IN VITRO PRODUCTION OF SECONDARY METABOLITES FROM CULTURED CELLS/TISSUES AND MOLECULAR CHARACTERIZATION OF *CALOPHYLLUM INOPHYLLUM* (L).

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AUGUST, 2008

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BY

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Dedicated

to my late maternal grand mother

(Tahnu Aaji)

CONTENTS

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(Mr. Kiran D. Pawar)

Date :
Place: Pune

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"*In vitro* production of secondary metabolites from cultured cells/tissues and molecular characterization of *Calophyllum inophyllum* (L)"** submitted by Mr. Kiran D. Pawar was carried out by the candidate under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

(Dr. Mrs. S. R. Thengane)
Guide

Pune

Date:

DECLARATION BY THE CANDIDATE

I declare that the thesis entitled ***"In vitro* production of secondary metabolites from cultured cells/tissues and molecular characterization of *Calophyllum inophyllum* (L)"** Submitted by me for the degree of Doctor of Philosophy is the record of work carried out by me under the guidance of **Dr. (Mrs.) S. R. Thengane** and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other university or other institute of higher learning.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Date- **(Mr. Kiran D. Pawar)**

Key to abbreviations

ANOVA	Analysis Of Variance
BAP	6-Benzyl amino purine
°C	Degree Celsius
$CaCl_2$	Calcium Chloride
$CdCl_2$	Cadmium Chloride
CF	Culture Filtrate
cm	Centimeter
$CuSO_4$	Copper sulphate
DCP	Dried Cell Powder
DDW	Double Distilled water
DMSO	Di-Methyl Sulf Oxide
DNA	Deoxyribose Nucleic Acid
FW	Fresh Weight
g	Gram
GA_3	Gibberellic acid
GR	Growth Regulators
$HgCl_2$	Mercuric Chloride
H_2O_2	Hydrogen Peroxide
HPLC	High Performance Liquid Chromatography
ISSR	Inter Simple Sequence Repeats
$K_2Cr_2O_7$	Pottasium dichromate
L	Liter
LOD	Limit Of Detection
LOQ	Limit Of Quantification
mg	Milligram

mg%	mg/100 g biomass
mm	Milimeter
mM	Milli Molar
MS	Murashige and Skoog medium (1962)
MSL	Mean Sea Level
NAA	α-Naphthalene acetic acid
NaOCl	Sodium hypo chlorite
NaOH	Sodium Hydroxide
NNRTI	Non Nucleoside Reverse Transcriptase Inhibitor
NR	No Result
NS	Non Significant
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
Picloram	4-amino-3,5,6-trichloro picolinic acid
PPB	Percent Polymorphic Bands
PVP	Polyvinyl pyrrolidone
rpm	revolution per minute
RSD	Relative Standard Deviation
RT	Retention Time
S D	Standard Deviation
S E	Standard Error.
TDZ	Thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-ylurea)
UV	Ultra violet
v/v	Volume/volume (concentration)
WPM	Woody Plant Medium
w/v	Weight/ volume (concentration)
µM	Micro molar
2,4-D	2,4-di chlorophenoxy acetic acid

Abstract

Rationale of the study

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are the group of compounds that inhibit the activity of reverse transcriptase Type-1 enzyme of HIV-1 by the virtue of their ability to bind irreversibly at the non-substrate binding, allosteric site (De Clercq, 2000). Few of such compounds namely calanolides A and B (IC_{50}, 20 and 15 µM, respectively) were isolated from *Calophyllum lanigerum* (Kashman et al., 1992) and inophyllum B and P (IC_{50}, 38nM and 130 nM, respectively) were isolated from *Calophyllum inophyllum* Linn, Clusiaceae, (Patil et al., 1993). Beside anti-HIV-1 inophyllum B and inophyllum P, few other dipyranocoumarins viz. inophyllum A, inophyllum C, inophyllum D and calophyllolide isolated from *C. inophyllum* have been reported to exhibit cancer chemopreventive activity against Epstein-Barr Virus early antigen activation induced by 12-*O*-tetradecanoylphorbol-13-acetate in Raji cells (Itoigawa et al., 2001), while inophyllum C and calophyllolide were also shown to have antimicrobial and cytotoxic activity (Yimdjo et al., 2004). Chemistry of *C. inophyllum* has been well studied and several classes of secondary metabolites like xanthone, triterpene and coumarins have been isolated and characterized. *C. lanigerum* from which potential NNRTIs calanolides were isolated has not been reported from India so far, while *C. inophyllum* from which NNRTIs inophyllums (dipyranocoumarins) were isolated grows at several locations along The Western Ghats of India. This plant produces a good quality timber that can be used in boat and ship making because of which it is cut fairly on large scale. Due to its large scale cutting for obtaining timber, this plant species has been included in IUCN's red list of threatened species (Stevens et al., 1998) and needs to be conserved.

Presently, many other natural products are derived by extracting and isolating solely from massive quantities of whole plant parts. Often the source plants are either wild or cultivated in tropical or subtropical and geographically remote areas which are subject to political instability, drought, disease and changing land use patterns and other environmental factors. In addition, the long cultivation periods between planting and extraction make selection of high-yielding plants difficult, thus resulting in expensive drugs. Cultivation periods may range from several months to decades. In spite of these difficulties and costs, the extraction of medicinally important secondary metabolites from cultivated plants or plants in the wild continues because of a lack of credible alternatives. Clearly, the development of alternative and complimentary methods to whole plant extraction for the production of clinically important secondary metabolites is an issue of considerable socioeconomic importance. These facts have generated great interest in the use of plant cell culture technologies for the production of inophyllums like pharmaceuticals and other plant derived secondary metabolites. Indeed, the plant cell culture technology is now sufficiently advanced to allow for large quantities of relatively homogeneous, undifferentiated cells to be

produced. When compared to whole plant extraction, plant cell and tissue culture systems are complementary and may provide competitive metabolite production systems.

Objectives

The present study "*In vitro* production of secondary metabolites from cultured cells/ tissues and molecular characterization of *Calophyllum inophyllum*" was taken up with the following objectives.

1. To develop and validate HPLC method for analysis of dipyranocoumarins in *in vitro* growing cultured cells / tissues and *in vivo* growing plant materials.
2. To study the chemo diversity and genomic diversity in *C. inophyllum.*
3. To establish micropropagation protocol for *C. inophyllum.*
4. To establish callus cultures using different media, hormones and explants for studying the *in vitro* expression pattern of dipyranocoumarins.
5. To establish cell suspension culture for increased biomass and dipyranocoumarins, expression.
6. To study the effects of different biotic and abiotic elicitors on *in vitro* expression of dipyranocoumarins.

Chapter 1. General Introduction

This chapter will cover the general introduction to the importance of medicinal plants and plant secondary metabolites, use of biotechnological approaches for studying secondary metabolite production. This chapter will also cover general introduction to genetic diversity and use of different molecular markers to investigate genetic diversity. An introduction to *Calophyllum inophyllum* and its phytochemical and medicinal importance are also covered. The aims and objectives of the study will be presented.

Chapter 2. Materials and Methods

The materials used and various general techniques adopted for studying chemo diversity and genetic diversity, *in vitro* micropropagation, callus cultures, cell suspension culture and elicitation of cell suspension culture will be described. The general materials and techniques used in development of HPLC methods, isolation of endophytic fungi, preparation of biotic elicitors will also be discussed.

Chapter 3. Development of HPLC method for analysis of dipyranocoumarins and diversity studies in *Calophyllum inophyllum*.

The chapter will deal with development and validation of HPLC method for analysis of dipyranocoumarins, development of extraction protocol and investigation of chemo diversity *C. inophyllum*. This chapter will also deal with investigation of genetic diversity by using ISSR markers and finding out association between chemo diversity and genetic diversity. Based on chemo diversity and genetic diversity, identification of elite plants or location will be discussed.

Chapter 4. *In vitro* propagation of *Calophyllum inophyllum*.

The chapter will describe the results on plant micropropagation system in *C. inophyllum.* Results on the optimization of sterilization protocol, influence of basal medium MS and WPM on seed germination, influence of BAP, TDZ and IBA on multiple shoot induction, shoot elongation and root induction will be presented.

Chapter 5. Establishment of callus cultures in *Calophyllum inophyllum.*

The chapter will deal with the establishment of callus cultures of *C. inophyllum*. Results on the influence of different basal media, explants, auxins and cytokinins on callus induction will be presented. Results on influence of various combinations and concentrations on callus induction and analysis of these callus cultures with HPLC to study the expression pattern of dipyranocoumarins will be described.

Chapter 6. Establishment of cell suspension cultures for increased biomass and dipyranocoumarins' expression.

This chapter will deal with establishment of cell suspension cultures using callus cultures induced from different explants, effects of different hormones, their combinations and concentrations on biomass and expression of dipyranocoumarins in cell suspension cultures. This chapter will include results on influence of various medium components like total nitrate, sulphate, vitamins, sucrose and $CaCl_2$ on biomass and expression of dipyranocoumarins.

Chapter 7. Study of effect of different elicitors on dipyranocoumarins' expression in cell suspension cultures of *Calophyllum inophyllum*.

This chapter will deal with the elicitation of cell suspension cultures in *C. inophyllum*. Results on isolation and identification of fungi endophytic to *C. inophyllum*, effects of isolated endophytic fungi as biotic elicitors on dipyranocoumarins' expression will be covered. This chapter

will include results on influence of various heavy metals like Copper, Cadmium and Chromium as a biotic elicitors in cell suspension culture.

Summary

This section will contain salient findings and conclusions of the present study.

