00	29.00	25	21	27.00	29.00	Brown	Brown

Contd.

Table 3. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
71	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25+1	L+P	15.00	27.00	21	23	15.00	27.00	Brown	Brown
		Leaf seg	28.00	31.00	25	22	28.00	31.00	Brown	Brown
72	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25+2.5	L+P	17.50	21.00	25	22	17.50	21.00	Brown	Brown
		Leaf seg	31.00	35.00	24	21	31.00	35.00	Brown	Brown
73	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+0.25	L+P	0.00	31.00	23	22	0.00	31.00	-	Brown
		Leaf seg	0.00	27.30	25	21	0.00	27.30	-	Brown
74	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0 .00	-	-	0.00	0.00	-	-
	1+1	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
75	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+2.5	L+P	0.00	27.00	25	21	0.00	27.00	-	Brown
		Leaf seg	31.00	44.00	23	22	31.00	44.00	Brown	Brown
76	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5+0.25	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
77	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25+0.25	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-

Contd.

Table 3. Continued

Treat-ment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
78	IBA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5+2.5	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
79	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	13.00	21.00	22	23	13.00	21.00	Brown	Brown
	0.25+0.25	L+P	12.00	27.00	24	26	12.00	27.00	Brown	Brown
		Leaf seg	41.00	60.00	23	24	41.00	60.00	Brown	Brown
80	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	13.00	27.00	27	20	13.00	27.00	Brown	Brown
	0.25+1	L+P	22.00	27.00	20	21	22.00	27.00	Brown	Brown
		Leaf seg	41.00	30.00	22	19	41.00	30.00	Brown	Brown
81	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	2.00	9.00	27	28	2.00	9.00	Brown	Brown
	0.25+2.5	L+P	35.00	7.00	25	22	35.00	7.00	Brown	Brown
		Leaf seg	17.00	23.00	26	23	17.00	23.00	Brown	Brown
82	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	12.00	21.00	24	23	12.00	21.00	Brown	Brown
	1+0.25	L+P	41.00	53.00	26	21	41.00	53.00	Brown	Brown
		Leaf seg	69.00	71.00	28	22	69.00	71.00	Brown	Brown
83	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25+0.25	L+P	21.00	17.00	23	26	21.00	17.00	Brown	Brown
		Leaf seg	27.00	8.00	25	20	27.00	8.00	Brown	Brown
84	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+2.5	L+P	20.00	10.00	23	21	20.00	10.00	Brown	Brown
		Leaf seg	21.00	27.00	24	22	21.00	27.00	Brown	Brown

Contd.

Table 3. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
85	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	27.00	3.00	25	24	27.00	3.00	Brown	Brown
	2.5+0.25	L+P	13.00	15.00	25	23	13.00	15.00	Brown	Brown
		Leaf seg	20.00	20.00	24	21	20.00	20.00	Brown	Brown
86	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	28.00	33.00	24	18	28.00	33.00	Brown	Brown
	2.5+1	L+P	51.00	60.00	21	17	51.00	60.00	Brown	Brown
		Leaf seg	67.00	71.00	19	16	67.00	71.00	Brown	Brown
87	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	21.00	33.00	22	24	21.00	33.00	Brown	Brown
	2.5+2.5	L+P	27.00	33.30	25	21	27.00	33.30	Brown	Brown
		Leaf seg	34.00	42.00	24	22	34.00	42.00	Brown	Brown
88	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	25.00	23.00	25	22	25.00	23.00	Brown	Brown
	0.25+0.25	L+P	25.00	27.00	23	21	25.00	27.00	Brown	Brown
		Leaf seg	13.00	33.00	24	20	13.00	33.00	Brown	Brown
89	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25+1	L+P	3.50	7.00	25	22	3.50	7.00	Brown	Brown
		Leaf seg	10.00	10.00	21	22	10.00	10.00	Brown	Brown
90	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5+2.5	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	7.00	0.00	23	-	7.00	0.00	Brown	-
91	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+0.25	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-

Contd.

Table 3. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
92	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+1	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	17.00	23.00	22	24	17.00	23.00	Brown	Brown
93	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+2.5	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	7.00	-	22	0.00	7.00	-	Brown
94	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5+0.25	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
95	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5+1	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
96	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	2.50	3.00	22	21	2.50	3.00	Brown	Brown
	2.5+2.5	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	7.50	10.00	27	26	7.50	10.00	Brown	Brown
97	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25+0.25	L+P	2.50	7.00	29	26	2.50	7.00	Brown	Brown
		Leaf seg	3.00	9.00	26	23	3.00	9.00	Brown	Brown
98	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25+1	L+P	3.00	7.00	28	25	3.00	7.00	Brown	Brown
		Leaf seg	5.00	11.00	25	22	5.00	11.00	Brown	Brown

Contd.

Table 3. Continued

Treat-ment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
99	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25+2.5	L+P	3.00	7.00	24	22	3.00	7.00	Brown	Brown
		Leaf seg	5.00	13.00	21	20	5.00	13.00	Brown	Brown
100	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+0.25	L+P	13.00	21.00	29	28	13.00	21.00	Brown	Brown
		Leaf seg	17.00	21.00	27	27	17.00	21.00	Brown	Brown
101	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+1	L+P	21.00	27.00	27	25	21.00	27.00	Brown	Brown
		Leaf seg	30.00	33.30	26	23	30.00	33.30	Brown	Brown
102	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1+2.5	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
103	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	2.00	0.00	25	-	2.00	0.00	Brown	Brown
	2.5+0.25	L+P	3.30	0.00	21	25	3.30	0.00	Brown	Brown
		Leaf seg	7.00	10.00	20	29	7.00	10.00	Brown	Brown
104	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5+1	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
105	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5+2.5	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-

* Stem - Nodal and internodal segments
** Petiole - Petiole segments
*** L + P - Leaf segments attached with petiole bases
****Leaf seg - Leaf segments

which gave a high callus index of 70 for leaf cultures under dark incubated conditions in treatment T_5. As seen in Table 3 here also (Table 4) culturing in dark favoured callus initiation

4.2.3 Effect of cytokinin and auxin combinations on callusing in solid MS media

As presented in Table 5, a combination of auxin and cytokinin favoured the callusing. The treatments T_{34}, T_{35}, T_{36} and T_{37} had callus indices 21, 25, 31 and 50 respectively (under light) for leaf cultures. Treatment T_{37} (IAA 2.5 mg l^{-1} and BA 1 mg l^{-1} in MS medium) showed the highest callus index of 66 when incubated under dark condition.

4.2.4 Effect of cytokinin and auxin combinations on callusing in ½ MS solid media

The best treatments obtained in full strength MS media were tried with half strength MS media. The treatments T_3 and T_5 showed a very high callus index of 273 and 261 under light and 261 and 249 under dark incubated conditions respectively for leaf cultures. Compact calli were also obtained from leaf segments with petiole bases in treatments T_3 and T_5 with a very high callus index of 243 and 252 under light and 231 and 242 under dark incubated conditions respectively. The number of days taken for callus induction was also reduced to 19 days in treatment T_3 receiving a combination of IAA 2 mg l^{-1} and BA 1 mg l^{-1} (Plate 2) and treatment T_5 receiving a combination of IAA 2 mg l^{-1} and 2,4-D 1 mg l^{-1} (Plate 2a). The growth of the callus was rapid and the callus was yellowish green in colour and friable in nature (Table 6). Culturing under dark favoured earlier callusing but callus indices were higher under coninuous illumination (1000 lux) than in dark for foliar explants (Treatments T_3 and T_5 in Table 6). Thus it can be seen (Tables 4, 5 and 6) that though culturing under dark was congenial for earlier callus induction,

Table 4. Effect of cytokinins on callusing in solid MS media

Treat-ment No.	Treatment concentration MS media ($mg\ l^{-1}$)	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
1	Kinetin	*Stem	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	**Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		***L+P	0.00	0.00	-	-	0.00	0.00	-	-
		****Leaf seg	6.00	2.00	25	21	6.00	2.00	Brown	Brown
2	Kinetin	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	1	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	3.00	10.00	26	23	3.00	10.00	Brown	Brown
3	Kinetin	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	21.00	22	21	0.00	21.00	Brown	Brown
4	BA	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Petiole	20.00	21.00	25	23	20.00	21.00	Brown	Brown
		L+P	10.00	16.00	24	21	10.00	16.00	Brown	Brown
		Leaf seg	20.00	33.00	26	22	20.00	33.00	Brown	Brown
5	BA	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	1	Petiole	10.00	19.00	21	23	10.00	19.00	Brown	Brown
		L+P	30.00	48.00	26	21	30.00	48.00	Brown	Brown
		Leaf seg	49.00	70.00	27	22	49.00	70.00	Brown	Brown
6	BA	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Petiole	9.00	10.00	23	20	9.00	10.00	Brown	Brown
		L+P	20.00	39.00	24	21	20.00	39.00	Brown	Brown
		Leaf seg	30.00	46.00	26	20	30.00	46.00	Brown	Brown
7	2iP	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-

Contd.

Table 4. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
8	2iP	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	1	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
9	2iP	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
10	Kinetin +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Leaf seg	7.50	8.00	23	21	7.50	8.00	Brown	Brown
11	Kinetin +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	4.00	21.00	25	22	4.00	21.00	Brown	Brown
12	Kinetin +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	7.00	21.00	26	23	7.00	21.00	Brown	Brown
	2.5	Leaf seg	6.00	33.00	25	22	6.00	33.00	Brown	Brown
13	Kinetin +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	12.00	31.00	24	21	12.00	31.00	Brown	Brown
	0.25	Leaf seg	20.00	33.00	26	23	20.00	33.00	Brown	Brown
14	Kinetin +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	12.00	32.00	23	21	12.00	32.00	Brown	Brown
	1	Leaf seg	19.00	10.00	24	23	19.00	10.00	Brown	Brown

Contd.

Table 4. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
15	Kinetin +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	12.00	17.00	25	22	12.00	17.00	Brown	Brown
	2.5	Leaf seg	10.00	16.00	26	23	10.00	16.00	Brown	Brown
16	Kinetin +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
17	Kinetin +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
18	Kinetin +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-

* Stem - Nodal and internodal segments
** Petiole - Petiole segments
*** L + P - Leaf segments attached with petiole bases
****Leaf seg - Leaf segments

Table 5. Effect of auxins and cytokinins on callusing in solid MS media

Treatment No.	Treatment concentration MS media ($mg\ l^{-1}$)	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
1	NAA +	*Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	**Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	***L+P	17.00	26.00	27	23	17.00	26.00	Brown	Brown
	0.25 b	****Leaf seg	21.00	33.3	24	22	21.00	33.30	Brown	Brown
2	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	18.00	24.00	26	22	18.00	24.00	Brown	Brown
	0.25	Leaf seg	22.00	33.30	24	21	22.00	33.30	Brown	Brown
3	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	13.00	21.40	24	21	13.00	21.40	Brown	Brown
	1	Leaf seg	17.00	26.00	26	22	17.00	26.00	Brown	Brown
4	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	23.00	22.00	25	21	23.00	22.00	Brown	Brown
	2.5	Leaf seg	12.00	21.00	24	20	12.00	21.00	Brown	Brown
5	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Leaf seg	7.00	12.00	25	21	7.00	12.00	Brown	Brown
6	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	10.00	29.00	21	20	10.00	29.00	Brown	Brown
7	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	12.00	29.00	24	22	12.00	29.00	Brown	Brown

Contd.

Table 5. Continued

Treat-ment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
8	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Leaf seg	7.00	15.00	23	20	7.00	15.00	Brown	Brown
9	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	12.00	13.00	25	21	12.00	13.00	Brown	Brown
10	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
11	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	11.00	27.5	26	23	11.00	27.50	Brown	Brown
	0.25	Leaf seg	12.00	33.00	27	22	12.00	33.00	Brown	Brown
12	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	10.00	21.00	25	20	10.00	21.00	Brown	Brown
	1	Leaf seg	11.00	22.00	22	21	11.00	22.00	Brown	Brown
13	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	8.00	13.6	24	20	8.00	13.60	Brown	Brown
	2.5	Leaf seg	7.00	35.00	23	21	7.00	35.00	Brown	Brown
14	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	21.00	16.00	22	20	21.00	16.00	Brown	Brown
	0.25	Leaf seg	21.00	24.80	26	21	21.00	24.80	Brown	Brown

Contd.

Table 5. Continued

Treat-ment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
15	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	12.10	16.70	25	23	12.10	16.70	Brown	Brown
	1	Leaf seg	17.00	21.00	24	21	17.00	21.00	Brown	Brown
16	NAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	21.00	20.00	24	20	21.00	20.00	Brown	Brown
	2.5	Leaf seg	17.10	19.20	26	21	17.10	19.20	Brown	Brown
17	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	25.00	22.00	25	22	25.00	22.00	Brown	Brown
	0.25	Leaf seg	26.00	23.00	24	21	26.00	23.00	Brown	Brown
18	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	10.00	9.00	26	23	10.00	9.00	Brown	Brown
	1	Leaf seg	24.00	33.10	27	22	24.00	33.10	Brown	Brown
19	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	22.00	23.40	21	26	22.00	23.40	Brown	Brown
20	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	2.00	33.00	22	24	2.00	33.00	Brown	Brown
	0.25 +	L+P	2.00	5.00	21	19	2.00	5.00	Brown	Brown
	1	Leaf seg	10.00	10.00	25	24	10.00	10.00	Brown	Brown
21	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	3.00	5.50	22	21	3.00	5.50	Brown	Brown
	0.25 +	L+P	5.00	7.50	21	20	5.00	7.50	Brown	Brown
	2.5	Leaf seg	17.00	36.00	23	22	17.00	36.00	Brown	Brown

Contd.

Table 5. Continued

Treat-ment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
22	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	2.00	7.00	24	23	2.00	7.00	Brown	Brown
	1 +	L+P	5.00	7.00	25	24	5.00	7.00	Brown	Brown
	0.25	Leaf seg	10.00	12.00	26	25	10.00	12.00	Brown	Brown
23	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	9.00	7.30	25	26	9.00	7.30	Brown	Brown
	1	Leaf seg	10.00	13.00	25	24	10.00	13.00	Brown	Brown
24	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	0.00	12.00	24	23	0.00	12.00	-	Brown
25	IAA +	Stem	0.00	0.00	-	-	0.0	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	9.00	7.30	25	24	9.00	7.30	Brown	Brown
	0.25	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
26	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	3.50	7.00	25	24	3.50	7.00	Brown	Brown
	1	Leaf seg	10.00	7.00	26	25	10.00	7.00	Brown	Brown
27	IAA +	Stem	0	0	-	-	0	0	-	-
	Kinetin	Petiole	0	0	-	-	0	0	-	-
	2.5 +	L+P	0	0	-	-	0	0	-	-
	2.5	Leaf seg	2	3.3	23	24	2.00	3.30	Brown	Brown
28	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-

Contd.