CHAPTER 1. GENERAL INTRODUCTION

1.1. Introduction to medical plants and secondary metabolites:

Since time immemorial, humans have relied on nature for their needs of food, clothing, shelter, mean of transportations, fertilizers, flavors, fragrances, and medicines. Plant based systems of medicine continue to play an important role in health care. World Health Organization (WHO) estimates that 80 % of the world's populations rely mainly on traditional system of medicine for their health care (Fakim et al., 2006). Plant products also play an important role in health care system of remaining 20 % population, mainly residing in developed countries. Analysis of the data on prescriptions dispensed from community pharmacies in the US from 1959 to 1980 indicates that 25% of drugs prescribed contained plant extracts or active principles derived from higher plants. At least 119 chemical substances derived from 90 plant species can be considered as important drugs and are currently in use in one or more countries (Farnsworth et al., 1985). Of these 119 drugs, 74% were discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine. These compounds have the same or related use as the plants from which they were derived. Natural products and their derivatives represents more than 50 % of all the drugs in clinical use today to which higher plants contribute no less than 25 % (Fakim et al., 2006). A recent investigation of natural products as a source of new drugs over the period 1981–2002 shows that 39% of the 877 molecules can be classified as truly synthetic in origin. Out of remaining 61 %, 28.4 % are semi synthetic molecules and 32.6 % are of natural origin. In the area of the anti-infectives (anti-bacterial, anti-fungal, parasitic, and viral), close to 70% are naturally derived or inspired, while in the cancer treatment area, 67% are in this category (Fakim et al., 2006).

It has been estimated that about 3,10,000 - 4,22,000 number of higher plants are growing on earth and about 1,20,000 are tropical endemic species (Pitman and Jorgersen, 2002). Approximately half of the world's flowering plant species live in the tropical forests which continue to support a vast reservoir of potential drug species. These flowering plants provide natural product chemist with a invaluable source of new compounds as starting points for development of new drugs. As on today, only about 1 % of tropical species have been studied for their pharmaceutical potential and possibilities for finding more compounds is enormous. To date about 50 % drugs have come from tropical plants. Up to 1985, 2600 out 3500 structures identified came from higher plants (Payne et al., 1991). During the last 40 years, at least a dozen potent drugs have been derived from flowering plants.

People who use traditional remedies may not understand the scientific rationale behind their medicines, but they know from their personal experience that some medicinal plants can be highly effective if used at therapeutic doses. Today, modern science has the better understanding of how the body functions because of which we are in a better position to understand the healing

power of the plants and their potential as multi- functional chemical entities for treating complicated health problems. Medicinal plants typically contain mixture of different chemical compounds that may act individually, additively or synergistically to improve health. According to OPS (Arias, 1999), medicinal plant is any plant used to cure, prevent or relieve disease or to alter physiological and pathological process. Also medicinal plant is a plant used as a source of drug or their precursor.

Natural products, particularly secondary metabolites have formed the basis of natural product derived medicines, but the presence of these in the biochemistry of the plant is very often difficult to justify. Metabolic system of the plants may be regarded as being constituted of regulated processes within which biochemical conversions and mass transfers take place. In the field of phytochemistry, understanding has progressed to a stage in which metabolic processes, biosynthetic pathways and their interconnections are distinguished and studied in the context of their function and genetic control. It has been well established fact that plant secondary metabolites are synthesized by the plants as a part of their defense system e. g. plants are known to produce phytoalexins as a response to attacks by bacteria and fungi. Plants also produce secondary metabolites in response to extremes of physical and environmental conditions like draught, high salinity, extremes of pH and temperature of soil, and high concentrations of heavy metals in soil etc. These secondary metabolites help plants survive in such adverse conditions.

Metabolites being formed in plants can be distinguished into primary metabolites and secondary metabolites. Primary metabolites are metabolites or intermediates involved in metabolism associated with fundamental life processes common to all plants. These comprise processes such as photosynthesis, pentose cycle, glycolysis, the citric acid cycle, electron transport, phosphorylation, energy regulation and management. In contrast to primary metabolites, secondary metabolites represent features that can be expressed in terms of ecological, taxonomical and biochemical differentiation and diversity. Plant secondary metabolites have no recognized role in the maintenance of fundamental life processes in the plant but they do have important role in the interaction of the plant with its environment. The biosynthesis and accumulation of secondary metabolites provide not only a basis for biochemical systematics and chemosystematics but also is extremely rich biogenic resources for discovery and development of novel drugs and for developing novel drugs. Higher plants are rich source of bioactive constituents and phyto-pharmaceuticals that can be used in pharmaceutical industry. Some of the plant derived natural products include drugs like morphine and codeine (*Papaver somniferum*), cocaine (*Erythroxylum coca*), quinine (*Cinchona spp*), anti cancer vincristine, vinblastine (*Catharanthus roseus*), atropine (*Belladonna spp*), colchicines, phytostiminine, pilocarpine, reserpine (*Rauwolfia serpentina*), and steroids like diosgenin (*Dioscorea spp*), digoxin, and digitoxin (*Digitalis species*).

Plant secondary metabolites belong to three major chemical classes as

A. Alkaloids- derived from amino acids.

B. Phenolics- derived from carbohydrates.

C. Terpene- derived from lipids.

1.1.1. Alkaloids:

Alkaloids are cyclic organic compounds containing nitrogen in a negative oxidation state. Based on their structures, alkaloids are divided into subgroups as non-heterocyclic alkaloids and heterocyclic alkaloids, which are again divided into 12 major groups according to their basic ring structures. Alkaloids are having marked physiological action on humans and animals. Alkaloids are involved in plant protection and germination. They are more common in dicot than in monocots.

1.1.2. Phenols and phenolic glycosides:

Phenols are among the largest group of secondary metabolites ranging from simple structures with one aromatic ring to complex polymers such as tannins and lignins. Phenolic compounds are mostly biosynthesized via the Shikimic acid or acetate pathway. Following are the sub groups of phenols and phenolic glycosides.

1.1.2.1. Simple phenolic compounds:

These compounds have a monocyclic aromatic ring with an alcoholic, aldehydic or carboxylic group. They may have a short hydrocarbon chain. Capsaicin, isolated from *Capsicum sp*., is a vanillyl amide of isodecenoic acid and is marketed as an analgesic. Eugenol is widely used in dentistry due to its anti-bacterial and anti-inflammatory and local anaesthetic activities.

1.1.2.2. Tannins:

The chemistry of these compounds is very complex. They can be distinguished as hydrolysable and condensed tannins. This grouping of tannins is based on whether acids or enzymes can hydrolyse the components or whether they condense the components to polymers.

1.1.2.3. Coumarins and their glycosides:

Coumarins are shikimate-derived, benzo-apyrone derivatives that can be present in plants, both in a free state and as glycosides. They have limited distribution in the plant

kingdom and have been used as chemotaxonomic markers in order to classify plants. They give a characteristic odour of new-mown. Some coumarins are phytoalexins and are manufactured by the plant in the event of an infection by bacteria and fungi.

1.1.2.4. Quinones:

Quinones are oxygen-containing compounds that are oxidized homologues of aromatic derivatives and are characterized by a 1, 4-diketo-cyclohexa- 2, 5-diene pattern (paraquinones) or by a 1, 2-diketo-cyclohexa-3, 5-diene pattern (ortho- quinones). In naturally occurring quinones, the dione is conjugated to an aromatic nucleus (benzoquinones), or to a condensed polycyclic aromatic system e.g. naphthalene (naphthoquinones), anthracene (anthraquinones), 1, 2-benzanthacene (anthracyclinones), naphthodianthrene (naphthodianthrone), pyrelene, phenanthrene and abietane-quinone. Naphthoquinones are yellow or orange pigments from plants. Biosynthetically, the naphthoquinones are derived almost exclusively from the Shikimic acid pathway. The pharmaceutical significance of this group is limited except for few examples like Plumbagin isolated from *Plumbago species* that exhibit anti-bacterial and cytotoxic properties.

1.1.2.5. Flavonoids:

Flavonoids are compounds that are responsible for the colour of flowers, fruits and sometimes leaves. Some may contribute to the colour by acting as co-pigment. Flavonoids have important role of protecting the plant from UV-damaging effects and play role in pollination by attracting animals by their colours. Structurally flavonoids are 2-phenyl chromane or an Ar–C3–Ar skeleton. Biosynthetically they are derived from a combination of the Shikimic acid and the acetate pathways. Small differences in basic substitution patterns give rise to several subgroups. In the plant, flavonoids can either occur as aglycones or as O- or C-glycosides. Recently, flavonoids have attracted interest due to the discovery of their pharmacological properties such as anti-inflammatory, analgesic, anti-tumour, anti-HIV, anti infective (anti-diarrhoeal, anti-fungal), anti-hepatotoxic, anti-lipolytic, anti-oxidant, vasodilator, immunostimulant and anti-ulcerogenic.

1.1.2.6. Anthocyanins:

These compounds impart bright colours to most flowers and fruits. These are water-soluble pigments that occur as glycosides (anthocyanins *sensu stricto*) and their aglycones (anthocyanidins). Chemically they are composed of the 2-phenyl benzopyrylium cation, more commonly referred to as the flavylium cation. Cyanidin is an example of an anthocyanin. The pharmacological activities are similar to flavonoids; for instance for decreasing capillary

permeability and fragility, and as anti-oedema. The application of anthocyanins is as food additives, e.g. in beverages, jams and confectionary products.

1.1.2.7. Lignans and related compounds:

Chemically, lignans are formed by copolymerization of alcohol with a p-hydroxycinnamic structure (p-hydroxycinnamyl, coniferyl or sinapyl alcohol). Lignans and related compounds are derived from condensation of phenyl propane units. Neolignans are also condensation products of phenylpropanoid units. Lignans are always combined with polysaccharides and are substances deposited at the end of the primary and secondary cell walls formation. Pharmacologically lignans exhibit anti-tumour activity and lignans like Kadsurenone, a neolignan, exhibits anti-allergic and anti-rheumatic activity. The major application of lignin is as a precursor of vanillin, which is widely used in the food industry.