Table 5. Continued

Treatment No.	Treatment concentration MS media ($mg\ l^{-1}$)	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
29	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
30	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	
	BA	Petiole	21.00	22.00	27	23	21.00	22.00	Callus yellow	
	0.25 +	L+P	23.00	23.00	24	21	23.00	23.00	first, then	
	0.25	Leaf seg	25.00	37.00	25	22	25.00	37.00	turned black	
31	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	
	BA	Petiole	23.00	25.00	21	21	23.00	25.00	Callus yellow	
	0.25 +	L+P	22.00	25.00	20	16	22.00	25.00	first, then	
	1	Leaf seg	25.00	33.30	19	17	25.00	33.30	turned black	
32	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	
	BA	Petiole	22.00	27.00	26	22	22.00	27.00	Callus yellow	
	0.25 +	L+P	24.00	30.00	16	20	24.00	30.00	first, then	
	2.5	Leaf seg	21.00	33.30	18	18	21.00	33.30	turned black	
33	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	
	BA	Petiole	21.00	25.00	23	24	21.00	25.00	Callus yellow	
	1 +	L+P	24.00	27.00	26	21	24.00	27.00	first, then	
	0.25	Leaf seg	17.00	36.00	21	18	17.00	36.00	turned black	
34	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	
	BA	Petiole	21.00	25.00	23	22	21.00	25.00	Callus yellow	
	1 +	L+P	24.00	27.00	21	18	24.00	27.00	first, then	
	1	Leaf seg	21.00	30.00	19	19	21.00	30.00	turned black	
35	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	
	BA	Petiole	17.00	15.00	20	18	17.00	15.00	Callus yellow	
	1 +	L+P	20.00	24.00	19	21	20.00	24.00	first, then	
	2.5	Leaf seg	25.00	27.00	16	18	25.00	27.00	turned black	

Contd.

Table 5. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
36	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	12.00	13.00	21	18	12.00	13.00	Callus yellow	
	2.5 +	L+P	11.00	20.00	19	20	11.00	20.00	first, then	
	0.25	Leaf seg	31.00	27.00	23	20	31.00	27.00	turned black	
37	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	23.00	22.00	21	21	23.00	22.00	Callus yellow	
	2.5 +	L+P	36.00	57.00	19	17	36.00	57.00	first, then	
	1	Leaf seg	50.00	66.00	16	17	50.00	66.00	turned black	
38	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	12.00	12.00	19	20	12.00	12.00	Callus yellow	
	2.5 +	L+P	6.60	21.00	22	19	6.60	21.00	first, then	
	2.5	Leaf seg	21.00	25.60	16	16	21.00	25.60	turned black	
39	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	13.00	17.00	21	18	13.00	17.00	Callus yellow	
	0.25 +	L+P	1.00	10.00	19	19	1.00	10.00	first, then	
	0.25	Leaf seg	7.00	8.90	16	17	7.00	8.90	turned black	
40	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
41	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	7.00	21.00	25	21	7.00	21.00	Brown	Brown
42	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	5.00	7.50	26	23	5.00	7.50	Brown	Brown

Contd.

Table 5. Continued

Treat-ment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
43	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Leaf seg	5.00	3.30	27	24	5.00	3.30	Brown	Brown
44	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	3.00	7.50	22	21	3.00	7.50	Brown	Brown
45	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	3.00	9.00	26	23	3.00	9.00	Brown	Brown
46	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Leaf seg	4.00	7.30	25	21	4.00	7.30	Brown	Brown
47	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
48	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
49	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	12.00	17.00	27	23	12.00	17.00	Brown	Brown
	0.25	Leaf seg	21.00	18.00	26	22	21.00	18.00	Brown	Brown

Contd.

Table 5. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
50	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	17.00	15.00	21	20	17.00	15.00	Brown	Brown
	1	Leaf seg	21.00	18.00	26	26	21.00	18.00	Brown	Brown
51	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	21.00	13.00	26	22	21.00	13.00	Brown	Brown
	2.5	Leaf seg	16.00	11.30	24	21	16.00	11.30	Brown	Brown
52	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Leaf seg	10.00	13.30	23	21	10.00	13.30	Brown	Brown
53	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	12.00	13.30	24	22	12.00	13.30	Brown	Brown
54	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	11.00	20.30	25	22	11.00	20.30	Brown	Brown
55	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Leaf seg	8.00	19.60	23	20	8.00	19.60	Brown	Brown
56	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	9.00	12.3	24	20	9.00	12.30	Brown	Brown

Contd.

Table 5. Continued

Treat-ment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
57	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	6.00	12.10	25	22	6.00	12.10	Brown	Brown
58	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
59	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
60	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	Kinetin	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
61	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
62	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
63	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-

Contd.

Table 5. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
64	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
65	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
66	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
67	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
68	2,4,5-T +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	2.00	7.00	21	23	2.00	7.00	Brown	Brown
69	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Leaf seg	8.00	12.30	21	23	8.00	12.30	Brown	Brown
70	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	7.00	14.10	24	21	7.00	14.10	Brown	Brown

Contd.

Table 5. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
71	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
72	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	0.25	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
73	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
74	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
75	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
76	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	1	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
77	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	2.5	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-

* Stem - Nodal and internodal segments
** Petiole - Petiole segments
*** L + P - Leaf segments attached with petiole bases
**** Leaf seg - Leaf segments

Table 6. Effect of different concentrations of auxins on callus induction of explants under light and dark conditions in solid ½ MS media

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
1	IAA + BA 1 + 1	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	11.40	13.30	27	26	11.40	13.30	Brown	Brown
		L+P	17.00	13.80	25	22	17.00	13.80	Brown	Brown
		Leaf seg	20.00	22.60	26	23	20.00	22.60	Brown	Brown
2	IAA + BA 2.5 + 1	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	10.00	22.00	28.00	27	10.00	22.00	Brown	Brown
		L+P	12.30	17.60	26.00	25	12.30	17.60	Brown	Brown
		Leaf seg	27.00	18.10	23.00	24	27.00	18.10	Brown	Brown
3	IAA + BA 2 + 1	Stem	70.00	60.00	29	26	70.00	60.00	Brown	Brown
		Petiole	87.00	73.00	28	24	87.00	73.00	Brown	Brown
		L+P	81.00	77.00	16	20	243.00	231.00	Yellow	White
		Leaf seg	91.00	87.00	19	18	273.00	261.00	Yellow	Yellow
		Fruit	-	-	-	-	-	-	-	-
4	IAA + BA 0.25 + 1	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	12.00	13.40	29	28	12.00	13.40	Brown	Brown
		L+P	21.40	22.30	23	22	21.40	22.30	Yellow	White
		Leaf seg	33.30	27.80	25	25	33.30	27.80	White	White
		Fruit	-	-	-	-	-	-	-	-
5	IAA + 2,4-D 2 + 1	Stem	61.00	72.00	30	33	61.00	72.00	Brown	Brown
		Petiole	71.00	62.00	29	29	71.00	62.00	Cream	Cream
		L+P	84.10	80.20	18	17	252.00	242.00	Yellow	White
		Leaf seg	87.30	83.10	19	19	261.00	249.00	White	White
		Fruit	-	-	-	-	-	-	-	-
6	IAA + 2,4-D 2.5 + 1	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	13.10	11.30	28	28	13.10	11.30	Brown	Brown
		L+P	18.90	14.00	21	22	18.90	14.00	Brown	Brown
		Leaf seg	31.30	27.00	26	24	31.30	27.00	Brown	Brown

Contd.

Table 6. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
7	IAA +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D	Petiole	9.00	8.50	27	26	9.00	8.50	Brown	Brown
	1 + 1	L+P	13.00	17.00	25	24	13.00	17.00	Brown	Brown
		Leaf seg	29.00	33.00	26	27	29.00	33.00	Brown	Brown
8	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	7.00	8.50	27	27	7.00	8.50	Brown	Brown
	2 + 1	L+P	12.10	17.30	26	25	12.10	17.30	Brown	Brown
		Leaf seg	23.20	28.10	27	26	23.20	28.10	Brown	Brown
9	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T	Petiole	12.30	11.70	28	27	12.30	11.70	Brown	Brown
	2 + 2.5	L+P	13.00	17.10	25	24	13.30	17.10	Brown	Brown
		Leaf seg	12.50	9.30	26	27	12.50	12.50	Brown	Brown
10	2,4-D +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	NAA	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2 + 1	L+P	13.40	17.80	21	26	13.40	17.80	Brown	Brown
		Leaf seg	21.30	21.60	28	25	21.30	21.60	Brown	Brown
11	IAA 2	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA 1	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	For stem	L+P	0.00	0.00	-	-	0.00	0.00	-	-
	charcoal 0.5	Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
12	IAA 2	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	BA 0.5	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
13	IAA 2	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D 1	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-

Contd.

Table 6. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
14	2,4,5-T 2	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D 1	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
15	2,4-D 0.25	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
16	2,4-D 1	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
17	2,4-D 2.5	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
18	2,4-D 5	Stem	0.00	0.00	-	-	0.00	0.00	-	-
		Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
19	NAA 2	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4,5-T 1	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-

Contd.

Table 6. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
20	NAA 2	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2,4-D 1	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
21	Kinetin	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 0.25	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	4.00	-	26	0.00	8.00	-	Brown
22	Kinetin	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 1	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	2.10	-	25	0.00	4.20	-	Brown
23	Kinetin	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 2.5	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	10.00	-	24	20.00	0.00	-	Brown
24	Kinetin	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 0.25	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	11.00	-	25	0.00	22.00	-	Brown
25	Kinetin	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 1	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	2.00	-	23	0.00	4.00	-	Brown
26	Kinetin	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	1 +	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 2.5	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-

Contd.

Table 6. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
27	Kinetin	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 0.25	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
28	Kinetin	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 1	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
29	Kinetin	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2.5 +	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 2.5	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
30	Kinetin	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	0.25 +	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 0.25	L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
31	BA 2.5 +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 1	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	7.00	8.50	9	20	7.00	8.50	Brown	Brown
32	BA 0.25 +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 2.5	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	3.30	7.00	18	21	3.30	7.00	Brown	Brown
33	BA 1 +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 0.25	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	5.00	8.00	26	23	5.00	8.00	Brown	Brown

Contd.

Table 6. Continued

Treatment No.	Treatment concentration MS media (mg l^{-1})	Explant used	% culture initiating callus		Days to initiate callus		Callus index		Nature of callus	
			light	dark	light	dark	light	dark	light	dark
34	BA 1 +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 1	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
35	BA 1 +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 2.5	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
36	BA 2.5 +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 0.25	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
37	BA 2.5 +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 1	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	
		Leaf seg	0.00	0.00	-	-	0.00	0.00	-	-
38	BA 2.5 +	Stem	0.00	0.00	-	-	0.00	0.00	-	-
	2iP 2.5	Petiole	0.00	0.00	-	-	0.00	0.00	-	-
		L+P	0.00	0.00	-	-	0.00	0.00	-	-
		Leaf seg	10.00	33.30	25	22	10.00	33.30	Brown	Brown
39	¼ PO_4 + IAA 2 + BA 1	Fruit	33.30	0.00	24	-	33.30	0.00	Yellow Friable	- -

* Stem - Nodal and internodal segments
** Petiole - Petiole segments
*** L + P - Leaf segments attached with petiole bases
**** Leaf seg - Leaf segments
***** Activated charcoal at 0.5mgl^{-1} was added to media in which stem segments were cultured

Plate 2. Compact callus obtained from leaf segment cultured on solid ½ MS medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}

Plate 2a. Compact callus obtained from leaf segment cultured on solid ½ MS medium supplemented with IAA 2 mg l^{-1} and 2,4-D 1 mg l^{-1}

Plate 2

Plate 2a

culturing under continuous illumination (1000 lux) favoured the callus proliferation (Table 4, 5 and 6).

In other treatments, callusing was obtained but the callus turned brown and died later on. Callusing was obtained from the immature fruit explants (Plate 3) with a callus index of 66 when cultured on solid ½ MS medium with phosphate ions reduced to 25 per cent supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1} (Treatment T_{39} in Table 6).

4.2.5 Standardisation of basal media for inducing calli

Response of the explants to various growth regulator combinations for initiation of the callus are given in Tables 3, 4, 5 and 6.

It can be seen from these Tables that half strength MS basal media was suitable for callus induction. Callus induction was observed initially from the cut edges of the leaf, from the midrib of leaf segments and from the basal portion of the stem explants. In MS media, brown coloured callus was observed from the explant, which however did not continue its growth.

4.2.6 Suitability of explants employed in initiation and proliferation of calli

It can be seen from Tables 3, 4, 5 and 6 that the leaf segments and leaf segments attached with petiole bases readily induced callus than other explants (stem and petiole). The callus obtained from leaf segment and leaf segment attached with petiole base are presented in Plates 4 and 5 respectively. Cultures obtained from leaf segments exhibited the highest callus growth rate in ½ MS solid media (Treatment T_3 and T_5 in Table 6). Hence leaf segments were most suitable as explants for induction and proliferation of calli.

Plate 3. Callusing from immature fruit of *C. fenestratum*

Plate 3

Plate 4. Green coloured callus obtained from leaf segment

Plate 5. Callus obtained from leaf segment with petiole base

Plate 4

Plate 5

4.3 Influence of culture environment on callusing

Induction of callus was better under dark with respect to the number of days taken for callus initiation, percentage of cultures initiating calli for the explants (stem, petiole, leaf segments attached with petiole bases and leaf segments) and callus indices in solid MS media (Table 3, 4 and 5).

Callus indices were found to be maximum under light for leaf segments when cultured on solid ½ MS media (Table 6). Culturing under dark favoured callus induction and reduced the number of days taken for callus initiation for all the explants when cultured on solid ½ MS media. The percentage of cultures initiating callus was also found to be higher under light for all the explants in most of the treatments in solid ½ MS media (Table 6).

4.4 Regeneration of calli

Calli initiated from ½ MS medium enriched with IAA 2 mg l^{-1} + BA 1 mg l^{-1} and IAA 2 mg l^{-1} + 2,4-D 1 mg l^{-1} were used for attempting regeneration from calli.

4.4.1 Effect of different growth regulators and additives on callus regeneration

The results of the attempts to induce regeneration are presented in Tables 7, 8 and 9.

The calli though subcultured into media with different growth regulator combinations did not respond through regeneration of adventitious shoots or through somatic embryogenesis. Treatments with BA 0.25 and 0.5 mg l^{-1} incubated under light resulted in the callus mass acquiring a greenish colouration and a compact nature (Plates 6 and 7 respectively).

Table 7. Effect of different auxins and auxin synergist on callus regeneration

Treatment No.	Media in which explants were raised originally ½ MS solid + growth regulator (mg l^{-1})	Explant	Callus induction media ½ MS + growth regulators (mg l^{-1})	Subculture No.	Response of callus
1	2 IAA + 1 BA	Leaf bit	IAA 0.25	1	Callus turned brown & dried
2	2 IAA + 1 BA	,,	IAA 0.50	1	,,
3	1 IAA + 1 BA	,,	IAA 10.00	1	,,
4	2 IAA + 1 2,4-D	,,	IBA 0.25	1	,,
5	2 IAA + 1 2,4-D	,,	IBA 5.00	1	,,
6	2 IAA + 1 2,4-D	,,	1BA 10.00	1	,,
7	2 IAA + 1 2,4-D	,,	NAA 0.25	2	Callus turned to green then to brown & dried
8	2 IAA + 1 2,4-D	,,	NAA 5.00	1	,,
9	2 IAA + 1 2,4-D	,,	NAA 10.00	1	Callus turned brown & dried
10	2 IAA + 1 BA	,,	IAA + 1BA 0.5+0.5	1	,,
11	2 IAA + 1 BA	,,	IAA + 1BA 1.0+0.5	1	,,
12	2 IAA + 1 BA	,,	IAA + NAA 0.5+0.5	1	,,
13	2 IAA + 1 BA	,,	IAA + NAA 0.5+1.0	2	Callus turned to green then to brown & dried
14	2 IAA + 1 BA	,,	1BA + NAA 0.5+0.5	1	Callus turned to brown & dried
15	2 IAA + 1 BA	,,	IBA + NAA 1.0+5.0	1	,,
16	2 IAA + 1 BA	,,	1BA + Phloroglucinol 1.0+50.0	1	,,
17	2 IAA + 1 BA	,,	1BA + Phloroglucinol 0.5+100.0	1	,,
18	Basal medium (½ MS)	,,	,,		,,

Table 8. Effect of different cytokinins on callus regeneration

Treatment No.	Media in which explants were raised orginally ½ MS solid + growth regulator (mg l^{-1})	Explant	Callus induction media ½ MS + growth regulators (mg l^{-1})	Subculture No.	Response of callus
1	2 IAA + 1 BA	Leaf bit	Kinetin 0.25	2	Callus turned green and then yellow
	,,				
	,,		Kinetin 5.00	1	Callus turned green
	,,		Kinetin 10.00	1	Callus turned green followed by browning
2	2 IAA + 1 2,4-D	Leaf bit	Kinetin 0.25	1	Callus turned green & then brown
	,,				
	,,		Kinetin 5.00	1	,,
	,,		Kinetin 10.00	1	,,
3	2 IAA + 1 BA		BA 0.25	3	Callus turned
	,,		BA 0.50	2	green & then yellow
	,,		BA 5.00	1	Callus brown
	,,		BA 5.00	3	Callus turned green & then yellow
	,,		BA 5.00	1	Callus turned
	,,		BA 10.00	1	brown & then died
4	2 IAA + 1 2,4-D	Leaf bit	Kinetin+BA 0.25+0.5	1	Callus turned
	,,		Kinetin+BA 0.5+1.0	1	brown
	,,		Kinetin+BA 1.0+1.0	1	,,
5	2 IAA + 1 BA	Leaf bit	Kinetin+2iP 1.0+0.5	1	,,
6	2 IAA + 1 BA	Leaf bit	2iP 0.25	1	,,
	,,		2iP 5.00	1	,,
	,,		2iP 10.00	1	,,
7	Basal medium (½ MS)	Leaf bit	-	2	Callus turned green ,then white & then brown

Table 9. Response of growth regulators and different media additives on callus regeneration

Treatment No.	Media in which explants were raised orginally ½ MS solid + growth regulator (mg l^{-1})	Explant	Callus induction media ½ MS + growth regulators (mg l^{-1})	Subculture No.	Response of callus
1	2 IAA + 1 BA	Leaf bit	2,4-D 2.5	1	Callus turned brown
2	,,	,,	2,4-D 0.25	1	,,
3	,,	,,	2,4-D 0.5	1	,,
4	,,	,,	2,4-D 5.0	1	,,
5	,,	,,	2,4-D+BA 0.25+0.25	1	,,
6	,,	,,	BA 0.25	3	Callus turned yellow
7	,,	,,	BA 0.5	3	,,
8	,,	,,	BA 2.5	1	Callus turned brown
9	,,	,,	BA 5.0	1	,,
10	,,	,,	Kinetin 0.25	3	Callus turned yellow
11	,,	,,	Kinetin 2.5	1	Callus turned brown
12	,,	,,	Kinetin 5.0	1	,,
13	,,	,,	Kinetin+BA 1.0+1.0	1	,,
14	,,	,,	CCC 0.1	1	,,
15	,,	,,	CCC 0.2	1	,,
16	½ MS basal	,,	-	1	,,

Plate 6. Green coloured callus obtained from leaf segment cultured on solid ½ MS medium supplemented with BA 0.25 mg l^{-1}

Plate 7. Green coloured callus obtained from leaf segment cultured on solid ½ MS medium supplemented with BA 0.5 mg l^{-1}

Plate 6

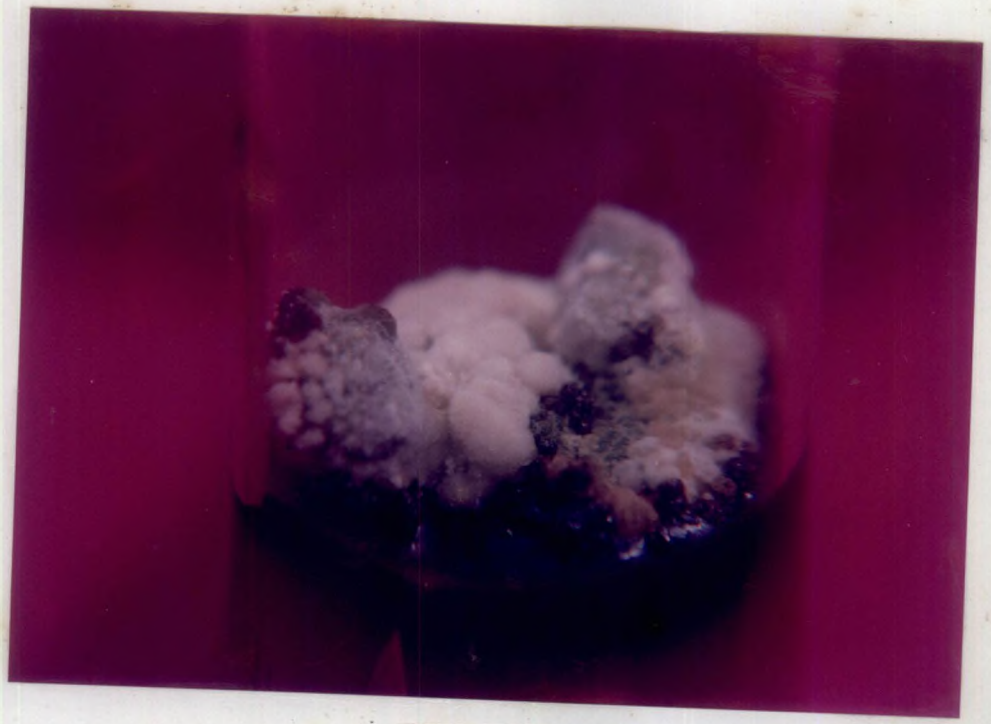


Plate 7

4.4.2 Effect of different carbohydrate sources and osmoregulants on callus regeneration

The results of the effect of different carbohydrate sources and osmoregulants on callus regeneration are presented in Tables 10 and 11. None of the treatments were able to produce callus regeneration.

4.5 Effect of various media on nodal segments

Among the different media tried for regeneration from nodal segments, half strength SH medium enriched with BA 2.5 mg l^{-1} elicited a yellow callus growth from the base of the nodal segments. The browning was highest in the case of nodal segments comparing other explants viz., leaf segments, leaf segments attached with petiole bases and petioles.

4.5.1 Effect of media supplements on nodal and internodal segments

The addition of phloroglucinol, activated charcoal and adenine sulphate at different concentrations failed to elicit regeneration (Table 13).

4.5.2 Effect of liquid media on nodal and internodal segments

Different liquid media tried with growth regulators at varying levels failed to induce any growth and there was no yellow colouration in the liquid medium (Table 14).

4.6 Screening of calli for berberine production obtained from solid medium

4.6.1 Preliminary tests

Qualitative tests were conducted for the presence of alkaloids in methanol extracts of calli obtained from different treatments in ½ MS medium. It

Table 10. Effect of different carbohydrate sources on callus regeneration

Source of carbon	Concentration per cent	Media in which explants were raised originally ½ MS solid + growth regulators (mg l^{-1})	Explant	Subculture No.	Response of callus
Maltose	3.0	2 IAA + 1 BA	Leaf bit	1	Callus turned brown
Lactose	3.0	,,	,,	2	Callus turned yellow then to brown
Glucose	3.0	,,	,,	1	Callus turned to brown
Sucrose	5.0	,,	,,	2	,,
Sucrose	8.0	,,	,,	1	,,
Maltose+sucrose	1.5+1.5	,,	,,	1	,,
Lactose+sucrose	1.5+1.5	,,	,,	1	,,
Lactose+sucrose	2.0+1.0	,,	,,	1	,,
Basal medium (½ MS)	-	-	,,	1	,,

Table 11. Effect of various osmoregulants on callus regeneration

Source of carbon	Concentration per cent	Media in which explants were raised originally ½ MS solid + growth regulators (mg l^{-1})	Explant	Subculture No.	Response of callus
Sorbitol	1.5	2 IAA + 1 BA	Leaf bit	1	Callus turned brown
	2.0	,,	,,	2	Callus turned yellow then to brown
	3.0	,,	,,	2	,,
D Mannitol	1.5	,,	,,	1	Callus turned brown
	2.0	,,	,,	1	,,
	3.0	,,	,,	1	,,
PEG	1.5	,,	,,	1	,,
	2.0	,,	,,	1	,,
	3.0	,,	,,	1	,,
Basal medium (½ MS)	-	-	,,	1	,,

Table 12. Effect of various media on nodal and internodal segments of *Coscinium fenestratum*

Treatment No	Treatment Growth regulator	concentration + charcoal 0.5 mg l^{-1}	Basal media	Response of nodal and internodal segments
1	BA	0.25	½ SH	Yellow callusing from the base of nodal segments later dried up
2	BA	1.0	½ SH	,,
3	BA	2.5	½ SH	Yellow callus from the base which sustained callus growth
4	BA	5.0	½ SH	Yellow callusing from the base of nodal segments later dried up
5	IAA+2,4-D	2+1	½ SH	,,
6	IAA+2,4-D	1+2	½ SH	,,
7	CCC + BA	2+1	½ SH	,,
8	BA	0.5	½ SH	,,
9	BA	7.5	½ SH	,,
10	BA	0.25	SH	,,
11	BA	5.0	SH	,,
12	BA	1.0	SH	,,
13	BA	1.5	SH	,,
14	BA	2.5	SH	,,
15	BA	5.0	SH	,,
16	IAA+2,4-D	2+1	SH	,,
17	IAA+BA	2+1	SH	,,
18	Kin	0.25	½ SH	,,
19	Kin	2.5	½ SH	Brown callus from the base of nodal segments
20	Kin	1.0	½ SH	,,
21	BA	0.25	½ WPM	,,
22	BA	0.5	½ WPM	,,
23	BA	1.0	½ WPM	,,
24	BA	2.5	½ WPM	,,
25	BA	5.0	½ WPM	,,
26	BA	0.25	WPM	,,
27	BA	0.5	WPM	,,
28	BA	1.0	WPM	,,
29	BA	2.5	WPM	,,
30	½ MS basal	-	-	,,

Table 13. Effect of different media supplements on nodal and internodal segments

Additives	Concentrations (mg)	Basal media	Response
Phloroglucinol	100.00	½ MS + 2 IAA + 1 BA	-
,,	125.00	,,	-
Activated charcoal	0.50	,,	Yellow callusing at the base of node which later dried up
,,	0.25	,,	,,
Adenine sulphate	2.00	,,	,,
,,	1.00	,,	,,
Basal medium (½ MS)	,,	,,	Callus turned brown

Table 14. Effect of different liquid media on establishment of shoot tip explants of *Coscinium fenestratum*

Treatment No.	Liquid meida	Treatment		Establishment
		Growth regulator	Concentration (mg l^{-1})	
1	MS	IAA + BA	2 + 1	Nil
2	MS	IAA + 2,4-D	2 + 1	Nil
3	MS	BA	0.5	Nil
4	M S	BA	1	Nil
5	MS	BA	2.5	Nil
6	½ MS	BA	0.5	Nil
7	½ MS	BA	1	Nil
8	½ MS	BA	2.5	Nil
9	½ MS	IAA + BA	2 + 1	Nil
10	½ MS	IAA + 2,4-D	2 + 1	Nil
11	½ WPM	IAA + BA	2 + 1	Nil
12	½ WPM	IAA + 2,4-D	2 + 1	Nil
13	½ WPM	BA	0.5	Nil
14	½ WPM	BA	1	Nil
15	½ WPM	BA	2.5	Nil
16	½ SH	IAA + 2,4-D	2+1	Nil
17	½ SH	IAA + BA	2+1	Nil
18	½ SH	BA	0.5	Nil
19	½ SH	BA	1	Nil
20	½ SH	BA	2.5	Nil
21	WPM	BA	0.5	Nil
22	WPM	BA	1	Nil
23	WPM	BA	2.5	Nil
24	SH	BA	0.5	Nil
25	SH	BA	1	Nil
26	SH	BA	2.5	Nil
27	Basal medium (½ MS)	-	-	Nil

can be observed that the Treatments T_2, T_4, T_6, T_{12} and T_{13} indicated the presence of berberine (Table 15).

As is evident from Table 16, yellow spots identical to that of standard berberine hydrochloride were observed in TLC at 365 nm. Treatments T_2, T_3, T_5, T_{12}, T_{13}, T_{14}, T_{15}, T_{16}, T_{17}, T_{18} gave yellow spots and had an Rf value of 0.44 which is similar to that of standard berberine hydrochloride indicating the presence of berberine in the callus tissues (Plate 8).

4.7 Optimisation of ½ MS solid medium for *in vitro* metabolite production

4.7.1 Effect of incorporation of media additives

Incorporation of media additives like phloroglucinol to the basal medium (½ MS) followed by subsequent subculture did not result in callus induction (Table 17).

4.7.2 Incorporation of growth retardants/inhibitors

Incorporation of growth retardant, cycocel (CCC) or growth inhibitor abscissic acid (ABA) each at 0.25, 0.75 and 1 mg l^{-1} were incorporated to the production medium and subsequent subculture was done. A low growth of callus was observed in the production medium enriched with 0.25 mg l^{-1} ABA (Table 18 and Plate 9). The methanol extract of the above callus gave positive results in the qualitative and chromatographic test for berberine (Tables 15 and 16).

4.7.3 Effect of increasing concentration of agar

Higher levels of agar added to the medium did not sustain the callus development (Table 19).