1.1.3. Terpenoids:

Terpenoids and steroids are biosynthesized from acetate via mevalonic acid and are derived from the isoprene (5-C unit). Terpenoids can be further devided into following groups.

1.1.3.1. Monoterpenes:

Monoterpenes arise from the head to tail coupling of two isoprene units. These are most simple constituents in the terpene series and are C10 compounds. They are commonly found in essential oils. Iridoids and pyrethrins are included in this group.

1.1.3.2. Sesquiterpenes:

Sesquiterpenes are constituents of essential oils of many plants, e.g. bisabolol, humulene and caryophyllene. Sesquiterpene lactones are well known as bitter principles. These compounds possess a broad range of activities due to the a-methylene- lactone moiety and epoxides. Their pharmacological activities are anti-bacterial, anti-fungal, anthelmintic, anti-malarial and molluscicidal. Examples are Santonin, which is used as an anthelmintic and as an anti-malarial.

1.1.3.3. Diterpenes:

Diterpenes constitute a vast group of C20 compounds arising from the metabolism of 2E, 6E, 10E-geranylgeranyl pyrophosphate. They are present in animals and plants. These compounds have some therapeutic applications. For example, Taxol and its derivatives are anti-

cancer drugs. Other examples are Forskolin, which has anti-hypertensive activity. Zoapatanol is an abortifacient while Stevoside is a sweetening agent.

1.1.3.4. Triterpenes:

Triterpenes are C30 compounds arising from the cyclization of squalene. The basic skeleton arises from the cyclization of 3S-2, 3-epoxy, 2,3-squalene. Oleanane is an example of a pentacyclic triterpenes and testosterone of a steroid. Tetracyclic terpenes and steroids have similar structures but have different biosynthetic pathway.

1.1.3.5. Carotenoids:

Carotenoids contain 8 isoprene (C40) units that are responsible for the orange and yellow colours of some vegetables and fruits. Among these compounds, the hydrocarbons are collectively referred to as carotenes and the hydroxylated derivatives as xanthophylls. Carotenoids are either acyclic (e.g. lycopene) or comprise of one or two pentacyclic or hexacyclic rings at one end or the other (e.g. b,w-carotene) or at both ends (e.g. b,b-carotene). Carotenoids became interesting agents after the discovery of a negative correlation between the plasma concentration or b-carotene and the prevalence of certain forms or cancer. Some doctors prescribe β-carotene for cancer patients. Furthermore, in the intestine b-carotenes are converted to retinol (Vitamin A). They can be used for the treatment or photosensitization, retinal diseases and glaucoma. Carotenoids are also safe coloring agents for food substances and cosmetics (Padua et al., 1999).

1.1.3.6. Steroids:

Steroids contain a ring system of three 6-membered and one 5-membered ring. Because of the profound biological activities encountered, many natural steroids together with a considerable number of synthetic and semi-synthetic steroidal compounds are employed in medicine (e.g. steroidal saponins, cardioactive glycosides, corticosteroid hormones and mammalian sex hormones).

(i) Saponins:

Saponins constitute a vast group of glycosides, which occur in many plants. They are characterized by their surfactant properties; they dissolve in water and when shaken, form a foamy solution. Saponins are classified by their aglycone structure into triterpenoids and steroid saponins. Most triterpenoid saponins are derivatives of one of the triterpenes oleanane, ursane and lupane, while steroid saponins generally possess the typical steroid skeleton with 2 extra rings E, a furan structure and F, a pyran structure respectively.

(ii) Cardiac glycosides:

The aglycone part of cardiac glycosides is a tetracyclic steroid with an attached unsaturated lactone ring that may have 5 or 6 members. Cardiac glycosides are classified into two groups according to the lactone ring: the C23 cardenolides with an a,b-unsaturated d-c-lactone (= butenolide), and the C24 bufadienolides with a di-unsaturated c-lactone (= pentadienolide). The sugar moiety is normally attached via the C-3 hydroxyl group or the aglycone. The majority or the saccharides found in cardiac glycosides are highly specific. They are 2,6-dideoxyhexoses, such as D-digitoxose, L-oleandrose or D-diginose. Cardiac glycosides have been used as drugs for the treatment of cardiac insufficiency. An example is digitoxin from *Digitalis*, where the sugar moiety is attached to the aglycone digitoxigenin via the C-3 hydroxyl group (Padua et al., 1999).

1.2. Biodiversity and medicinal plants:

Biodiversity is the variety and differences among living organisms from all sources, including terrestrial, marine, and other aquatic ecosystems and the ecological complexes of which they are a part. This includes genetic diversity within and between species and of ecosystems. Thus, in essence, biodiversity represents all life.

The nature is a potential source of useful drugs and its importance has been recognized since ancient time. This has resulted in large number of medicinal plants are used to treat various diseases and some drugs in today's medicine are based on their traditional use (Verpoorte, 1998). Clearly, plants hold a prominent position in the availability of resources of natural bioactive molecules. The sessile character of plants required the development of large array of molecules to effectively respond to biotic and abiotic stimuli *in situ*. The chemical diversity of plants has made them the source of choice for the isolation of pharmacologically important metabolites. Metabolites derived from plants can be used in several different ways in the development of drugs.

Biodiversirty, "the diversity of living forms" has attracted the great interest and concern because of its ability to provide large array of molecules for the development of drugs. Decline in the biodiversity is largely the result of human activities such as drastic transformation of natural landscape and deforestation. The consequence of a reduction in biodiversity through loss of species constitutes the serious threat to human survival. The loss of plant species could reduce the availability of natural products used for drug developments. In medical science, loss of species could reduce the opportunity for treatment of disease through the loss of medical model and a new medicine.

Because secondary metabolites represent features that can be expressed in terms of ecological, taxonomical and biochemical differentiation and diversity, plants growing at

different locations with different ecological conditions accumulate different levels of secondary metabolites. From conservation point of view and *in vitro* production of secondary metabolites, starting plant material needs to be an elite in terms of higher levels of secondary metabolites of interest. For selecting such elite plants, chemodiversity studies have to be carried out thereby collecting the plant material from different locations with different ecological conditions and analyzing with different analytical techniques like TLC, HPTLC, GC, HPLC, GC-MS, HPLC-MS for highest initial levels of secondary metabolites. Based on such chemodiversity studies, plants or locations of plants with higher levels of secondary metabolites of interest can be selected as elite plants and be used for conservation as well as for taking *in vitro* secondary metabolite production. Selection based on chemodiversity as well as genetic diversity is always considered authentic and proper, especially in medicinal plants. In genetic diversity study, plant material like leaves more often are collected from different locations, their genomic DNAs are isolated and PCR based molecular markers like RFLP, AFLP, RAPD, SSR, ISSR, SNP are established and studied.

1.2.1. Genetic Diversity:

Genetic fingerprinting is a techniques used to distinguish between individuals of the same species using only samples of their DNA. Its invention by Sir Alec Jeffreys at the University of Leicester was announced in 1985. Two plants of same species and genus will have the vast majority of their DNA sequence in common. Genetic fingerprinting exploits highly variable repeating sequences called microsatellites. Two unrelated plants will be unlikely to have the same numbers of microsatellites at a given locus. Recently, number of PCR based molecular markers are available and were used to assess the genetic variations in different plant species (Vieira et al., 2001; Casasoli et al., 2001; Xiao et al., 2004; Ray et.al., 2006;). Of these, most reliable are inter simple sequence repeats (ISSR) marker. ISSR markers are well reproducible, has less operational and development cost, and do not require knowledge of genomic sequence to design primers.

1.2.2. Introduction to DNA markers:

Genetic polymorphism is classically defined as the simultaneous occurrence of two or more discontinuous variants or genotypes of a trait in the same population (Joshi, 2001). Although DNA sequencing is a straightforward approach for identifying variations at a locus, it is very expensive and laborious. A wide variety of techniques have, therefore been developed in the past few years for visualization of DNA sequence polymorphism.

The term DNA fingerprinting was introduced to describe bar code-like DNA fragment patterns generated by hybridization with multilocus probes after eletrophoretic

separation of genomic DNA fragments. The emerging pattern make up a unique feature of the analysed individual and are currently considered to be the ultimate tool for biological individualization. DNA fingerprinting / DNA marker system is used in describing the combined use of several single locci and is one of those techniques used in detection of polymorphism.

1.2.3. Properties of DNA markers:

DNA markers are ubiquitous, innumerable, discrete, nondeleterious, inherited by Mendelian laws, unaffected by the environment, and are free of epistatic and pleiotropic interactions (Beckman and Soller 1986; Tanksley et al., 1989). These markers offer several advantages over morphological and isozyme markers (Gupta PK, 1996; Joshi et al., 1999):

1. They are unlimited in number because the RFLP and PCR techniques are able to detect differences at the DNA level in both translatable and non translatable DNA regions.
2. They are phenotypically and environmentally neutral because sequence polymorphisms are detected directly at the DNA level and not by assaying gene products which may be influenced by alterations in gene coding sequence or by changes in the level of gene expression.
3. DNA markers mediated selection in breeding programs can be carried out in a non destructive process to the original plant because only a small amount of tissue is required.
4. They enable selection for specific recessive genotypes in a heterozygous form where the recessive genotype cannot express because a dominant allele masks a recessive allele. This advantage can be achieved with markers which are co-dominant in nature.
5. They do not show either dominant recessive interaction or epistasis and are devoid of pleotropic effect.
6. DNA markers in the genome can be considered as entry point to the genes. Thus, they facilitate the chromosome walking techniques for gene cloning.
7. DNA markers are not tissue specific and can be used at any stage of the plant growth.