Table 15. Influence of hormonal combinations on expression of alkaloids in calli of *Coscinium fenestratum*

No.	Basal medium	Hormonal combination	Concentration (mg l^{-1})	Dragendorff's reagent	Mayer's reagent
1	½MS	NAA+BA	2+1	-ve	-ve
2	½MS	BA	0.25	+ve	+ve
3	½MS	BA	2.5	-ve	-ve
4	½MS	BA	0.5	+ve	+ve
5	½MS	IAA+2,4-D	2+1	+ve	+ve
6	½MS	Kinetin	0.25	+ve	+ve
7	½ MS	Kinetin	2.5	-ve	-ve
8	½ MS	Kinetin	0.5	-ve	-ve
9	½ MS	2,4-D	0.25	-ve	-ve
10	½ MS	2,4-D	2.5	-ve	-ve
11	½ MS	2,4-D	5	-ve	-ve
12	½ MS	IAA+BA	2+1	+ve	+ve
13	½ MS	IAA+BA+ABA	2+1+0.25	+ve	+ve

Dragendorff's reagent +ve - orange precipitate
Mayer's reagent +ve - white precipitate

Table 16. Effect of media on production of alkaloids as detected by TLC

Sl. No.	Basal media	Amount of callus (g)	Growth regulators	Concentration (mg l^{-1})	Age of callus (months)	Rf value	Colour of spot at 365 nm	Berberine
1	½ MS	2.50	NAA + BA	2 + 1	5	0.000	No spot	Absence
2	,,	1.00	BA	0.25	5	0.442	Yellow	Present
3	,,	1.00	BA	0.50	5	0.446	Yellow	Present
4	,,	1.00	BA	2.50	5	0.000	No spot	Absent
5	,,	1.30	Kin	0.25	5	0.446	Yellow	Present
6	,,	1.29	Kin	0.50	5	0.396	Feeble yellow	Absent
7	,,	1.00	Kin	2.50	5			
8	,,	2.50	IAA+2,4-D	2+1	4	0.412	No spot	Absent
9	,,	2.50	IAA+2,4-D	2+1	8	0.344	No spot	Absent
10	,,	2.50	IAA+BA	2+1	3	0.421	Feeble spot	Absent
11	,,	2.50	IAA+BA	2+1	5 ½	0.445	Yellow	Present
12	,,	2.50	IAA+BA	2+1	7	0.443	Yellow	Present
13	,,	2.50	IAA+BA	2+1	9	0.443	Yellow	Present
14	,,	2.50	IAA+BA	2+1	12	0.447	Yellow	Present
15	,,	2.30	IAA+BA	2+1	15	0.441	Yellow	Present
16	,,	2.50	IAA+BA	2+1	17	0.440	Yellow	Present
17	,,	1.30	IAA+BA+ABA	2+1+0.25	5	0.440	Yellow	Present
18	Standard Berberine	0.01	-	-	-	0.440	Yellow	Present

*Solvents system - Propanol:acetic acid:water (7:1:2)

Plate 8. TLC profile of the extracts of leaf induced calli screened for berberine content

1. Standard berberine hydrochloride
2. 5 months old callus obtained from solid ½ MS medium + BA 0.25 mg l^{-1}
3. 5 months old callus obtained from solid ½ MS medium + BA 0.50 mg l^{-1}
4. 5 months old callus obtained from solid ½ MS medium + Kin 0.25 mg l^{-1}
5. 5 months old callus obtained from solid ½ MS medium + Kin 0.50 mg l^{-1}
6. 5 months old callus obtained from solid ½ MS medium + IAA 2 mg l^{-1} + BA 1 mg l^{-1} + ABA 0.25 mg l^{-1}
7. 7 months old callus obtained from solid ½ MS medium + IAA 2 mg l^{-1} + BA 1 mg l^{-1}
8. 12 months old callus obtained from solid ½ MS medium + IAA 2 mg l^{-1} + BA 1 mg l^{-1}
9. 15 months old callus obtained from solid ½ MS medium + IAA 2 mg l^{-1} + BA 1 mg l^{-1}
10. 9 months old callus obtained from solid ½ MS medium + IAA 2 mg l^{-1} + BA 1 mg l^{-1}
11. 4 months old callus obtained from solid ½ MS medium + IAA 2 mg l^{-1} + 2,4-D 1 mg l^{-1}
12. 8 months old callus obtained from solid ½ MS medium + IAA 2 mg l^{-1} + 2,4-D 1 mg l^{-1}
13. 17 months old callus obtained from solid ½ MS medium + IAA 2 mg l^{-1} + BA 1 mg l^{-1}

Plate 9. Yellowish callus obtained from solid ½ MS medium supplemented with IAA 2 mg l^{-1}, BA 1 mg l^{-1} and ABA 0.25 mg l^{-1}

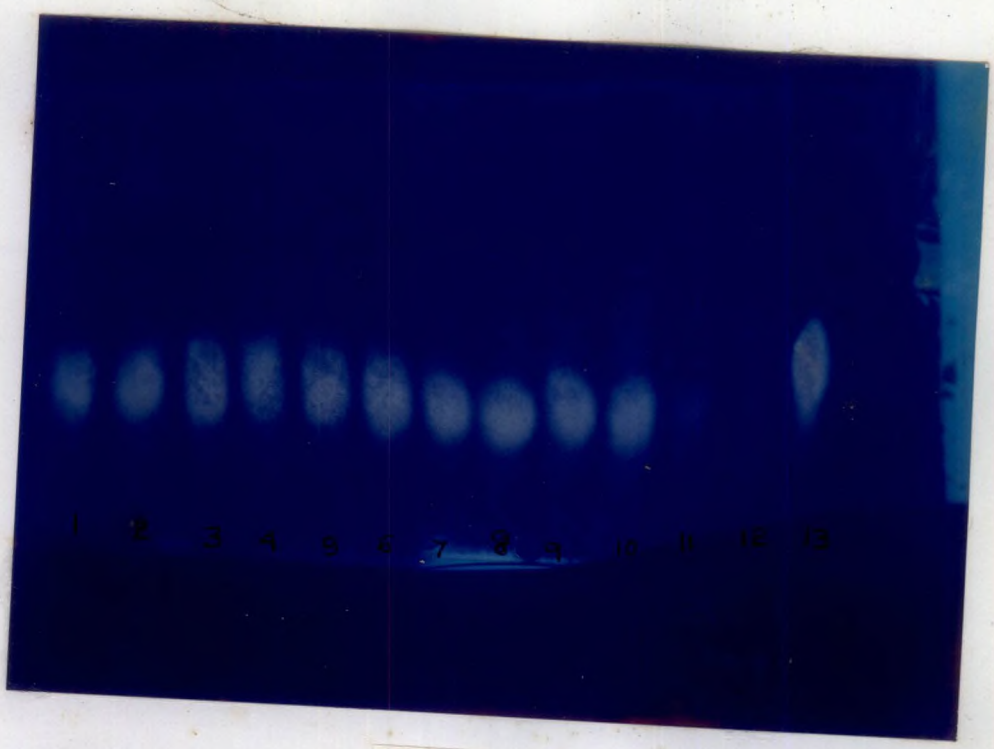


Plate 8

Plate 9

Table 17. Effect of different concentrations of media additives

Treat-ment No.	Media additive	Concentration (mg l^{-1})	Media in which explants were raised originally ½ MS solid + growth regulator (mg l^{-1})	Explant	Subculture No.	Response of callus
1	Phloroglucinol	100.00	½ MS + 2 IAA + 1 BA	Leaf bit	1	Callus turned brown
2	Phloroglucinol	125.00	,,	Leaf bit	1	,,
3	Adenine sulphate	0.50	,,	Leaf bit	1	,,
4	Adenine sulphate	1.00	,,	Leaf bit	1	,,
5	Adenine sulphate	1.50	,,	Leaf bit	1	,,
6	Adenine sulphate	2.00	,,	Leaf bit	1	,,
7	Adenine sulphate	2.50	,,	Leaf bit	1	,,
8	Basal medium (½ MS)	-	-	Leaf bit	1	,,

Table 18. Effect of different concentrations of growth retardants / inhibitors

Treat-ment No.	Growth retardant	Concentration (mg l^{-1})	Media in which explants were raised originally ½ MS solid + growth regulator (mg l^{-1})	Explant	Subculture No.	Response of callus
1	Cycocel	0.25	,,	Leaf bit	1	Callus turned brown
2	Cycocel	0.75	,,	Leaf bit	1	,,
3	Cycocel	1.00		Leaf bit	1	,,
4	ABA	0.25	½ MS + 2 IAA + I BA	Leaf bit	2	Yellow Callus
5	ABA	0.75	,,	Leaf bit	2	Callus turned brown
6	ABA	1.00	,,	Leaf bit	1	Callus turned brown
7	Basal medium (½ MS)	-	-	Leaf bit	1	Callus turned brown

4.7.4 Effect of modification of carbon source

Increasing the levels of sucrose in basal medium (½ MS) (4.0, 5.0 and 8.0 per cent) and substituting sucrose with maltose totally or in proportions of 1:1 or 1:2 did not even sustain the callus development (Table 20).

4.7.5 Effect of withdrawal of inorganic nutrients

Withdrawing nitrogen and phosphorus from the ½ MS medium to half and ¼ of their original concentration did not sustain callus development (Table 21).

4.7.6 Effect of addition of osmoregulants in solid ½ MS medium

None of the osmoregulants, added to the basal growth medium such as polyethylene glycol, mannitol and sorbitol (1:5-3%) could sustain callus growth as evident from Table 11.

4.7.7 Effect of addition of spermidine

Spermidine (0.25, 0.5 and 2.5 mg l^{-1}) added to the basal growth medium failed to sustain callus growth (Table 22).

4.8 Optimisation of liquid medium for *in vitro* metabolite production

Suspension cultures were established by subculturing friable callus (4 to 12 months age) in liquid media supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}

4.8.1 Effect of modification of carbon source in liquid ½ MS medium

The carbon source in the liquid medium was modified by increasing levels of sucrose to four, five and eight per cent. Sucrose was replaced by maltose, lactose and galactose (each at 3%, 5% and 8% respectively). However they failed to sustain callus growth and calli turned brown and died (Table 23).

Table 19. Effect of different concentrations of agar

Treatment No.	Agar concentration (%)	Media in which explants were raised originally ½ MS solid + growth regulator (mg l^{-1})	Explant	Subculture No.	Response of callus
1	2.0	½ MS + 2 IAA + I BA	Leaf bit	1	Callus turned brown
2	2.5	,,	Leaf bit	1	,,
3	3.0	,,	Leaf bit	1	,,
4	3.5	,,	Leaf bit	1	,,
5	5.0	,,	Leaf bit	1	,,
6	Basal medium (½ MS)	-	Leaf bit	1	,,

Table 20. Effect of different carbon sources

Treatment No.	Source of carbon	Concentration (%)	Media in which explants were raised originally ½ MS solid + growth regulator (mg l^{-1})	Explant	Subculture No.	Response of callus
1	Sucrose	4.0	2 IAA + 1 BA	Leaf bit	1	Callus turned brown
2	,,	5.0	,,	Leaf bit	1	,,
3	,,	8.0	,,	Leaf bit	1	,,
4	Maltose+Sucrose	1.5 + 1.5	,,	Leaf bit	1	,,
5	Lactose+Sucrose	1.5 +1.5	,,	Leaf bit	1	,,
6	Maltose+Sucrose	1.0+2.0	,,	Leaf bit	1	,,
7	Basal medium (½ MS)	-	-	Leaf bit	1	,,

Table 21. Effect of withdrawal of inorganic nutrients

Treatment	Media	Reduction of inorganic nutrients	Media in which explants were raised originally ½ MS solid + growth regulator (mg l^{-1})	Explant	Subculture No.	Response of callus
1	½ MS	½ NO_3	2 IAA+1 BA	Leaf bit	2	Callus turned brown
2	½ MS	1/3 NO_3	,,		1	,,
3	½ MS	¼ NO_3	,,		2	,,
4	½ MS	½ PO_4	,,		1	,,
5	½ MS	1/3 PO_4	,,		1	,,
6	½ MS	¼ PO_4	,,		1	,,
7	½ MS	½ NO_3+½ PO_4	,,		1	,,
8	Basal medium (½ MS)	-	-		1	,,

Table 22. Effect of different concentration of spermidine

Treatment No.		Concentration (mg l^{-1})	Basal solid medium	Subculture No.	Response of callus
1		0.25	½ MS+2 IAA + 1 BA	1	Callus turned brown
2		0.50	,,	1	,,
3		1.00	,,	1	,,
4		1.50	,,	1	,,
5		2.00	,,	1	,,
6		2.50	,,	1	,,
7	Control - Basal medium (½ MS)	-	-	1	,,

Table 23. Effect of different concentrations of carbon sources in liquid medium

Treatment No.	Stress inducer	Concentration (mg l^{-1})	Response of callus
1	Sucrose	4	Callus turned brown
2	,,	5	,,
3	,,	8	,,
4	Maltose	4	,,
5	,,	5	,,
6	,,	8	,,
7	Lactose	4	,,
8	,,	5	,,
9	,,	8	,,
10	Galactose	4	,,
11	,,	5	,,
12	,,	8	,,
13	Control - Basal medium (½ MS)	-	,,

Basal medium - ½ MS liquid medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}
Subculture number 2.

4.8.2 Effect of addition of spermidine to the liquid ½ MS medium

The effect of addition of spermidine at 10 µg, 30 µg, 50 µg, 60 µg and 100 µg to the basic production medium and also to the medium supplemented with NAA 2 mg l^{-1} and BA 1 mg l^{-1} was tested. A yellow exudate was visible in the liquid medium. The yellow coloured extract was separated from the liquid medium by adsorption chromotgraphy and TLC was performed which indicated the presence of berberine (Table 24 and Plate 10).

4.8.3 Effect of withdrawal of inorganic nutrients from the liquid ½ MS medium

Nitrate and phosphate ion supply of ½ MS basal liquid medium was reduced to 75, 50 and 25 per cent and calli grown on them for 4 weeks. The adsorption chromatography was carried out and TLC performed for the detection of berberine which indicated the presence of berberine (Table 26 and Plate 11).

4.9 Quantification of the active principle

Callus underwent darkening and died after one week for many of the treatments tried for optimisation of berberine production. There were only few treatments which sustained the callus growth. The callus obtained from these treatments were subjected to qualitative tests for detection of berberine. As the callus growth was slow, the quantification of berberine was done from small quantities of callus.