An ideal DNA marker should have the following properties (Weising et al., 1995):

- ❖ High polymorphism
- ❖ Co-dominant inheritance
- ❖ Frequent occurence in genome
- ❖ Selective neutral behaviour
- ❖ Easy access
- ❖ Easy and fast assay

- ❖ High reproducibility
- ❖ Easy exchange of data between laboratories

It is not easy to find a molecular marker which would meet all the above criteria. Depending upon the type of study to be undertaken, a marker system can be identified that would fulfil at least a few of the above characteristics (Weising et al., 1995).

1.2.4. Types of DNA markers:

Various types of molecular markers are utilized in evaluation of DNA polymorphism and are generally classified as hybridization based markers and polymerase chain reaction based markers. In hybridization based markers, the DNA profiles are visualized by hybridizing the restriction enzyme digested DNA, to a labelled probe, which is a DNA fragment of known origin or sequence. PCR based markers involve *in vitro* amplification of particular DNA sequences or loci with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme. The amplified fragments are separated electrophoretically and banding patterns are detected by different methods such as staining and autoradiography. Information about few representative marker systems is as given below (Joshi et al., 1999).

1.2.4.1. Single or Low copy probes:

Restriction Fragment Length Polymorphism (RFLP) are simply inherited Mendelian characters and codominant in nature. They have their origin in the DNA rearrangements that occur due to the evolutionary processes, point mutations within the restriction enzyme recognition site sequences, insertions or deletions within the fragments and unequal crossing over (Shlotterer and Tautz, 1992). Botstein et al., (1980) used RFLPs for the first time to construct a genetic map. In RFLP analysis, restriction enzyme digested genomic DNA is resolved by gel electrophoresis and then blotted on to a nitrocellulose membrane. Specific banding patterns are then visualized by hybridization with radioactive labelled probe. The utility of RFLPs has been hampered due to the requirement of large amount of DNA and specific radioactive labelled probe. Moreover, the assay is time consuming and labor intensive. RFLP markers converted to PCR based markers are Sequence Tagged Sites (STS), Allele Specific Associated Primers (ASAP), Expressed Sequence Tag markers (EST) and Single Strand Conformation Polymorphism (SSCP) (Orita et al., 1989).

1.2.4.2. Arbitrary sequence markers:

Random Amplified Polymorphic DNA (RAPD) marker assay in which nucleotide sequence polymorphism is detected by using a single primer of arbitrary nucleotide sequence. Some variations from the RAPD technique includes DNA amplification fingerprinting (DAF), Arbitrary primed PCR (AP-PCR), Sequence characterized amplified regions for amplification of specific band (SCAR), Cleaved amplified polymorphic sequences (CAPs), Randomly amplified microsatellite polymorphisms (RAMPO) and Amplified Fragment Length Polymorphism (AFLP).

1.2.4.3. Multilocus probes:

About 30-90% of the genomes of virtually all species is constituted of repetitive DNA, which are highly polymorphic in nature. These regions contain genetic loci comprising several alleles differing from each other with respect to length, sequence or both. The repetitive DNA play an important role in absorbing mutations in a genome and inherited mutations forces together from the basis for a number of marker systems that are useful for several applications in plant genome analysis. The major forms of repetitive DNA are

(i) Microsatellites and Minisatellites:

They are multilocus probes creating a complex banding patterns usually nonspecies specific and occur ubiquitously. The patterns generated by these probes are known as Oligonucleotide prints. Minisatellites are tandem repeats with a monomer repeat length of about 11-60 bp, while microsatellites or short tandem repeats/simple sequence repeats (STRs / SSRs) consist of 1-6 bp long monomer sequence that is repeated several times. These loci contain tandem repeats that vary in the number of repeat units between genotypes and are referred as variable number of tandem repeats (VNTRs). Microsatellites and minisatellites form an ideal marker system creating complex banding patterns by detecting multiple DNA loci simultaneously. They are dominant fingerprinting markers and codominant STMS markers. They exist as many alleles in a population, their level of heterozygosity is high and they follow Mendelian inheritance. Minisatellite and Microsatellite sequences converted in to PCR based markers are Sequence Tagged Microsatellite Sites (STMS), Directed amplification of Minisatellite region DNA (DAMD), Inter Simple Sequence Repeats (ISSR). STMS method includes DNA polymorphism using specific primers designed from a sequence data of a specific locus. Where ISSRs can be used in the species with unknown sequence data. The potential of ISSR markers have been exploited in the present study.

(ii) Inter simple Sequence Repeats (ISSR) and its applications:

Inter simple sequence repeats are the regions which lie within the microsatellite repeats. Zietkiewicz et al., (1994) reported this technique for the first time. The technique involves use of a single primer composed by microsatellite sequences plus a short arbitrary sequence (anchor) which target to a subset of 'simple sequence repeats' (SSRs) or microsatellites and amplify the region between two closely spaced and oppositely oriented SSRs. The primers can be dinucleotide, tri-nucleotide, tetra- nucleotide or penta-nucleotide and number of combinations could be higher with increasing number of nucleotides e.g. for dinucleotide (4)^2= 16, (4)ˆ3=64, (4)ˆ4=256 etc. The primers used can be either unanchored or more usually anchored at 3' or 5' end with 1 or 4 degenerate bases extended into the flanking sequences. ISSRs segregate mostly as dominant markers following simple Mendelian inheritance (Gupta et al., 1994; Tsumura et al., 1996; Wang et al., 1998; Joshi et al., 1999). However, they have also been shown to segregate as co-dominant markers in some cases thus enabling distinction between homozygotes and heterozygotes (Wang et al., 1998; Reddy et al., 2002). This technique combines most of the benefits of AFLP and microsatellite analysis with the universality of RAPD. ISSRs have high reproducibility possibly due to the use of longer primers (16-25 mers) as compared to RAPD primers (10- mers) which permits the subsequent use of high annealing temperature (45-60º C) leading to higher stringency. This technique is found to be stable across wide range of PCR parameters (Bornet et al., 2001; Reddy et al., 2002) and the potential for integrating ISSR-PCR into programs of plant improvement is enormous.

1.3. Biotechnology and plant secondary metabolites:

Because of over exploitation of natural resources for fulfilling the ever increasing demands of six billion population of the world, all forests including some biodiversity hotspots are undergoing major habitat loss. Also because of over harvesting, many plant species containing high value bioactive compounds are becoming endangered and are difficult to cultivate. Furthermore, the chemical synthesis of plant derived bioactive compounds in laboratory is often not economically feasible because of their complex structures and specific stereochemistry. There is justifying concern about the impending loss of medicinal plants as some of these species may not be available to future generations of natural product drug discovery scientists (Cox, 2000). Production of secondary metabolites by plants is often very low, restricted to species or genus, and is always activated during a particular growth or developmental stage, or under specific seasonal, stress or nutritional availability conditions. For these reasons, a lot of efforts have been put into several biotechnological approaches as a possible production method for plant secondary metabolites. Plant biotechnology is an emerging branch of life sciences helping to solve some of

the major issues in agriculture, food and medicine industry. Biotechnology offers an opportunity to exploit the cell, tissue, organ or entire organism by growing them *in vitro* and to manipulate them physiologically and genetically to get desired products. Following are the aspects of biotechnological approaches for plant secondary metabolites' production.

Aspects of biotechnological approaches to plant derived secondary metabolites.

1. Micropropagation of medicinal plants
   - Endangered plants.
   - High yielding varieties
   - Metabolically engineered plants.
2. Organ culture
   - Shoot culture
   - Root culture
3. Cell culture
   - Callus culture
   - Cell suspension culture
4. Transgenic plants
   - Metabolic engineering.
   - Heterologous expression.
   - Molecular farming.

1.3.1. Plant cell culture:

Plant cell cultures are an attractive alternative source to whole plant for the production of high value secondary metabolites. Because plant cells are biosynthetically potent, each cell in a culture retains complete genetic information and is able to produce complete range of chemicals found in parent plants. Plant cell cultures are independent of geographical, seasonal and environmental variation and it offers defined production system that ensures continuous supply of products, uniform quality and yield. Novel compounds that are not produced by parent plants can also be produced by plant cell cultures. Plant cell cultures facilitate easy downstream processing, recovery and rapid productions.

To ensure the maximum yield and to further enhance the production of desired plant secondary metabolites, different strategies that can be employed are as

1. Screening and selection of highly productive cell lines
2. Manipulation of nutrients
3. Optimization of culture conditions
4. Elicitation

1.3.1.1. Screening and selection of highly productive cell lines:

This begins with selection of elite parent plant with high content of desired product for callus induction to obtain high producing cell lines. High producing cell lines from highly heterogenous population of callus cell lines can be screened and selected by employing different analytical as well as quantitative chromatographic techniques like thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC), high performance liquid chromatography (HPLC), gas chromatography (GC), liquid chromatography- mass spectrometry (LC-MS), gas chromatography- mass spectrometry (GC-MS) etc. Mutation strategy can also be used to screen overproducing cell lines where large population of cells is exposed to a toxic inhibitor or environmental stress. The cells able to resist can only grow and can be selected. *P*-Fluorophenylalanin (PFP), an analogue of phenylalanine was extensively used to select high yielding cell lines with respect to phenolics.

1.3.1.2. Manipulation of nutrients:

Once high yielding callus lines have been selected, expression of desired compounds can further be increased by manipulating different medium components like carbon source, nitrate levels, phosphate levels, growth regulators, and precursor feeding. Optimization of culture environment like pH, temperature, illumination, agitation and aeration is needed in some instances.