4.9.1 Quantification of berberine from liquid medium enriched with varying concentration of spermidine

For studying the effect of different concentration of spermidine on berberine synthesis, the cells grown in production medium for 25 days after

Table 24. Effect of different concentrations of spermidine on alkaloid expression in ½ MS liquid medium

No.	Liquid media with growth hormones	Concentration of Spermidine (µg)	Rf value	Berberine
1	*P	10	0.442	Present
2	P	30	0.443	Present
3	P	50	0.443	Present
4	P	60	0.441	Present
5	P	100	0.442	Present
6	*Q	10	0.443	Present
7	Q	30	0.441	Present
8	Q	50	0.443	Present
9	Q	60	0.441	Present
10	Q	100	0.438	Present

*P = ½ MS liquid medium + IAA 2 mg l^{-1} + BA 1 mg l^{-1}
*Q = ½ MS liquid medium + NAA 2 mg l^{-1} + BA 1 mg l^{-1}
Solvent system used - propanol:acetic acid:water (7:2:1)
Quantity of liquid medium for P and Q - 25ml

Plate 10. Effect of different concentration of spermidine on berberine synthesis in the production medium as detected by TLC

P - ½ MS liquid medium + IAA 2 mg l^{-1} + BA 1 mg l^{-1}
Q - ½ MS liquid medium + NAA 2 mg l^{-1} + BA 1 mg l^{-1}

1. Q + 30 µg spermidine
2. Q + 50 µg spermidine
3. Q + 60 µg spermidine
4. Q + 100 µg spermidine
5. P + 30 µg spermidine
6. P + 50 µg spermidine

7 and 12. P + 60 µg spermidine

8. P + 100 µg spermidine
9. Standard berberine hydrochloride
10. P + 10 µg spermidine
11. Q + 10 µg spermidine

Plate 11. Effect of withdrawal of inorganic nutrients on berberine synthesis in the production medium as detected by TLC

P - ½ MS liquid medium + IAA 2 mg l^{-1} + BA 1 mg l^{-1}
Q - ½ MS liquid medium + IAA 2 mg l^{-1} + 2,4-D 1 mg l^{-1}

1. 1 and 1a, P¼ NO_3 7 month old callus
2. 2 and 2a, P½ NO_3 7 month old callus
3. 3 and 3a, P¼ PO_4 7 month old callus
4. 4, P½ PO_4 7 month old callus
5. 5 and 5a, P 1 year old callus
6. 6 and 6a, Q 1 year old callus
7. 7 and 7a, Standard berberine hydrochloride

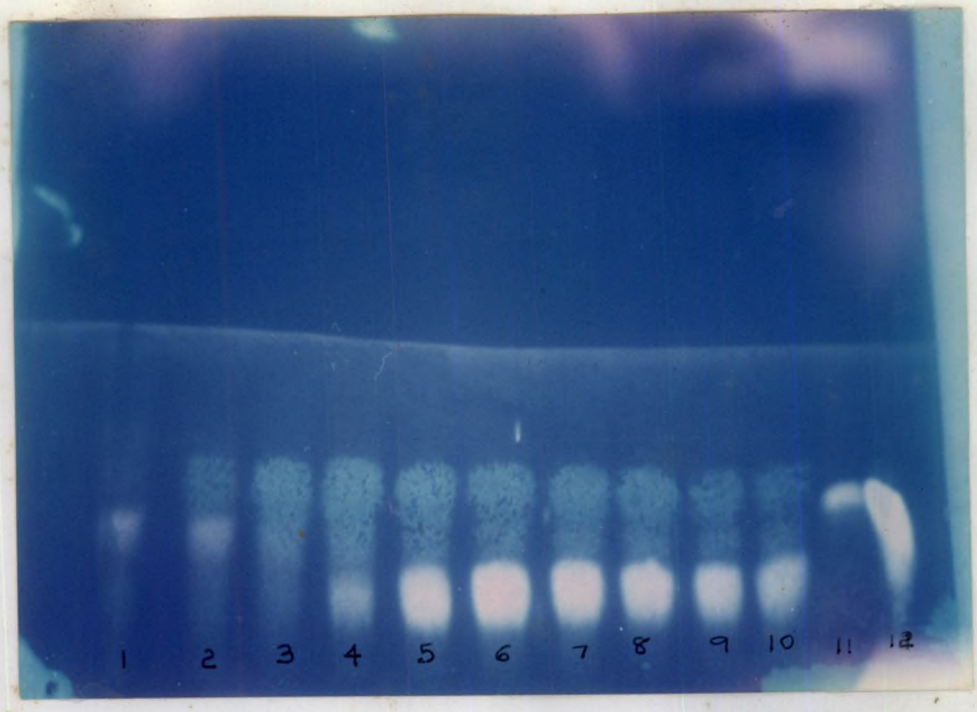


Plate 10

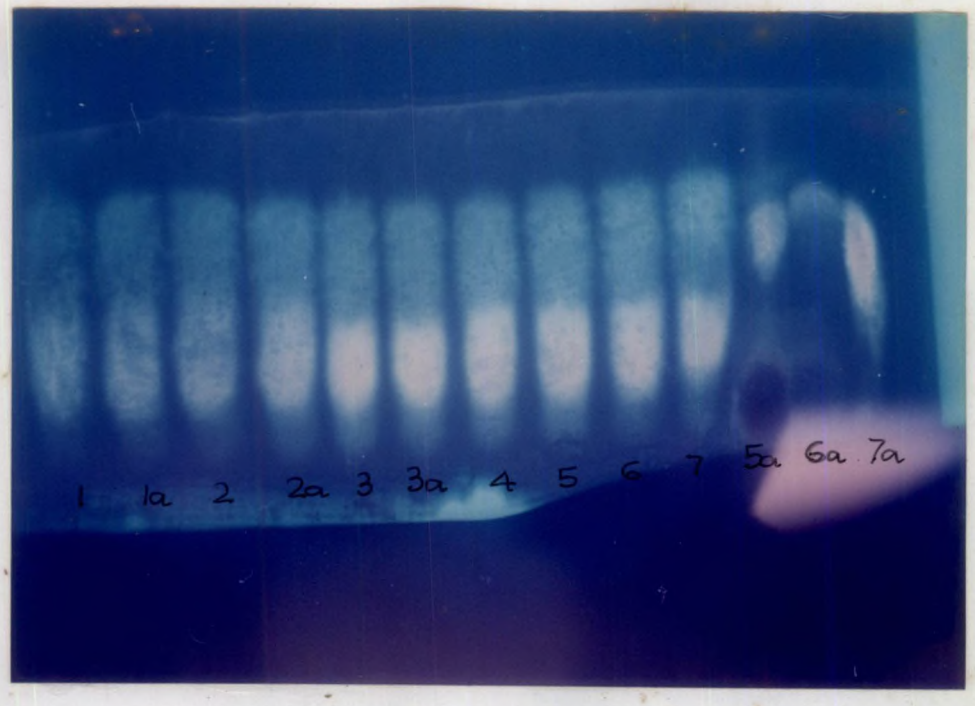


Plate 11

inoculation were used. Spermidine at 60 µg concentration added to the production medium had a berberine yield of 5.015 µg per g callus (Table 25 and Fig. 3). When spermidine was added to the culture medium, a yellow coloured substance was developed in the medium.

Berberine yield was high when 100 µg concentration of spermidine in half strength MS liquid medium supplemented with NAA 2 mg l^{-1} and BA 1 mg l^{-1} (0.729 µg per g callus) Yellow coloration was comparatively less in this medium after spermidine administration. Considering the two production media (Table 25), half strength liquid MS medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1} at 60 µg concentration of spermidine was found to be the optimum for berberine harvesting (5.015 µg per g callus, Table 25). A portion of the quantity of berberine isolated when 60 µg of spermidine was added to the production medium is shown in Plate 12.

4.9.2 Quantification of berberine from half strength MS liquid medium with varying concentration of nitrogen and phosphorus sources

Friable callus tissues of 7 to 12 months were transferred to ½ MS liquid medium with varying concentration of nitrogen and phosphorus sources containing IAA 2 mg l^{-1} and BA 1 mg l^{-1}. The berberine content in the cells and medium are shown in Table 26. Maximum quantity of berberine was recovered when phosphorus ion sources were reduced to 25 per cent in the ½ MS liquid medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}. The recovery was 10.079 µg from g callus tissue. When nitrate ions were reduced to its quarter strength in the ½ MS liquid medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}, berberine content was 2.402 µg berberine from g callus. A portion of the quantity of berberine isolated when phosphorus ion sources were reduced to 25 per cent in the production medium is shown in Plate 13.

Table 25. Quantification of berberine from liquid medium enriched with different concentration of spermidine

Treatment No.	Concentration of spermidine (µg)	Media	Quantity of callus weight (g)	Berberine (µg)	Berberine in callus µg/g
1	10	*P	0.029	$6.348x10^{-3}$	0.219
2	30	P	0.033	0.040588	1.230
3	50	P	0.041	0.077962	1.901
4	60	P	0.022	0.11032	5.015
5	100	P	0.039	0.041664	1.068
6	10	*Q	0.029	$1.7408x10^{-4}$	$6.003x10^{-3}$
7	30	Q	0.038	$2.48832x10^{-3}$	0.065
8	50	Q	0.037	$3.2448x10^{-3}$	0.088
9	60	Q	0.031	$3.8702z10^{-3}$	0.125
10	100	Q	0.039	$2.8416x10^{-4}$	0.729

*P = ½ MS liquid medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}
*Q = ½ MS liquid medium supplemented with NAA 2 mg l^{-1} and BA 1 mg l^{-1}
Quantity of liquid medium P and Q - 25 ml
Age of the callus - 7 months

FIG. 3 EFFECT OF SPERMIDINE ON BERBERINE PRODUCTION IN ½ MS LIQUID MEDIUM SUPPLEMENTED WITH IAA 2 mg l^{-1} AND BA 1 mg l^{-1}

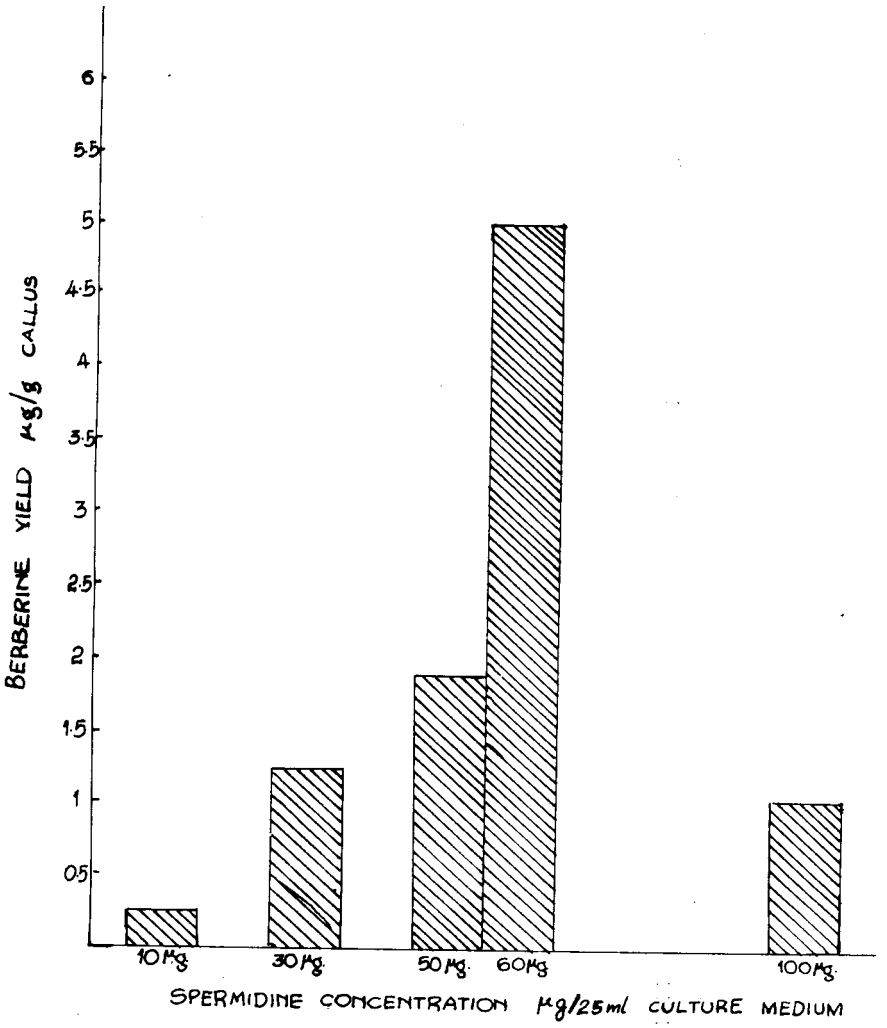


Plate 12. A portion of the quantity of berberine recovered when 60 µg of spermidine was added to the production medium

Plate 13. A portion of the quantity of berberine isolated when phosphorus ion sources were reduced to 25 per cent in the production medium

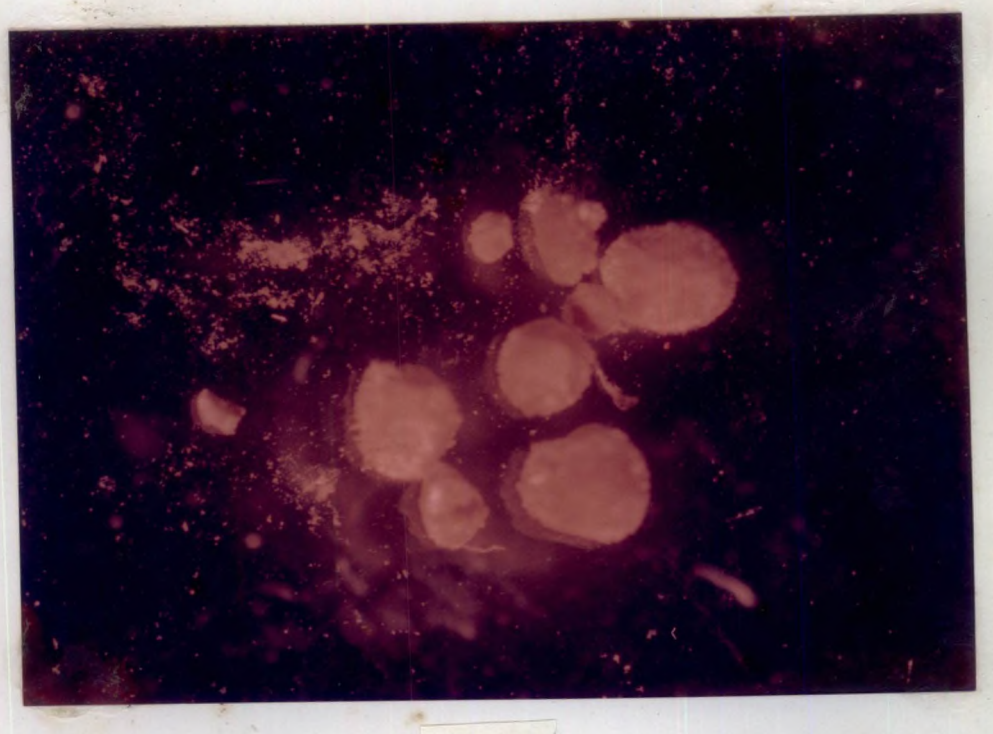


Plate 12

Plate 13

Table 26. Quantification of berberine from half strength MS liquid medium with varying concentrations of nitrogen and phosphorus sources

Treatment No.	Media	Quantity of callus weight (g)	Berberine in µg	Berberine in callus µg/g	RF value	Berberine
1	P ¼ NO_3 7 months old callus	0.027	0.065	2.402	0.443	presnt
2	P ½ NO_3 7 months old callus	0.041	$3.526x10^{-3}$	0.086	0.441	presnt
3	P ¼ PO_4 7 months old callus	0.023	0.232	10.079	0.442	presnt
4	P ½ PO_4 7 months old callus	0.042	0.032	0.768	0.443	presnt
5	P 1 year old callus	0.032	0.041	1.281	0.446	presnt
6	Q 1 year old callus	0.035	0.024	0.685	0.441	presnt

P = ½ MS liquid medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}
Q = ½ MS liquid medium supplemented with IAA 2 mg l^{-1} and 2,4-D 1 mg l^{-1}
Culture period - 21 days
Quantity of medium - 23 ml

Table 27. Quantification of berberine from callus obtained from ½ MS solid media with different growth regulators

Treatment No.	Media + growth regulators (mg)	Age of callus (months)	Weight of callus (g)	Berberine (µg)	Berberine µg/g callus
1	½ MS + 0.25 BA	7	1.086	$4.309x10^{-3}$	$3.968x10^{-3}$
2	½ MS + 0.5 BA	7	1.421	0.019	0.013
3	½ MS + 0.25 Kin	7	1.410.	0.263	0.019
4	½ MS + 0.25 ABA	7	1.250	0.118	0.095
5	½ MS + 2 IAA + 1 BA	12	5.000	0.748	0.150
6	½ MS + 2 IAA + 1 2, 4-D	12	5.500	0.136	0.025

Considering the effect of the different treatments in ½ MS liquid medium, the berberine recovery was found to be highest when the phosphate ion source was reduced to 25 per cent. A graphic representation of quantity of berberine recovered from ½ MS liquid medium is shown in Fig. 4.