(i) Carbon source:

Plant cell cultures grow heterotrophically using simple sugars as a carbon source. Types of sugars and their concentrations have been shown to affect the secondary metabolite accumulation. Carbohydrate, especially sucrose is an important carbon source for most of the plant cells. The sucrose plays dual role as carbon source and osmotic agent. The osmotic stress created by sucrose alone and with other osmotic agents were found to regulate anthocyanin production in *Vitis vinifera* cell suspension culture. In some cases, glucose or fructose can also be used as carbon source.

(ii) Nitrate level:

Plant tissue culture medium such as Murashige and Skoog's (MS, 1962), Linsmaier and Skoog's (LS, 1965), Gamborg's B5 (1968) and Woody Plant Medium (WPM, Lloyd, and McCown, 1980) has nitrate and ammonium as a source of nitrogen. The ratio of ammonium/ nitrate- nitrogen and overall level of total nitrogen markedly affect the total production of

secondary metabolites in plant cell cultures. Nitrogen level was also found to affect the level of proteinaceous or amino acid products in cell suspension culture.

(iii) Phosphate level:

Phosphate concentration in the culture medium does have major role and can effect the production of secondary metabolites. Higher levels of phosphate were found to enhance the cell growth, where it had negative influence on secondary product accumulation. Sasse et al. (1982) have given a number of examples to show that a medium limited in phosphate either induces or stimulates both the product and the levels of key enzymes leading to the product. Thus, reduced phosphate levels induced the production of ajmalicine and phenolics in *Catharanthus roseus*, of caffeoyl putrescines in *Nicotiana tabacum* and of Harman alkaloids in *Peganum harmala.*

(iv) Growth regulators:

Growth regulators and their concentrations are often critical factors in secondary product accumulation (Deus and Zenk, 1982; DiCosmo and Towers, 1984). The type and concentration of auxin or cytokinin or the auxin/cytokinin ratio alters dramatically both the growth and the product formation in cultured plant cells (Mantell and Smith, 1984). The growth regulator 2, 4-D has been shown to inhibit the production of secondary metabolites in a large number of cases. In such cases, elimination of 2, 4-D or replacement of 2,4-D by naphthalene acetic acid (NAA) or indole acetic acid (IAA) has been shown to enhance the production of anthocyanins in suspension cultures of *Populus* and *Daucus carota*, of betacyanins in suspension cultures of *Portulaca*, of nicotine in suspensions of *Nicotiana tabacum*, of shikonin in suspensions of *Lithospermum erythrorhizon* and of anthraquinones in *Morinda citrifolia* (Zenk et al., 1975; Sahai and Shuler, 1984; Seitz and Hinderer, 1988; Tabata, 1988; Rajendran et al., 1992.).

(v) Precursor feeding:

Concept of precursor feeding is based on the idea that any compound which is an intermediate, in or at the beginning of secondary metabolite biosynthetic pathway stands a good chance of increasing the yield of final product. Production of plant secondary metabolites can be increased by supplying such precursors or intermediate compounds and this approach has been successful in many cases. Phenyalanine as precursor led to improved rosmanic acid yield in *Coleus blumei* cell cultures (Ibrahim, 1987) and in *Salvia officinalis* cell suspension cultures.

1.2.1.3. Optimization of culture conditions:

Culture environmental conditions such as light, temperature, medium pH and oxygen have their effect upon secondary metabolite accumulation and have been examined for in many types of cultures.

(i) Temperature:

The optimum temperature range for the induction of callus tissues and growth of cultured cells is 17–25º C and is normally used. However, in some cases, cultured cells of some plant species may favour a different temperature. Lowering the cultivation temperature was found to increase the total fatty acid content per cell in dry weight (Toivonen et al., 1992). Biotransformation of digitoxin to digoxin was favoured when the temperature was maintained at 19ºC whereas 32º C favoured purpureaglycoside A formation in *Digitalis lanata* cell cultures (Kreis and Reinhard, 1992).

(ii) Illumination:

Illumination has been known to strongly alter the accumulation pattern of few metabolites. Illumination stimulated accumulation of anthocyanin in cell cultures of *D. carota* and *Vitis* hybrids (Seitz and Hinderer, 1988). Illumination was found to affect the composition of sesquiterpenes in callus cultures of *Marticaria chamomilla* (Mulder-Krieger et al., 1988). Accumulation of monoterpenes in callus cultures of *Citrus limon* was prompted by exclusion of light (Mulder-Krieger et al., 1988).

(iii) Medium pH*:*

pH of the medium is known to play key role in the production and accumulation pattern of secondary metabolites. pH of the medium between 5 and 6 is the optimum pH range and is usually adjusted before autoclaving the medium. Extremes of pH are avoided. The concentration of hydrogen ions in the medium changes during the development of the culture. The medium pH decreases during ammonia assimilation and increases during nitrate uptake (McDonald and Jackman, 1989).

1.3.1.4. Elicitation:

Elicitation is the induced or enhanced biosynthesis of metabolites due to addition of trace amounts of elicitors and elicitors are the substances when introduced in small concentrations to a living cell system initiates or improves biosynthesis of specific compounds (Ramdan et al., 2003). Since plants produce secondary metabolites in nature as a defense mechanism against attack by pathogens, they have been found to elicit the same response as the pathogen itself when challenged by compounds of pathogenic origin (elicitors). Elicitors are signals triggering the formation of secondary metabolites. Secondary pathways are activated in response to

stress. Elicitors can be of two types as biotic and abiotic. Elicitors of fungal, bacterial and yeast origin, viz. polysaccharides, glycoproteins, inactivated enzymes, purified curdlan, xanthan and chitosan, are biotc elicitors (DiCosmo et al., 1985; Funk et al., 1987; Johnson et al., 1991; Furze et al., 1991; Robbins et al., 1991; Guo et al., 1992; Rajendran et al., 1992; Ramachandra Rao et al., 1996a,b); while salts of heavy metals, UV light, detergent, fungicides, herbicides, non essential media components, denaturated proteins are abiotic elicitors (Namdeo et al., 2007). Elicitors are used to stimulate secondary metabolite product formation in plant cell cultures, thereby reducing the process time to attain high product concentrations and increased culture volumes (DiCosmo and Tallevi, 1985; Eilert, 1987; Barz et al., 1986). Production of many bioactive secondary metabolites have been successfully taken through plant cell cultures (reviewed in Tables 1.1 and 1.2).

Table 1.1. Reports on secondary metabolite production through callus cultures

Plant Name	Bioactive secondary Metabolite	Culture Medium	Reference
Agave amaniensis	Saponins	MS + Kinetin (23.2 µM), 2, 4- D (2.26 µM), KH_2PO_4 (2.50 µM), Sucrose (87.64 mM)	Andrijany et al., 1999.
Allium sativum L	Alliin	MS + IAA (11.4 µM), NAA (10.8 µM), Kinetin (9. 3 µM), Coconut Water (15 %).	Malpathak and David, 1986.
Ambrosia tenuifolia	Altamisine	MS + Kinetin (10 µM), 2, 4-D (1 µM) Ascorbic acid and Cystine (10 µM).	Goleniowski and Trippi, 1999.
Bupleurum falcatum	Saikosaponin	LS + 2, 4- D (2 mg/L)	Wang and Huang, 1982.
Canavalia ensiformis	L- Canavanine	LS + NAA (1.8 mg /L) 2, 4-D (0.05 mg/ L), BA (4.5 mg/ L), Picloram (0.05 mg/ L).	Ramirez et al., 1992.
Chrysanthemum cinerarieafolium	Pyrethrins	MS + 2, 4- D (2 mg/ L), Kinetin (5 mg/ L), Sucrose (3 %)	Rajasekaran et al., 1991.
Citrus sp.	Naringin, Limonin	MS + 2, 4- D (0.66 mg/ L), Kinetin (1.32 mg/ L) , Coconut milk(100 ml)	Barthe et al., 1987.
Coffea Arabica L.	Caffeine	MS + Thiamine HCl (0.9×10^{3}) Cysteine. HCl (10×10^{2}), Kinetin (0.1×10^{3}), 2, 4- D (0.1×10^{3}), Sucrose (30×10^{3}).	Waller et al., 1983.
Corydalis ophiocarpa	Isoquinoline alkaloids	MS + 2, 4- D (1 mg/ L), Kinetin (0.1mg/ L).	Iwasa and Takao, 1982.
Croton subliratus Kurz	Plaunotol	MS + NAA (2 mg/L), BA (0.2 mg/ L), Sucrose (2 %).	Morimoto and Murai, 1989.
Cryptolepis buchanani Roem. & Schult.	Cryptisin	B5 + 2, 4- D (2 mg/ L), Kinetin (0.5mg/ L).	Venkateswara et al., 1987.
Duboisia leichharadtii	Tropane alkaloids	LS or B5 or White + NAA (5×10^{5} M), BA (5×10^{6} M),	Yamada and Endo, 1984.
Eriobotrya japonica	Triterpenes	LS + NAA (10 µM), BA (10 µM).	Taniguchi et al., 2002.
Eucalyptus tereticornis SM	Sterols and Phenolic compounds.	MS + 2, 4- D (2 mg/ L).	Venkateswara et al., 1986