4.9.3 Quantification of berberine from callus obtained from solid media

The quantity of berberine recovered from callus tissues obtained with different growth regulators of varying concentrations in ½ MS solid media are shown in Table 27.

The amount of berberine was high in the callus obtained from half strength MS solid medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1} which was maintained for 12 months through subcultures (0.748 µg from 5 g callus tissues which is equivalent to 0.150 µg/g callus). This was followed by 0.118 µg from 1.25 g callus (7 months old) ie. 0.095 µg from g callus obtained from ½ MS solid medium supplemented with IAA 2 mg l^{-1}, BA 1 mg l^{-1} and ABA 0.25 mg l^{-1}. The growth of callus was slow but the rate of berberine production was high in this treatment (Table 27). The 12 month old callus derived from solid ½ MS medium supplemented with IAA 2 mg l^{-1} and 2,4-D 1 mg l^{-1} had a berberine content of 0.136 µg from 5.5g callus i.e., 0.025 µg from g callus tissue which is more than the former. The calli obtained from ½ MS solid media supplemented with growth regulators BA 0.25, 5 and Kin 0.25 mg l^{-1} was found to have comparatively lesser content of berberine. A portion of the quantity of berberine recovered from 12 months old callus cultured on solid ½ MS medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1} is shown in Plate 14.

FIG. 4

QUANTITY OF BERBERINE FROM 1/2 MS LIQUID MEDIUM WITH VARYING CONCENTRATIONS OF NITROGEN AND PHOSPHORUS SOURCES

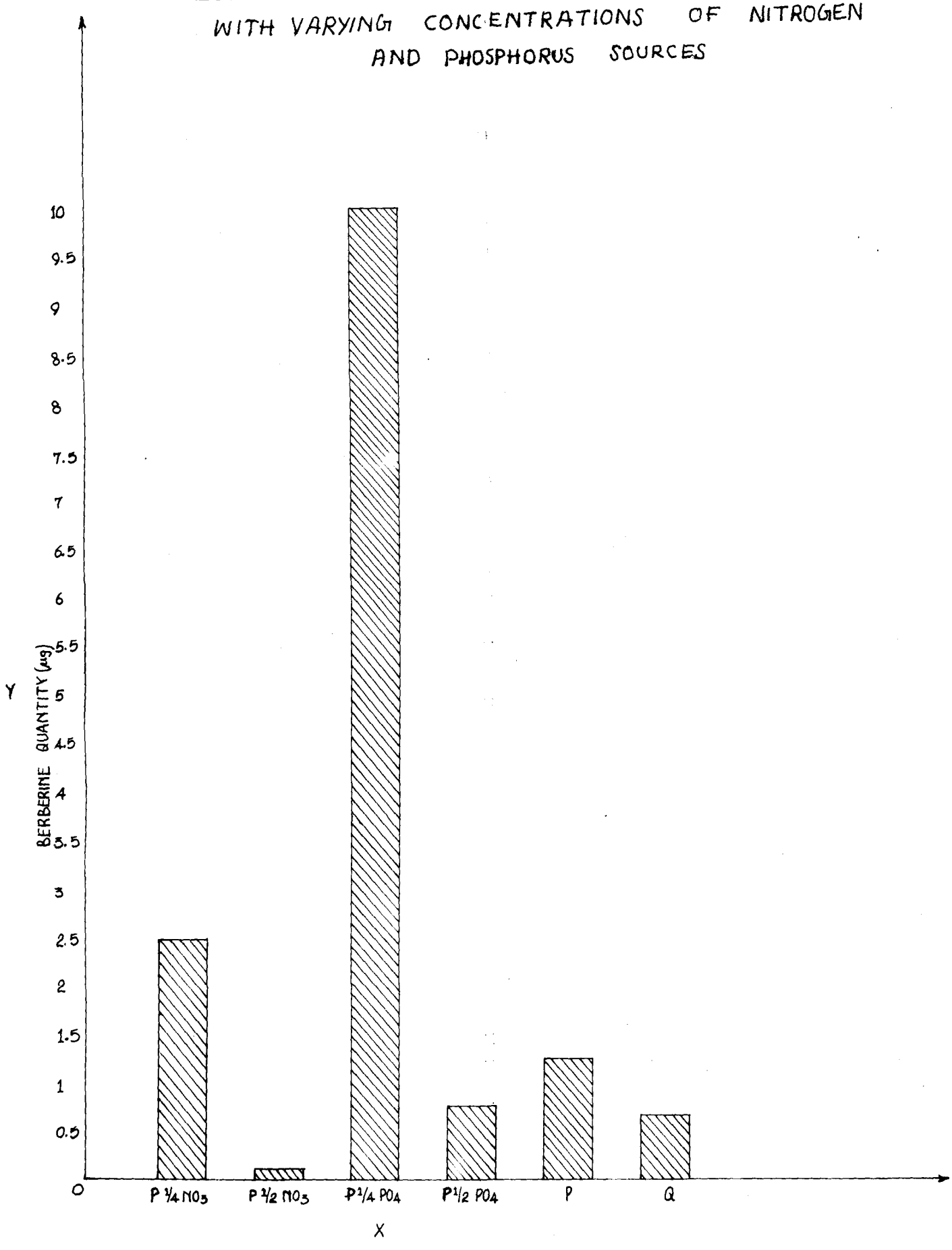


Plate 14. A portion of the quantity of berberine isolated from 12 months old callus cultured on solid ½ MS medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}

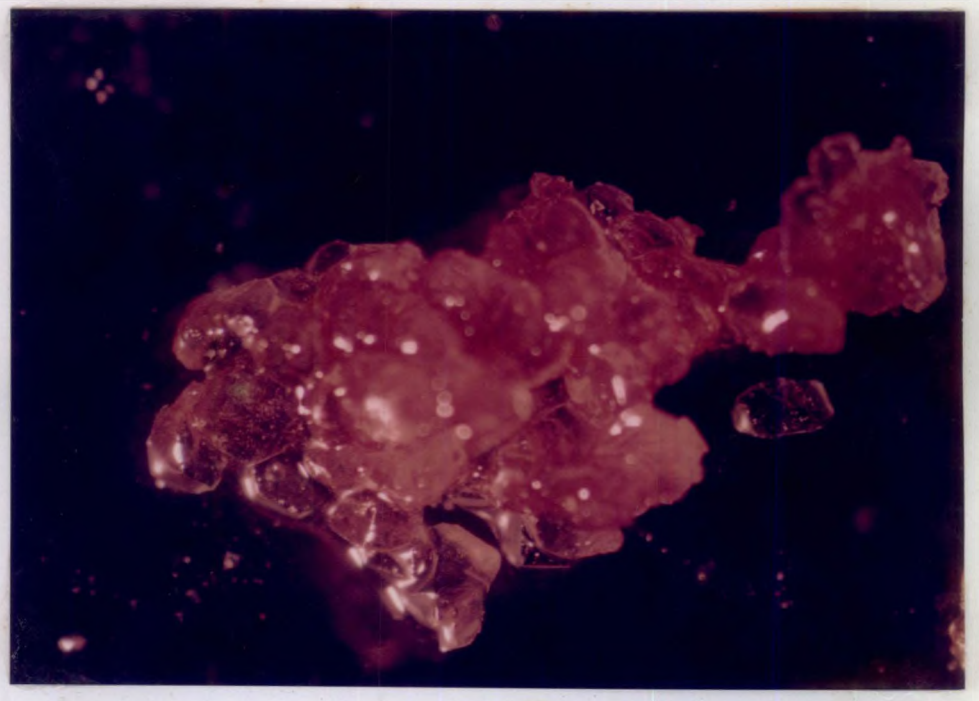


Plate 14

4.9.4 Screening of *ex vitro* samples for berberine by TLC

TLC was carried out for the detection of berberine for the *ex vitro* plant samples such as stem, mature leaves and tender leaves (Table 28). Berberine was detected in the stem, tender leaf and mature leaf extracts taken from the *ex vitro* plant (Plate 15). An unidentified compound with an Rf value of 0.66 was obtained from the stem extract. It was a brownish spot in visible light.

4.9.5 Quantification of berberine from *ex vitro* plant samples

The amounts of berberine recovered from different plant samples such as stem, tender leaves and mature leaves are shown in Table 29.

Tender leaves contained more quantity of berberine than the stem and mature leaves (0.320 µg/25 g tender leaves which is equivalent to 0.013 µg/g leaf). The berberine content in the stem was found to be 0.304 µg/30 g stem which is equivalent to 0.010 µg/g stem. The stem extract also gave another compound whose Rf value was 0.66 which gave a brownish spot in the visible light. A portion of the quantity of berberine isolated from 25 g stem is shown in Plate 16.

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

The *in vitro* derived callus gave berberine yield higher than the *ex vitro* samples (Tables 26 and 29). The highest berberine yield was obtained when phosphorus ion sources were reduced to 25 per cent in the ½ MS liquid medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}. The recovery was 10.079 µg from g callus tissue. The next highest yield of berberine (5.015 µg/g callus) was obtained when 60 µg of spermidine was added to 25 ml of the ½ MS liquid medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}.

Table 28. Detection of alkaloids from *ex vitro* samples by TLC*

Plant parts	Amount (g)	Rf value	Colour of the spot at 365 nm	Berberine
Tender leaf	25	0.441	Yellow spot	Present
Stem	30	0.440	Yellow spot	Present
Mature leaf	25	0.446	Faint yellow spot	Feebly present
Berberine standard	1	0.440	Yellow spot	Present

*Solvent system - propanol : acetic acid : water (7:1:2)
A brown spot was detected with an RF value of 0.66 in the visible light from the stem extract.

Table 29. Quantification of berberine from *ex vitro* plant samples

Treatment No.	Samples	Weight (g)	Quantity of berberine (µg)	Quantity of berberine (µg/g)
1	Stem	30	0.304	0.010
2	Mature leaf	25	0.131	0.005
3	Tender leaf	25	0.320	0.013

Plate 15. TLC profile of extracts taken from *ex vitro* sources

1. 1a, 1b and 1c - stem
2. 2 - mature leaf
3. 3a, 3b, 3c and 3d - tender leaf
4. Standard berberine hydrochloride

Plate 16. A portion of the quantity of berberine isolated from stem

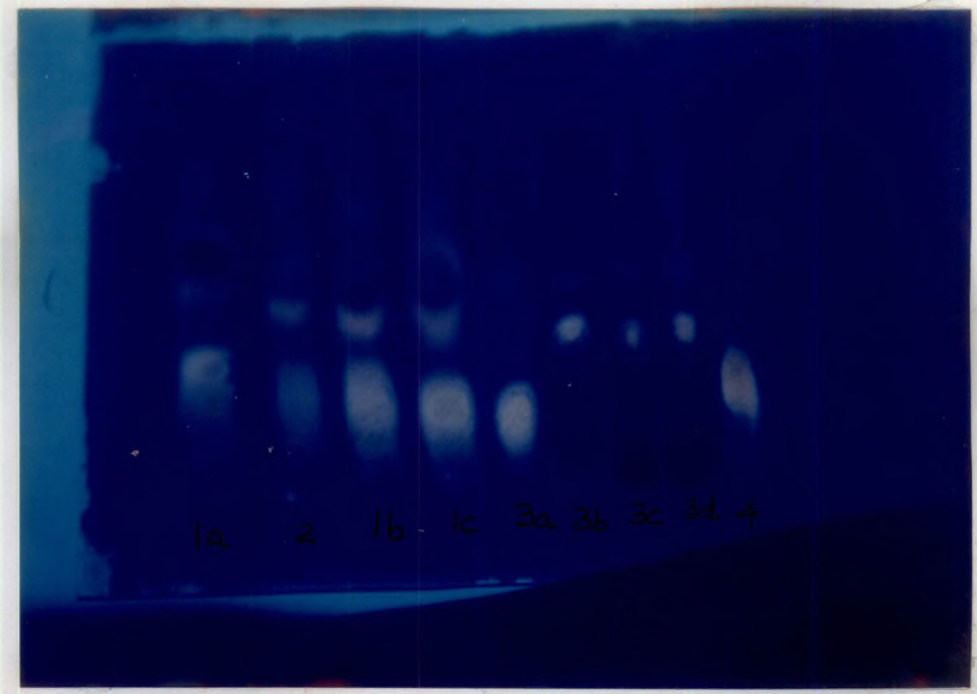


Plate 15

Plate 16

Discussion

DISCUSSION

The results of the studies on "*In vitro* callus induction and its exploitation in *Coscinium fenestratum* (Gaertn.) Colebr." are discussed in this chapter.

5.1 Standardisation of surface sterilisation of explants

5.1.1 Surface sterilisation of leaf and fruit explants

In the present study, the sterilisation procedure with respect to the concentration and duration of exposure was standardised, since the explants collected from the field harbour a variety of microorganisms. In the present study, among the different treatments tried, the least contamination rate was observed in cultures receiving treatments T_{17}, T_{18} and T_{10} (Table 1a). Treatment (T_{17}) with 0.03 per cent Cetrimide immersion for 5 minutes followed by mercuric chloride 0.1 per cent for 5 minutes and treatment (T_{18}) with 0.05 per cent Cetrimide immersion for 3 minutes followed by mercuric chloride 0.1 per cent for 5 minutes resulted in better establishment and growth rate (89.77% and 93.12% respectively) of the explants.

Treatments involving longer duration and higher concentration of $HgCl_2$ treatments resulted in higher percentage of bleached cultures and subsequent drying. None of the treatments with longer duration and lower concentration of mercuric chloride, were found to be effective in producing contamination free cultures.

The most commonly used surface sterilants are sodium hypochlorite (10%) and mercuric chloride (0.01-0.1%). In *Nardostachys jatamansi*, Mathur (1993) has reported that 2 per cent sodium hypochlorite treatment for 7 minutes was effective.

For surface sterilization of explants taken from the plants maintained in the glass house, treatment with 0.5 per cent mercuric chloride for 5 min or 10 min was found to be better in *Holostemma annulare* (John, 1996).

Combination of ethyl alchohol and mercuric chloride was effective in *Duboisia myoporoides* (Kukreja *et al.*, 1986).

5.1.2 Surface sterilisation of explants for the rainy season (May, June, July)

The percentage of contamination observed for the explants collected in the different months showed variation. Prabha (1993) reported that for *Ananas cosmosus,* the explants collected in the rainy months showed higher contamination rate. Likewise, the contamination rate was found to be higher in the present study when collected in the months of May, June and July. Among the treatments tried, Emisan and mercuric chloride combination tried were found promising. Treatment with 0.1 per cent Emisan for 5 minutes followed by 0.05 per cent Cetrimide for 5 minutes and then by 0.1 per cent $HgCl_2$ for 5 minutes was the best and gave higher percentage of establishment of cultures.