Gentiana sp.	Secoiridoid glucosides	B5 + Kinetin (1 mg/l), 2, 4-D (0.5 mg/ L)	Skrzypczak et at., 1993.
Glycirrhiza echinata	Flavanoids	MS + IAA (1 mg/l), Kinetin (0.1 mg/L), Sucrose (2 %).	Ayabe et al., 1990.
Glycirrhiza glabra var. glandulifera	Triterpenes	MS + IAA (5 ppm), or 2, 4- D (1 ppm), Kinetin (0.1 ppm).	Ayabe et al., 1990.
Hyoscymus niger	Tropane alkaloids	LS + NAA (10^{-5}M), BA (5×10^{-6} M)	Yamada and Hashimoto, 1982.
Mucuna pruriens	L-DOPA	MS + 2, 4- D (2.5 mg/ L), Coconut water (10%)	Brain, 1976.
Nandina domestica	Alkaloids	MS + 2, 4- D (1 mg/ L), Kinetin (0.1 mg/ L)	Ikuta and Itokawa, 1998.
Nicotiana rustica	Alkaloids	LS + 2, 4- D (1 µM), Kinetin (1 µM)	Tabata and Hiraoka, 1976.
Ophiorrhiza pumila	Camptothecin related alkaloids	LS + 2, 4- D (0.22 mg/ L), NAA (0.186 mg/ L), Sucrose (3 %)	Kitajima el al., 1998.
Pinax ginseng	Saponins and Sapogenins	MS (without glycine) + 2, 4- D (1 mg/ L).	Furuya et al., 1973.
Papaver bracteatum	Thebaine	MS + Kinetin (0.47 µM), 2, 4- D (4.52 or 0.45 µM), Sucrose (3 %).	Day et al., 1986.
Papaver somniferum	Alkaloids	MS (without Glycine) + Kinetin (0.1 mg/ L).	Furuya et al., 1972.
Polygala amarella	Saponins.	MS + IAA (1 mg/ L).	Desbene et.al., 1999.
Portulaca grandiflora	Betacyanin.	MS (without Glycine) + 2, 4- D (5 mg/ L), Kinetin (0.2 mg/ L).	Schroder and Bohm, 1984.
Ptelea trifoliate L.	Dihydrofuro (2, 3-b) quinolinium alkaloids.	MS + 2, 4- D (1 mg/ L), Kinetin (0.1 mg/ L), Coconut Water (5%).	Petit-Paly et al., 1987.
Rauvolfia serpentine X Rhazya stricta Hybrid plant.	3-Oxo- Rhazinilam.	LS Medium	Gerasimenko et al., 2001.
Ruta sp.	Acridone and Furoquinoline alkaloids and coumarins.	MS + 2, 4- D (1 mg/ L), Kinetin (1 mg/ L).	Baumert et al., 1992.

Salvia miltiorrhiza	Lithospermic acid B and Rosmarinic acid.	MS + 2, 4- D (0.5 mg/ L), BA (0.5 mg/ L).	Morimoto et al., 1994.
Scopolia parviflora	Alkaloids.	LS + 2, 4- D (10^{-6}M), IAA (10^{-5} M).	Tabata et al., 1972.
Scutelaria columnae	Phenolics	MS + 2, 4- D (0.3 mg/ L), Kinetin (1 mg/ L).	Stojakowska and Kisiel, 1999.

Table 1.2. Reports on production of plant secondary metabolites through cell suspension culture

Plant Name	Bioactive secondary Metabolite	Culture Medium	Reference
Ailanthus altissima	Alkaloids	MS + 2, 4- D (1 mg/ L), Kinetin (0.1 mg/ L), Sucrose (5 %).	Anderson et al., 1987.
Ailanthus altissima	Canthinone alkaloids	MS + 2, 4- D (1 mg/ L), Kinetin (0.1 mg/ L), Sucrose (5 %).	Anderson et al., 1986.
Aole saponaria	Tetrahydroanthracene glucosides	MS + 2, 4- D (1 mg/ L), Kinetin (2mg/ L).	Yagi et al., 1983.
Anchusa officinalis	Rosmarinic acid	B5 + 2, 4- D (1 mg/l), Kinetin (0.1 mg/ L).	De- Eknamkul and Ellis, 1985.
Brucea Javanica (L.) Merr.	Canthinone alkaloids	MS + 2, 4- D (1 mg/l), Kinetin (0.1 mg/ L), Sucrose (5 %).	Liu et al., 1990.
Camelia sinensis	Theamine, γ-glutamyl derivatives	MS + IBA (2 mg/ L), Kinetin (0.1 mg/ L), Sucrose (3 %), Agar (9g/L).	Orihara and Furuya, 1990.
Capsicum annuum	Capsaicin	MS + 2, 4- D (2 mg/l), Kinetin (0.5 mg/ L), Sucrose (3 %).	Johnson et al., 1990.
Casia acutifolia	Anthraquinone	MS + 2, 4- D (1 mg/ L), Kinetin (0.1 mg/ L), Sucrose (3 %), Myo-inosotol (100 mg/ L).	Nazif et al., 2000.
Catharanthus roseus	Indole alkaloids	MS + Sucrose (3 %).	Moreno et al., 1993.
Catharanthus roseus	Catharanthine	MS + NAA (2 mg/ L), IAA (2 mg/ L), Kinetin (0.1 mg/L), Sucrose (3 %).	Zhao et al., 2001b
Chrysanthemum cinerariaefolium	Chrysanthemic acid and pyrethrins	MS + Casein hydrolysate (1 g/L), 2, 4-D (0.5 mg/ L), Kinetin (0.75 mg/ L).	Kueh et al., 1985.
Cinchona L	Alkaloids	MS + Koblitz and Hagen vitamins & amino acids, 2, 4- D (4.52 µ mol /L), Kinetin (1µ mol/l), GA3 (0.3 µ mol/ L), Sucrose (0.09 µ mol/L)	Koblitz et al., 1983.
Cinchona robusta	Robustaquinone	MS + 2, 4- D (2 mg/ L), Kinetin (0.2	Schripsema et al., 1999.

		mg/ L), (Cystine 50 mg/ L), Sucrose (2 %).	
Cinchona spec.	Anthraquinone	B5 + 2, 4- D (1 mg/ L), Kinetin (0.2 mg/ L)	Wijnsma et al., 1985.
Cinchona succirubra	Anthraquinone	MS + 2, 4- D (1 mg/ L), Kinetin (0.1 mg/ L), Myo- Inositol (100mg/ L), Coconut milk (5%), Sucrose (2 %).	Khouri et al., 1986.
Cruciata glabra	Anthraquinone	LS + NAA (2 mg/l) Kinetin (0.2 mg/ L), Casein hydrolysate (1mg/L).	Dornenburg and Knorr, 1999.
Digitalis purpurea	Cardenolides	Ms + BA (1 mg/ L), IAA (1mg/ L), Thiamine HCL (1 mg/ L)	Hagimori et al., 1982.
Dioscorea dettoidea	Diosgenin	MS + 2, 4- D (0.1 mg/ L).	Heble and Staba., 1980.
Dioscorea doryophora Hance.	Diosgenin	MS + 2, 4- D (2 mg/l), BA (0.2 mg/ L).	Huang et al., 1993.
Ephedra spp.	L- Ephedrine D- Pseudoephedrine.	MS + Kinetin (0.25 µM),2, 4- D or NAA (5 µM), Sucrose (3%)	O'Dowd et al., 1993.
Fumaria capreolata	Isoquinoline alkaoids	LS medium	Tanahashi and Zenk, 1985.
Ginkgo biloba	Ginkgolide A	MS + NAA (1 mg/ L) Kinetin (0.1mg/ L), Sucrose (3 %).	Carrier et al., 1991.
Glehnia littoralis	Furarocoumarin	LS + 2, 4- D (1µM), Kinetin (1µM).	Kitamura et al., 1998.
Isoplexis isabellina	Anthraquinones	MS+2,4- D (5µM), Kinetin(10µM)	Arrebola et al., 1999.
Linum flavum L.	5- Methoxypodophyllotoxin	MS salts + B5 vitamins, Folic acids (0.88mg/ L), Glycine (2 mg/ L), Sucrose (2 %)	Uden et al., 1990.
Lithospermum erythrorhizon	Shikonin derivative	LS + IAA (10^{-6} M) Kinetin (10^{-5} M).	Fujita et al., 1981.
Lithospermum erythrorhizon	Shikonin derivative	LS + IAA (10^{-6} M) Kinetin (10^{-5} M).	Fukui et al., 1990.Jang et al., 1998.
Lycium chinense	Cerebroside	MS + 2, 4- D (1 mg/l), Kinetin (0.1 mg/ L).	Jang et al., 1998.

Morinda citrifolia	Anthraquinones	B5 + NAA (10^{-5} M), N-Z amine (0.2%), Sucrose (2 %)	Zenk et al., 1975.
Morinda citrifolia	Anthraquinones	B5 + NAA (10^{-5} M), Kinetin (0.2 mg/ L), Sucrose (4 %), Pluronic acid F- 68 (2 % w/v).	Bassetti et al., 1995.
Mucuna pruriens	L- DOPA	MS + IAA (1 mg/ L), BA (1 mg/ L), Sucrose (4 %).	Wichers et al., 1993.
Nicotiana tobacum L.	Nicotine	MS + NAA (2 mg/ L), Kinetin (0.2 mg/ L).	Mantell et al., 1983.
Pinax notoginseng	Ginsenosides	MS + 2, 4- D (2 mg/ L), Kinetin (0.7 mg/ L), Sucrose (3 %).	Zhong and Zhu, 1995.
Papaver somniferum	Morphine, Codeine.	MS + 2, 4- D (0.1 mg/ L), Kinetin (0.7 mg/ L), Cystine HCL (2.5 mg/ L), Sucrose (3 %).	Siah and Doran, 1991.
Peganum harmala L.	β-Carbiline alkalods	MS + 2, 4- D (2 µM)	Sasse et al. 1982.
Phytolacca americana	Betacyanin	MS + 2, 4- D (5 µM), Sucrose (3 %).	Sakuta et al., 1987.
Picrasma quassioides Bennet	Quassin	B5 medium + 2, 4- D (1 mg/ L), Kinetin (0.5 mg/l), Glucose (2 %).	Scragg and Allan, 1986.
Podophyllum hexandrum royle	Podophyllotoxin	B5 + NAA (4 mg/ L), Coconut Water (5%), Sucrose (4%).	Uden et al., 1989.
Polygonum hydropiper.	Flavanoids	MS+2,4-D (10^{-6}), Kinetin (10^{-6}), Casamino acid (0.1%), Sucrose (3%).	Nakoa et al., 1999.
Rauwolfia sellowii	Alkaloids	B5 + 2, 4- D (1 mg/ L), Kinetin (0.2 mg/l), Sucrose (3 %).	Rech et al., 1998.
Rauwlfia serpentina Benth	Reserpine	LS + NAA (10 µM), BA (1 µM)	Yamamoto and Yamada. 1986.
Salvia miltiorrhiza	Cryptotanshinone	MS + 2, 4- D (1mg/ L), Kinetin (0.1mg/l).	Miyasaka et al., 1989.
Solanum chrysotrichum	Spirostanol saponin	MS + 2, 4- D (2 mg/ L), Kinetin (0.5mg/ L), Sucrose (3-4 %).	Villarreal et al., 1997.

Solanum laciniatum Ait.	Solasodine	MS + 2, 4- D (1 mg/l), Kinetin (1 mg/ L), Sucrose (3 %).	Chandler and Dodds.,1983 a.
Solanum paludosum	Solamargine	MS + BA (10^{-6} M), NAA (10^{-6} M), or MS+Kinetin (10^{-6} M), 2, 4-D(10^{-6}M).	Badaoui, et al., 2001.
Tabernaemontana divaricata	Alkaloids	MS + NAA (2 mg/ L),BA (2.0 mg/ L)	Sierra et al., 1992.
Taxus spp.	Taxol	B5 medium + 2, 4- D (0.2 mg/ L), BA (0.5 mg/ L), Casein hydrolysate (200 mg/ L), sucrose (3%).	Wu et al., 2001.
Taxus baccata	Taxol baccatin III	B5 salt + 3x B5 vitamins, 2, 4- D (2 x 10^{-2} mM), Kinetin (4 x 10^{-3} mM), GA3 (10^{-3} mM).	Cusido et al., 1999.
Thalictrum minus	Berberin	LS + NAA (60 µM), 2, 4- D (1 µM), BA (10 µM).	Kobayashi et al., 1987.
Thalictrum minus	Berberin	LS + NAA (60 µM), BA (10 µM).	Nakagawa et al., 1986.
Torreya nucifera var. radicans	Diterpenoids	MS + 2, 4- D (10mg/ L), Casamino acid (1 g/ L), Coconut milk (7 %), and K^+ instead of $NH4^+$.	Orihara et al., 2002.
Trigonella foenumgraecum	Saponins	MS + 2, 4- D (0.25 or 0.5 mg/ L), Kinetin (0.5 mg/ L).	Brain and Williams, 1983.

1.4. Introduction to *Calophyllum inophyllum*

1.4.1. Genus *Calophyllum*:

Genus *Calophyllum* (Clusiaceae), is one of the genus that is creating great interest in the scientific community, due to promising chemical and biological results. This genus is composed of a great group of tropical trees, with approximately 180 – 200 species restricted to hot and humid tropics (Noldin et al., 2006). As to the chemosystematics, the plants that belong to this genus mostly consist of coumarins, xanthones, steroids, triterpenes and biflavonoids. Many of these substances have pronounced and important biological actions. Some species are medicinal and are used against several diseases like gastric ulcers, infectious pathologies painful, inflammatory processes and molluscicidal. Some plant species are also reported with cytotoxic, microbial and anti- HIV activities (Noldin et al., 2006). In India, genus *Calophyllum* has been represented by eight species out of which four species namely *C. inophyllum, C. apetallum, C. polyanthum and C. austroindicum* grow along the Western Ghats of India and remaining four in Andaman and Nicobar islands of India.

Calophyllum inophyllum:

Kingdom	Plantae
Subkingdom	Tracheobionta
Super division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Dilleniidae
Family	Clusiaceae
Genus	*Calophyllum*

Calophyllum inophyllum belongs to genus *Calophyllum* which means “beautiful Leaf” from Greek word “Kalos” means “Beautiful” and “Phyllum” means “Tree”. In Hawai, *Calophyllum inophyllum* is known as Kamanu or Kamani and in English, it is known as Ballnut or confusingly Alexandrian Laurel. (it is not a Laurel nor native to Alexandria). *C. inophyllum* is known by different names in different countries like Ponyal (Bangladesh); Dignkaran (Indonesia); Polanga, Pinnai, Undi (India); Ponnyet (Myanmar); Bintangore laut, Penaga laut (Malaysia); Tamanu, Dilo, Kamani, Portia tree, Rekich (Pacific island); Palo maria, Bitaog (Philippines); Krathing, Saraphee naea, Naowakan (Thailand); Beach Calophyllum, Poon (Trade name). It is essentially coastal species that grows on sandy beaches or sea shores in coral sand and to a lesser

extend along river margins further inlands. It is found distributed in areas with 1000 – 5000 mm rainfall per year at altitude from 0- 200 m. (Species profile for Pacific island Agroforestry. www.traditionaltree.org). It is tolerant to salt spray, strong winds and brackish water tables. *C. inophyllum* grows throughout the tropics and it is uncertain from where it is originated. It is believed to be distributed in Tropical Asia, Southern Coastal India, East Africa, Malaysia, Vietnam, Indonesia, Philippines, Australia and several Pacific, Melanesia and Polynesia islands. In India *C. inophyllum* grows well especially in coastal South India, along the Western Ghats of India.

Fig. 1.1 Habit and habitat of *C. inophyllum*.

C. inophyllum is a large evergreen, slow growing, tall tree that grows with broad and irregular crown with spreading, twisted limbs that can reach 10 to 30 m height. Sometime it grows upto 35 m with diameter up to 150 cms with typically thick, dark, grayish-brown and cracked bark.

Fig. 1.2. Foliage and leaves of *C. inophyllum*

Fig. 1.3. Flower and floral buds of *C. inophyllum*

The branches are covered with shiny, dark-green oval, opposite, and tough leaves. Leaves are hairless with broadly elliptical blades of size 10 – 20 cm. Both the tip and base of the leaves are rounded and leaf veins run parallel to each other and perpendicular to midrib. It bears cluster of 4 – 15 small, white flowers about 2.5 cm across and 8 – 14 cm long with yellow centers arranged in axillary cyme on long stout stalk in leaf axil. Flowers have sweet, lime like fragrance. The blossom gives off a delightful, sweet perfume.

Fig. 1.4. Fruits of *C. inophyllum*

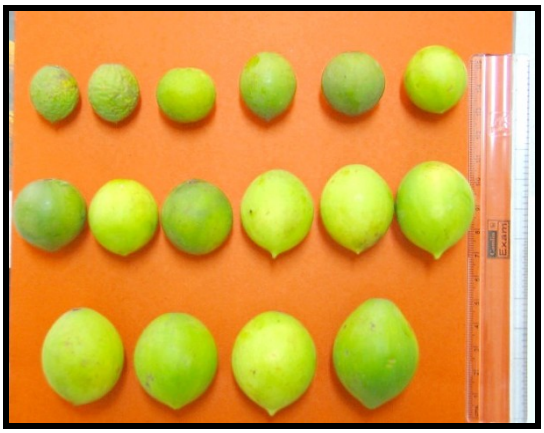

Fig. 1.5. Variable sizes of fruits of *C. inophyllum*

The tree can flower all the year but flowering is heaviest in late spring or early summer. The inedible mature green fruits or Ballnuts of the tree grow in cluster. Fruits are round, drupe measuring about 2- 5 cm in diameter with green colored skin that turns yellow and then brown and wrinkled when fruits are ripe.

Fig. 1.6. Dry fruits of *C. inophyllum*

The ripe fruit covers the thin pulp, the shell, a corky inner layer and a single seed kernel. Each fruit contains single large brown or yellow colored seed about 2 – 4 cm in diameter and is odorless when fresh. Seeds can be taken out by breaking open the skin and husk from the shell of the seed kernels. When chewed coats the mouth and emulsifies the saliva, and its insipid taste becomes bitter.

1.4.2.General uses of *Calophyllum inophyllum*:

1.4.2.1. Ornamental and religious importance:

C. inophyllum is a popular ornamental tree in the pacific and is tolerant to poor soil condition, salt spray and poor air quality. With its large size, the tree is impressive and is often planted as wide avenue (Muller, 1993). In some pacific island traditions, the plant is considered sacred and has been planted alongside temples.

1.4.2.2. Timber:

C. inophyllum produces hard, strong wood and is used in constructions and boat building. The wood is red, white when cut and ages to a reddish brown. Because of this interlocking grain, sawn surfaces tent to be wooly. The wood is moderately dense with specific gravity 0.6 – 0.8. Due to its interlocking grains, it is somewhat difficult to work. In Hawai, it is most valuable wood since it has been used for paneling and furniture. Because the wood is resistant to termite attacks, it is also used in cabinetry and construction. In Palau, it is favorite wood for carving traditional storyboards and is also prized for handicraft. The wood is also used for making food platters and calabashes, as it imparts no taste to food. The *C. inophyllum* wood price ranges

$6.00 – 15.00 / bf with logs fetching $3.00/bf. Wholesale lumber price may be $6.00 – $8.00 per board foot. (Species profile for Pacific island Agroforestry. www.traditionaltree.org).