5.1.3 Surface sterilisation of shoot buds, nodal and internodal segments

Surface sterilisation treatments tried for leaves were also done for stem and the contamination was cent percent. Another constraint was the exudation of phenols from the cut ends. Hence pre-treatment with antioxidants were carried out. Among the different antioxidants and mercuric chloride combinations tried for sterilisation of semi-mature and tender shoots, the best was with Cetrimide 0.1 per cent for 5 min, Emisan 0.1 per cent for 5 min, citric acid 0.1 per cent + ascorbic acid 0.1 per cent for 10 min and mercuric chloride 0.1 per cent for 3 min. The establishment percentage was higher and the exudation of phenol was minimised in this treatment.

In *Vanilla planifolia*, stem node sections with dormant axillary buds were washed with liquid detergent and then rinsed in running tap water for 10 min. The cleaned explants were surface sterilized with 0.15 per cent (w/v) mercuric chloride for 5 min followed by three rinses in sterile distilled water (George and Ravishankar, 1997).

Bhattacharya *et al.* (1990) reported in *Chrysanthemum morifolium* that the nodal and internodal segments were washed thoroughly with distilled water and disinfected with 90 per cent ethanol for 10 seconds, and subsequently surface sterilised with $HgCl_2$ solution (0.1% w/v) for 2 min followed by repeated rinsing with sterile double distilled water which was effective in minimising microbial contamination.

5.1.4 Seasonal variation in the rate of establishment of explants

In the present study, variations were noticed in the culture establishment and its sustained growth over a period of twelve months from both leaf and stem explants. Explants collected during the drier months of the year, that is, from October to January, showed lesser contamination with maximum establishment in November (96.1%) followed by October (94.4%) while the rate of contamination was higher in the rainy months. Better culture establishment has been observed in the summer months in *Holostemma annulare* by John(1996).

5.2 Standardisation of basal media for inducing calli

The best media for callus induction was identified based on the maximum initiation of callus in leaf and stem cultures. MS medium at half strength salt concentration, initiated calli to the maximum from leaf bit and stem cultures. The others, MS medium at full strength and Woody Plant medium failed to initiate callus in leaf bit and stem cultures. In MS media, brown callus was observed from the explant, but there was no further growth of callus.

Highest percentage of culture establishment in half strength MS medium over MS medium at full strength has been reported in *Kaempferia galanga* by Joseph (1997). MS medium is characterised by high concentration of minerals and hence its strength is reduced to half with beneficial effects in many crop species (Skirvin, 1980). Beneficial effects of MS medium at half strength over full MS medium in callus initiation was observed in *Papaver bracteatum* by Ilahi and Ghauri (1994). For the induction of callus tissues in *Thalictrum minus* var. *hypoleucum*, Linsmaier and Skoog medium supplemented with 2,4-D and BA was most suitable (Nakagawa *et al.*, 1984). In *Sida* spp., callus induction was highest in MS medium at half strength in leaf and stem cultures (Sankar, 1998).

5.2.1 Induction and proliferation of calli in solid medium

5.2.1.1 Effect of auxins on callusing

Auxins such as 2,4-D, 2,4,5-T, IAA, IBA and NAA at varying levels of concentrations were tried in the present study on full strength MS medium. Among the different treatments, solid MS medium supplemented with IAA and 2,4-D at 2.5 mg l^{-1} and 1 mg l^{-1} respectively gave a high callus index of 67 and 71 under light and dark conditions respectively. The number of days taken for callus initiation was reduced from 19 days in light to 16 days in the dark incubated conditions in solid full strength MS medium.

The best among the treatments tried in full strength MS medium were tried with ½ MS, SH and ½ SH media. Half MS medium enriched with a combination of IAA 2 mg l^{-1} and 2,4-D 1 mg l^{-1} recorded the highest percentage of callus initiating cultures registering 83.82 per cent for leaf bit cultures and 80.31 per cent for leaf segments attached with petioles.

Babu *et al.* (1992) reported that 2,4-D induced callus growth in ginger cv. Maran when explants taken from innermost leaves were cultured on revised MS medium.

5.2.1.2 Comparative performance of explants to callus induction and proliferation

Leaf segments and leaf segments attached with petiole bases exhibited highest callus growth rate in ½ MS media in the present study. Stem, unmature fruit and petiole explants did not respond to the callus promoting media.

Nair *et al.* (1992) reported the suitability of employing leaf petiole bases as explants to induce calli in *Coscinium fenestratum*.

The nodal and internodal segments and petiole bases produced callus, but the cells became black and its growth was retarded in the present study.

Kim *et al.* (1988) reported that in Thalictrum rugosum cell suspension cultures the cell mass of dark grown cultures decreased more rapidly than that of the light grown cultures. But the cells became black in the light grown cultures. The blackening could be due to the light induced production of polyphenols in the cells.

5.2.1.3 Effect of cytokinins on callusing

The incorporation of major cytokinins benzyladenine, kinetin and 2 isopentenyl adenine at varying levels to basal media did not induce any significant response. BA at 1 mg l^{-1} to basal media gave a high callus index of 49 and 70 respectively under light and dark incubated conditions. In *Holostemma annulare*, 2iP gave a higher callus index using leaf explants (John, 1996).

5.2.1.4 Effect of cytokinin and auxin combination on callusing in ½ MS solid medium

Nair *et al.* (1992) observed in *Coscinium fenestratum that* 2,4-D and BAP induced callus growth from leaf petiole bases cultured on solid MS medium.

In the present study, IAA 2 mg l^{-1} + BA 1 mg l^{-1} and IAA 2 mg l^{-1} + 2,4-D 1 mg l^{-1} showed a very high callus index of 273 and 261 under light and 261 and 249 under dark incubated conditions respectively on ½ MS solid medium. Compact calli were also obtained from leaf segments attached with petiole bases from the above treatments with a very high callus index of 243 and 252 under light and 231 and 242 under dark incubated conditions respectively. Auxin and cytokinin combination on MS solid medium did not produce profuse callus growth from leaf petiole bases in the present study as was reported by Nair *et al* (1992).

In the present study, a combination of IAA 2 mg l^{-1} and BA 1 mg l^{-1} was superior and it also reduced the number of days taken for callus induction to 19 days. The growth of callus was rapid and the callus was yellowish green and friable.

In other treatments, callusing was obtained but later the callus turned brown and dried. Nair *et al.* (1992) also reported that all the explants, except the leaf petiole bases, cultured on solid MS medium supplemented with 2,4-D and BAP underwent blackening and dried after one week of culture. In the present study, an yellow, friable callus was obtained from immature fruits with a callus index of 66 when cultured on solid ½ MS medium with phosphate ions reduced to 25 per cent supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}.

In *Thalictrum minus* var. *hypoleucum*, NAA and BA induced callus growth from leaf segments cultured on Linsmaier and Skoog (1965) medium for four weeks (Nakagawa *et al.*, 1984).

Gokul and Tejawathi (1997) found in *Cissampelos pareira*, a tropical climber used in Ayurvedic medicine belonging to Menispermaceae that stem explants formed callus in MS medium containing NAA and Kinetin.

5.2.1.5 Influence of culture environment on callusing in solid media

Culturing under dark was found beneficial for callusing with respect to the number of days taken for callus initiation, cultures initiating calli (percent), when leaf segments of *C. fenestratum* were cultured *in vitro*. But maximum callus indices were obtained when cultured under light.

Photoperiod had influence on callus initiation. Light stimulated the growth of friable yellowish calli than in the dark in the initial stage. But in the later stages, continuous illumination proved to be detrimental, as the cells became black and its growth was retarded than in dark incubated condition. Nair *et al* (1992) also reported that presence of light inhibited the growth but stimulated berberine synthesis in *C. fenestratum.*

Kim *et al.* (1988) reported that in *Thalictrum rugosum* cell suspension cultures, the cell mass of dark grown cultures decreased more rapidly than that of the light grown culture. Light helped to maintain the cells in viable state and also berberine production. In dark grown cultures, after the ceasure of growth there was a decrease in berberine level, whereas light grown cultures continued to produce berberine. Berberine production was maximum in light grown cultures (216.5 mg l^{-1}) than in dark grown cultures (159.0 mg l^{-1}).

5.3 Regeneration from callus

Calli initiated from ½ MS medium enriched with IAA 2 mg l^{-1} and BA 1 mg l^{-1} were used to attempt initiation of organoids. Among the different treatments tried for induction of organoids from the callus formed, no treatment was responsive

to the formation of organoids. Treatment with BA 0.5 mg l^{-1} on solid ½ MS media incubated under light resulted in the callus mass acquiring a greenish coloration and a compact nature. There was no *de novo* root and shoot formation. Various growth hormones and hormonal combinations were tried in solid half strength MS media and none of the treatments were found to be effective in inducing somatic embryogenesis.

5.4 Effect of other media on nodal and internodal segments

Among the different media tried for regeneration from nodal segments, half strength SH medium enriched with BA 2.5 mg l^{-1} elicited yellow callus growth from the base of the nodal segments. The exudation of brown coloured material from the cut ends was highest in the case of nodal segments comparing other explants that is, leaf segments, leaf segments attached with petioles and petiole segments. In the present study, it was found that stem contains a higher concentration of phenols which prevent the growth of callus and shoot buds.

Multiple shoots regenerated from *Woodfordia fruiticosa* (L.) nodal segments when cultured in SH medium supplemented with BA 0.2 mg l^{-1} for 4-5 weeks (Krishnan and Seeni, 1994).

Formation of shoots were reported in single node segments of *Aegle marmelos* when cultured in MS medium supplemented with 2.5 mg l^{-1} BA and IAA 1 mg l^{-1} (Ajithkumar and Seeni, 1998).

5.4.1 Effect of media supplements on nodal segments

The addition of phloroglucinol, activated charcoal and adenine sulphate at different concentrations failed to elicit regeneration in the present study. The rate of contamination was less but there was no regeneration in the present study.

5.4.2 Effect of liquid media on nodal segments

Different liquid media tried with growth hormones at various levels failed to stimulate any growth.

5.5 Regulation of *in vitro* metabolite production

5.5.1 Standardisation of production medium

Among the growth factor combinations attempted in the study, the combination of IAA 2 mg l^{-1} and BA 1 mg^{-1} proved beneficial to the synthesis of berberine. When 4 to 17 months aged calli were subjected to thin layer chromatography, spots identical to that of standard berberine hydrochloride were observed at 365 nm. The calli produced from leaf segments cultured on solid ½ MS medium supplemented with IAA 2 mg l^{-1} and 2,4-D 1 mg l^{-1} favoured production of berberine in the calli from 7 month onwards.

Cytokinins are known to stimulate the production of nicotine in callus cultures of *Nicotiana tabacum* (Tabata *et al.*, 1975) and that of phenolic compounds such as anthocyanins (Seitz and Hinderer, 1988), catechins, proanthocyanidins and lignins (Zaprometov, 1988). In the present study, also the cytokinins, BA and KIN at lower concentrations sustained the callus growth on ½ MS solid medium. Berberine was detected in callus obtained from ½ MS solid media supplemented with BA 0.25 mg l^{-1}, 0.5 mg l^{-1} and KIN 0.25 mg l^{-1}. The calli obtained from the above three treatments contained considerable amount of berberine. The maximum quantity of berberine was found to be 0.026 μg from 1.410 g calli obtained from solid ½ MS media supplemented with 0.25 kinetin among the cytokinin initiated callus cultures.

Ginkgolide B production in cultured cells derived from *Ginkgo biloba* leaves reached optimum levels with NAA 1 mg l^{-1} and kinetin 0.3 mg l^{-1} (Jeon *et al.*, 1995).

5.5.2 Addition of osmoregulants

The osmoregulant, polyethylene glycol at 2 per cent elicited a positive response in leaf calli of *Sida cordifolia* on biosynthesis of ephedrine (Sankar, 1998).

However in the present study none of the osmoregulants, added to the basal growth medium such as polyethylene glycol, mannitol, sorbitol (1.5-3%) could sustain callus growth and berberine synthesis.

5.5.3 Incorporation of growth retardants/inhibitors

Incorporation of the growth retardant, cycocel or growth inhibitor, abscissic acid could not sustain the callus growth. A low growth of callus was observed with 0.25 mg l^{-1} ABA. The methanol extract of the above callus was found positive in the chromatographic tests carried out, with respct to berberine. The calli obtained from solid ½ MS media supplemented with IAA 2 mg l^{-1}, BA 1 mg l^{-1} and abscissic acid 0.25 mg l^{-1} gave a berberine yield of 0.118 µg from 1.25 g callus. Although the growth of callus was slow, the rate of berberine production was high in this treatment.

5.5.4 Modifying carbon source

Rao and Narasu (1997) reported in *Artemesia annua* that artemisin content in callus cultures was three fold higher when maltose was used as carbon source and two fold higher in a combination of sucrose and maltose when compared to sucrose alone.

Contrary to reports wherein increasing sucrose concentration to 8.0 per cent stimulated anthraquinone yields in *Eschschottzia californica* (Berlin *et al.*, 1983) or stimulated taxol yield in *Taxus* spp. (Ellis *et al.*, 1996),

in the present study, higher concentration of sucrose retarded callus growth. Substituting sucrose totally with maltose or in proportions of 1:1 or 1:2 failed to sustain the growth of callus. Though generally recognized as the best carbon source for secondary product synthesis, sucrose when totally substituted with maltose in *in vitro* system increased the product synthesis as in *Digitalis lanata* (Kreis and Reinhard, 1986).

In *Thalictrum rugosum* cell suspension cultures, 8 per cent sucrose in the production medium significantly improved berberine production and cell biomass compared to MS medium supplemented with 2,4-D and 3 per cent sucrose (Kim *et al.*, 1991a).

5.5.5 Withdrawal of inorganic nutrients

Maintaining phosphate levels below optimum is a growth limiting factor in *in vitro* systems to stimulate product synthesis. In liquid ½ MS medium, reduction of ammonium phosphate to quarter strength increased the berberine production. This increase was more than that obtained by the reduction of nitrate source by 25 to 50 per cent. The berberine yield obtained when ammonium phosphate was reduced to quarter strength was 10.079 µg/g callus.

Cell suspension cultures of *Thalictrum minus* var. *hypoleucum* were found to produce a large amount of berberine (400-800 mg l^{-1}), when the cells were cultured in modified LS medium containing 20 mM KNO_3 and 40 mM NH_4Cl in place of 20.6 mM NH_4NO_3 as the nitrogen source. Most of the alkaloid crystallised to form berberine chloride instead of nitrate. Minor alkaloids, thalifendine and magnoflorine were also isolated from the medium and identified (Nakagawa *et al.*, 1984).

Yasaki *et al.* (1987) found that shikonin production in *Lithospermum erythrorhizon* cell suspension cultures were inhibited in the LS medium by the

presence of NH_4^+ which is contained in the form of NH_4NO_3 whereas it was not inhibited by NO_3^- which is contained in White's medium as the sole nitrogen source. Jeon *et al.* (1995) reported that for the maximal production of ginkgolide B alkaloid, cells were cultured in MS medium modified to contain 1.25 mM potassium phosphate with a molar ratio of ammonium to nitrate ions of 1:3.

5.5.6 Addition of stress inducers

Spermidine (0.25, 0.5 and 2.5 mg l^{-1}) added to the basal growth medium failed to sustain callus growth in solid agar medium whereas it increased the berberine yield in liquid cultures. There was an increasing trend in berberine content with increasing concentration of spermidine (upto 60 µg) to the production medium and then started declining. Spermidine at 60 µg concentration added to the production medium had a berberine yield of 5.015 µg/g callus (Table 25 and Fig. 3). Considering the second production medium (½MS liquid medium + NAA 2 mg l^{-1} + BA 1 mg l^{-1}) there was an increase in berberine production with increasing the concentration of spermidine, the highest being obtained at 100 µg concentration (0.729 µg/g callus). Considering the two production media (Table 25) ½ MS liquid medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1} at 60 µg concentration of spermidine was found to be optimum for berberine harvesting.

Hara *et al.* (1991) reported that berberine production was significantly increased (over 400%) by addition of spermidine in *Thalictrum minus* cell suspension cultures grown in LS medium supplemented with NAA and BA. Spermidine caused an increase in ethylene generation which was closely associated with berberine biosynthesis whereas other polyamines such as cadaverine, putrescine and spermine were ineffective.

5.6 Effect of age of the callus on berberine production

Age of the callus also influenced the berberine yield. The 12 month old callus which was maintained by subculture derived from solid ½ MS media with

IAA 2 mg l^{-1} and BA 1 mg l^{-1} gave a high yield of berberine, that is, 0.748 µg from 5 g of callus which is equivalent to 0.150 µg from 1 g of callus. This was followed by 0.118 µg from 1.25 g callus (7 months old) that is, 0.095 µg/g callus obtained from ½ MS solid medium supplemented with IAA 2 mg l^{-1}, BA 1 mg l^{-1} and ABA 0.25 mg l^{-1}. The growth of callus was slow, but the rate of berberine production was high in this treatment (Table 27). The 12 months old callus derived from solid ½ MS medium supplemented with IAA 2 mg l^{-1} and 2,4-D 1 mg l^{-1} had 0.136 µg berberine from 5.5 g callus, that is, 0.025 µg/g callus tissue. The growth of callus was comparatively fast but the rate of berberine production was slow in this treatment. The presence of berberine was detected from 7 months old calli onwards. Age of the callus had a profound influence in the liquid medium also. The quantity of berberine was more in cell suspension cultures initiated from one year old callus, that is, 1.278 µg/g callus, whereas the quantity of berberine was comparatively less in cell suspension cultures derived from 7 month old callus, that is, 0.024 µg/g callus.

5.7 Estimation of alkaloids from *ex vitro* sources

Bowen and Motawe (1985) first reported the isolation of alkaloid from *Tinosporia malabarica* (Menispermaceae). Three alkaloids were isolated from the stem methanol extract by flash column chromatography and were further purified by TLC.

Similarly an unknown yellow base, designated as menisporphine was isolated from the rhizome of *Menispermum dauricum* (Kunitomo *et al.*, 1983).

In the present study, the alkaloid berberine was detected from tender leaf, mature leaf and bark extract of *Coscinium fenestratum* and Rf value was confirmed with that of authentic sample. The stem extract also gave another compound whose Rf value was 0.66 and it gave a brownish spot under visible light.

The berberine yield was highest in the tender leaf, that is, 0.321 µg/25g leaves, which is equivalent to 0.013 µg/g of leaves. The quantity of berberine in the stem was found to be 0.010 µg/g of stem, that is, 0.304 µg/30g of bark. Alkaloids such as palmatine, isototrandrine, aromoline, jatrorrhizine, berberine, berberine chloride, columbamine, oxycanthine and magnoflorine were isolated from the root bark of *Berberis cartagena* (Basher *et al.*, 1996).

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

The *in vitro* derived callus gave berberine yield higher than the *ex vitro* samples (Tables 26 and 29). The highest berberine yield was obtained when phosphorus ion sources were reduced to 25 per cent in the ½ MS liquid medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}. The recovery was 10.079 µg/g callus tissue. The next highest yield of berberine (5.015 µg/g callus) was obtained when 60 µg of spermidine was added to 25 ml of the ½ MS liquid medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}.

Thus it can be seen from the present study that callus could be induced successfully from leaf segments and leaf segments attached with petiole bases of *Coscinium fenestratum*. The callus formed indicated the presence of berberine in significantly higher quantities than present in the tissues of the field grown plant. The *in vitro* techniques for the production of berberine can be a viable alternative for the production of berberine which may in turn help in conservation of this endangered plant.

Summary

SUMMARY

The present investigation entitled "*In vitro* callus induction and its exploitation in *Coscinium fenestratum* (Gaertn.) Colebr." was undertaken at the Plant Tissue Culture and Biochemistry Laboratories, College of Horticulture, Vellanikkara, Thrissur, during the period from 1997 to 1999. The study was aimed to standardise the *in vitro* techniques for callus induction, proliferation and regeneration. It was also intended to identify and quantify the active principle in the *in vitro* cultures.

The results obtained are summarised below:

1. Among the different surface sterilisation treatments tried, the best treatment varied with respect to the type of explant collected from the field.

2. The surface sterilisation treatment standardised for the immature fruits and leaves were found to be a combination treatment of 0.05 per cent Cetrimide immersion for 5 minutes followed by 0.1 per cent mercuric chloride for 5 minutes based on the least percentage of contamination, higher rate of establishment and growth of the explants obtained.

3. The exudation of polyphenols from the cut surfaces of shoot tip, nodal and internodal segments was minimised by pre-treatment with antioxidants. The pre-treatment followed was with Cetrimide 0.1 per cent for 5 minutes, Emisan 0.1 per cent for 5 minutes, ascorbic acid 0.01 per cent + citric acid 0.01 per cent for 10 minutes and mercuric chloride 0.1 per cent for 3 minutes.

4. The better establishment of cultures and the least contamination rate was obtained during the period from October to January.

5. For the better establishment of leaf explants during the rainy season, treatment with 0.05 per cent Cetrimide for 5 minutes followed by Emisan 0.1 per cent for 5 minutes and subsequent immersion in mercuric chloride 0.1 per cent for 5 minutes was found to be the best.

6. Among the different media tried, solid half strength MS medium was found best for the initiation of cultures.

7. Among the various explants used, leaf segments and leaf segments attached with petiole bases produced more healthy calli which were compact and yellowish in colour.

8. Of the various auxin combinations tried, IAA 2 mg l^{-1} and 2,4-D 1 mg l^{-1} combination was found to be good for callus production on solid ½ MS media from leaf segments and leaf segments with attached petiole bases.

9. Of the various auxin and cytokinin combinations tried, IAA 1 mg l^{-1} + BA 1 mg l^{-1} was found to be the best for initiation of callus cultures from the leaf segments and the leaf segments with petiole bases. The callus index was the highest for this treatment.

10. There was no growth from the callus in any of the treatments tried in the liquid medium.

11. Light stimulated the growth of yellowish, friable calli in the initial stages.

But in the later stages, continuous illumination proved to be detrimental as its growth was retarded.

12. There was no regeneration from callus in any of the treatments tried.

13. Callusing was obtained from immature fruits when cultured on solid ½ MS medium with phosphate ions reduced to its quarter strength supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}.

14. The shoot tips, nodal and internodal segments exhibited recalcitrancy and there was no initiation of growth from them.

15. The wavelength of marker berberine was recorded as 228 nm.

16. The alkaloid berberine was present in calli produced from leaf explants in solid ½ MS media in different treatments such as BA 0.25 mg l^{-1}, 0.5 mg l^{-1}, BA 0.25 mg l^{-1}, Kin 0.25 mg l^{-1}, IAA 2 mg l^{-1} + BA 1 mg l^{-1} and IAA 2 mg l^{-1} + 2,4-D 1 mg l^{-1}.

17. Incorporation of media additives, addition of osmoregulants, increasing concentration of agar, addition of stress inducers and modification of the carbon source in solid ½ MS medium failed to sustain the growth of callus for berberine production.

18. Addition of abscissic acid at low concentration of 0.25 mg l^{-1} was found to be the best among different treatments tried for berberine production, as it sustained callus development in solid ½ MS medium. Recovery of berberine was found to be 0.095 µg/g callus.

19. The highest amount of berberine (0.150 µg/g callus) obtained among the different treatments tried in solid ½ MS medium was with IAA 2 mg l^{-1} and BA 1 mg l^{-1} which was maintained for 12 months through subcultures.

20. Cell browning was reduced with the addition of spermidine to the ½ MS liquid medium with growth regulators. The liquid medium became deep yellow due to the release of the alkaloid. But there was no further cell growth.

21. Addition of spermidine at 60 µg concentration to the liquid ½ MS production medium gave a berberine yield of 5.015 µg/g callus. Lower and higher concentration of spermidine at 10 µg and 100 µg did not increase the yield of berberine in the production medium.

22. Considering different treatments in ½ MS liquid medium, the berberine recovery was found to be highest when the phosphate ion source was reduced to 25 per cent in the ½ MS liquid medium supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}. The recovery was 10.079 µg/g callus.

23. The highest berberine recovery obtained was from callus obtained from half strength MS liquid medium than in half strength MS solid medium.

24. Age of the callus tissue was found to influence the berberine production in ½ MS solid medium, since berberine was detected from 4 months aged calli onwards.

25. The alkaloid berberine was detected in the extract taken from the stem, tender leaves and mature leaves of the field grown plant. Screening of the different parts of the field grown plant revealed that the quantity of berberine

was highest in the tender leaf, that is, 0.320 µg/25g tender leaves which is equivalent to 0.013 µg/g leaf.

26. The berberine content in the stem was found to be 0.010 µg/g stem, that is, 0.304 µg/ 30 g stem. The stem extract also contained another compound whose Rf value was 0.66 and it gave a brownish spot under visible light.

27. The *in vitro* derived callus had higher amounts of berberine than the *ex vitro* samples. The highest berberine yield was obtained when phosphorus ion sources were reduced to 25 per cent in the ½ MS liquid medium supplemented with IAA $2mgl^{-1}$ and BA $1mgl^{-1}$. The recovery was 10.079 µg/g callus.

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* 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

IN VITRO CALLUS INDUCTION AND ITS EXPLOITATION IN *Coscinium fenestratum* (Gaertn.) Colebr.

By
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ABSTRACT OF THE THESIS

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ABSTRACT

The present investigations were carried out in the Plant Tissue Culture and Biochemistry Laboratories, College of Horticulture, Vellanikkara, Thrissur during the year 1997-1999. The study was undertaken with the objective to standardise the *in vitro* techniques for callus induction, proliferation and regeneration. It was also envisaged to identify and quantify the active principle in *in vitro* cultures.

Surface sterilisation treatments were standardised for the different types of explants from the field. For immature fruits and leaves, the sterilisation treatment standardised were found to be with 0.05 per cent cetrimide immersion for 5 minutes followed by 0.1 per cent $HgCl_2$ for 5 minutes with reference to least percentrage of contamination and higher rate of establishment and growth of the explants obtained.

The exudation of polyphenols from the cut surfaces of shoot tip, nodal and internodal segments was minimised by pre-treatment with antioxidants. The pre-treatment followed was with Cetrimide 0.1 per cent for 5 minutes, Emisan 0.1 per cent for 5 minutes, ascorbic acid 0.01 per cent + citric acid 0.01 per cent for 10 minutes and mercuric chloride 0.1 per cent for 3 minutes.

Though calli were obtained from leaf segments as well as leaf segments with petiole bases, leaf segments were found most suitable for callus cultures and produced profuse calli. The best treatment for callus induction was found to be solid ½ MS medium for leaf bit cultures. The treatment with IAA 2 mg l^{-1} and BA 1 mg l^{-1} on ½ MS solid medium was the best for callus induction from leaf segments and leaf segments with petiole bases. Similarly a combination of auxins such as IAA 2 mg l^{-1} and 2,4-D 1 mg l^{-1} on ½ MS solid medium also readily induced

callusing from leaf segments and leaf segments with petiole bases. The above treatments were superior with respect to callus index and the number of days taken for callus initiation.

Callusing was obtained from immature fruits when cultured on solid ½ MS medium with phosphate ions reduced to 25 per cent supplemented with IAA 2 mg l^{-1} and BA 1 mg l^{-1}.

The shoot tips, nodal and internodal segments exhibited recalcitrancy and there was no cell growth from these explants.

There was neither any proliferation nor any noticeable cell growth of calli in the liquid medium.

Light stimulated the growth of yellowish friable callus and berberine synthesis in the initial stages. But in the later stages, continuous illumination proved to be detrimental and its growth was retarded.

The calli did not respond to regeneration treatments being neither organogenic nor embryogenic.

The wavelength of marker berberine was recorded as 228 nm. Berberine was detected in calli produced from leaf explants of different treatments in solid ½ MS media supplemented with growth regulators such as BA 0.25 mg l^{-1}, BA 0.5 mg l^{-1}, Kin 0.25 mg l^{-1}, IAA 2 mg l^{-1} + BA 1 mg l^{-1} and IAA 2 mg l^{-1} + 2, 4-D 1 mg l^{-1}. Age of the callus had a profound influence on berberine production.

Employing special techniques for synthesis of berberine in *in vitro* cultures such as administration of osmoregulants, incorporation of media additives,

increasing concentration of agar, addition of stress inducers and modification of carbon source in solid ½ MS medium failed to sustain the growth of callus.

Incorporation of abscissic acid at low concentration of 0.25 mg l^{-1} sustained the callus development and berberine was detected by thin layer chromatography. The quantity of berberine recovered was 0.095 µg/g callus tissue.

Administration of spermidine to liquid ½ MS medium neither caused any noticeable cell browning nor any cell growth. The medium became deep yellow due to the release of the alkaloid. Spermidine at 60 µg concentration in the liquid ½ MS production medium had a berberine yield of 5.015 µg/g callus.

Berberine was detected from the field grown plant samples also. The quantity was the highest in the tender leaves, that is 0.320 µg from 25 g tender leaves which is equivalent to 0.013 µg/g leaf. The berberine content in the stem was found to be 0.010 µg/g stem, that is 0.304 µg/30 g stem. The stem extract also contained another compound whose Rf value was 0.66 and it gave a brownish spot under visible light.

The highest berberine recovery obtained was in callus obtained from half strength MS liquid medium than in half strength MS solid medium.

The *in vitro* derived callus had higher amounts of berberine than the samples from the field grown plant. The highest berberine yield was obtained when phosphorus ion sources were reduced to 25 per cent in the ½ MS liquid medium supplemented with IAA 2mgl^{-1} and BA 1mgl^{-1}. The recovery was 10.079 µg/g callus tissue. The next highest yield of berberine (5.015 µg/g callus) was obtained when 60 µg of spermidine was added to the 25 ml of the ½ MS liquid medium supplemented with IAA 2mgl^{-1} and BA 1mgl^{-1}.