1.4.3. Therapeutic uses:

Traditionally different plant parts are used as medicines to treat different ailments

1.4.3.1. Bark:

The bark of *C. inophyllum* contains tannins (11– 19 %), is astringent and its juice is purgative. In Asia, bark is considered highly medicinal. In India, it is used for orchitis (Quisumbing, 1951). In Indonesia, it is used after childbirth for vaginal discharge, the passing of blood and also for use in gonorrhoea (Burkill, 1994). It is used in decoction for internal haemorrhages and as a wash for indolent ulcers (Nadkarni and Nadkarni, 1999). When rubbed with water lime juice, bark makes useful application on armpits, groins and feet in bromidrosis. It also acts as antiseptic, disinfectant and expectoratnt when taken internally. The astringent juice of the bark is purgative and given in the form of a decoctions for internal haemorrhages. The bark is also useful in chronic bronchitis and phthisis. The oil that rises to surface after resin mixed with strips of bark and leaves stepped in water has household application for sore eyes. In Samoa, the plant is supposed to be virulent poison and the sap from the bark is used for preparation of an arrow poison (Jayweera, 1981).

1.4.3.2. Leaves:

The plant has rich history of use. Primitive native tribes of Papua New Guinea use the leaves frequently for different kinds of skin problems. On Manus, leaves made soft by heating over fire are applied to skin ulcers, boils, cuts, sores and pimples. While on Dobu island leaves are boiled and skin rashes are washed periodically with the solution, while natives in New Caledonia and in Samoa also use leaves for treating skin inflammations, leg ulcers and wounds. Water in which the macerated leaves have been soaked is used for haemorrhoids (Quisumbing, 1951). Leaves soaked in water yield a bluish colour and natural scent is applied to inflamed eyes in Linga and Fiji. For heartstroke, leaf infusion is taken internally and used in combination with an external application of the root decoction, whereas for stitch, hot poultice of leaves is applied externally and the root decoction taken internally (Quisumbing, 1951). In Combodia, the leaves oil is used for scabies, and are prescribed as an inhalation for migraine and vertigo. In Madagascar, the leaves are applied to sore eyes, the pounded bark for orchitis; gum resin is a vulnerary, resonant and anodyne. The leaves impart pleasant odour when soaked in water and used in Philippines as an astringent for haemorrhoid and is used in Indonesia as an eye lotion (Quisumbing, 1951, Burkill, 1994).

1.4.3.3. Flowers:

White, showy flowers are having pleasant fragrance and are used to make bouquets and wreath. Philippino women also wear these flowers in their hairs. The flowers and saps are used to scent bark cloth in old Hawai (Dweck and Meadows, 2002).

1.4.3.4. Fruits:

The fruits are more or less poisonous and only the endosperm of immature fruit is safe to eat. The mature fruit is poisonous enough to be ground and can be used as rat bait (Burkill, 1994). Fruits can also be burnt as mosquito repellent.

1.4.3.5. Gum and Sap:

Gum or latex from the cut bark is used to make into poison to kill rodents and rat fish. Gum extracted is emetic, purgative and also used for treatment of wound and ulcers. The resin is said to be responsible for colour and the odour of the oil and is poisonous; it is also believed to contain benzoic acid (Quisumbing, 1951). The gum resin has been considered to be good for old sores and wound. The resin may be useful in chronic cataract. The Balsam or oleoresin from the bark is having cicatrizing properties. In Philippines, an ointment prepared by mixing the plant sap with sulphur is applied on boils, open sores and wounds (Quisumbing, 1951).

1.4.3.6. Seeds:

C. inophyllum seed oil, unlike most vegetable oils is not contained in fresh ripe fruits. It forms in the course of nuts desiccation. Oil derived from seeds can be used an alternative to candlenut oil in lamps by some Polynesians and Indians. It can also be used for massage or as hair oil and a wood finish. In India, oil is applied on Cattles to get rid of cattle bugs. For extracting the oil, seeds are mechanically taken out of hard shell and exposed to sun. They must not be exposed to humidity to avoid the growth of some kernel moulds. During this desiccation process, kernel loose weight (from mean 7 g for fresh kernel to about 4.5 g for dry and oil rich one). On desiccation they turn brownish, develop colour and increase their oil content and also loose their germination power. For desiccation weather has to be dry and this process can take two months.

The yield of the oil from the seeds can be about 60% and sometime may be as high as 70-75% (Drury, 1873). The oil was once thought by the old pharmacologists to be Tacamahaca oil (*Balsam poplar* or *Populus balsamifera*). It is similar to myrhh (*Commiphora molmol*) and is also useful for indolent ulcers. It is sometimes called Domba oil in Europe and has proved useful in the treatment of rheumatism as well as in the treatment of itch or scabies. (Drury, 1873). The oil is also used in cases of gonorrhoea and gleet (Nadkarni and Nadkarni, 1999). Externally oil is used in

treatment of chronic rheumatism, inflammation of bones and joints, gout, scabies and ankylosis. In Fiji, natives value the oil as liniment for joint pains, arthritis, and bruises. It is also applied to suppurating wounds including coral sores. For strengthening the limbs of children who are slow in learning to walk, oil is rubbed on their limbs. It is occasionally used in place of chap stick for chapped, parched lips (Steiner, 1986). In Fiji, mothers rub the *C. inophyllum* oil onto their babies to prevent nappy/diaper rashes. In India, oil is used in dermal problems and is an ancient treatment for leprosy.

The oleoresin is applied externally on chronic ulcers, wound and taken internally for lung ailments. In southern India, the *Calophyllum* seed oil is used specifically for treating skin diseases and is also applied topically in cases of rheumatism (Oliver-Bever, 1986). The oil is also recommended for all kinds of burns, dermatoses, post surgical cicatrisation, certain skin allergies, ulcers, bad wounds, acne, psoriasis, herpes, chilblains, skin cracks, diabetes sores, dry skin, atonic wounds, physical and chemical burns, radiodermatitis, anal fissures, post surgical wounds, haemorrhoids, insomnia, hair loss etc. It is used in preparation of regenerative creams and soothing oils, soap making. Because of its healing, anti – inflammatory and antibiotic properties, oil is an excellent raw material for cosmetics, in regenerating and protective formulations (Muller, 1993). In southern sea island, missionaries observed leprous people coming to the leper house with the bottle of oil they used to rub over their wound. The idea suggested them to prepare solution of this oil with alcoholic ether and was used as injection that proved effective against neuritis due to leprosy or other origins, sciatica, and zona. *C. inophyllum* or Undi oil is also used after shaving, for face washing, for insect sting, to cure nappy rash, to heal cuts and graze, to treat athletes foot, to combat head lice, to treat ingrowing or infected nails, to heal blisters, to relieve sore throats, to eliminate dandruff, to fight acne, to prevent body odour, to treat burns, to relieve sunburns, on dry or scaly skins.

Chemically Undi oil or Tamanu oil contains basic classes of lipids such as (Kilham, 2004)

1. Neutral lipid (92 %).
2. Glycolipids. (6.4%)
3. Phospholipids. (1.6 %).

Neutral Lipids consisted of

Monoacylglycerols-	1.8 %
sn - 1,3 – Diaglycerides	2.4 %
sn - 1,3 (2,3) - Diaglycerides	2.6 %
Free fatty acids	7.4 %
Triacylglycerols	82.3 %
Sterols, sterolesters, & hydrocarbons	3.5 %.

Glycolipids consisted of	
Monogalactosyldiacylglycerol	11.4 %
Acylated steroglucoside	13.1 %
Monogalactosylmonoacylglycerol	22.2 %
Acylmonogalactosyldiacylglycerol	53.3 %
Phospholipids consisted of	
Phosphatidylethanolamine	46.3 %
Phosphatidylcholine	33.8 %
Phosphatidic acid	8.1 %
Phosphatidylserine	6.1 %
Lysophosphatidylcholine	5.7 %.

Source: (Herbal Gram, American botanical council, 63: 26-31).

1.5. Rationale and objectives of the present study:

1.5.1. Rationale:

Chemistry of *C. inophyllum* has been well studied and several classes of secondary metabolites like xanthone, triterpene and coumarins have been isolated and characterized. From *C. inophyllum,* anti HIV inophyllums B and P (Patil et al., 1993), anti cancerous inophyllums A, C. D and calophyllolide (Itoigawa et al., 2001) were isolated. Inophyllum C and calophyllolide were also shown to have antimicrobial and cytotoxic activity (Yimdjo et al., 2004). Inophyllums B and P (IC_{50}, 38nM and 130 nM, respectively) act as Non-nucleoside reverse transcriptase inhibitors (NNRTIs) and inhibit the activity of reverse transcriptase Type-1 enzyme of HIV-1 by the virtue of their ability to bind irreversibly at the non-substrate binding, allosteric site (De Clercq, 2000). *C. lanigerum* from which potential NNRTIs calanolides were isolated has not been reported from India so far, while *C. inophyllum* from which NNRTIs inophyllums (dipyranocoumarins) were isolated grows at several locations along The Western Ghats of India. This plant produces a good quality timber that can be used in boat and ship making because of which, it is cut fairly on large scale. Due to its large scale cutting for obtaining timber, this plant species has been included in IUCN's red list of threatened species (Stevens et al., 1998) and needs to be conserved.

Presently, many other natural products are derived by extracting and isolating solely from massive quantities of whole plant parts. Often the source plants are either wild or cultivated in tropical or subtropical and geographically remote areas which are subject to political instability, drought, disease and changing land use patterns and other environmental factors. In addition, the long cultivation periods between planting and extraction make selection of high-yielding plants difficult, thus resulting in expensive drugs. Cultivation periods may range from several months to

decades. In spite of these difficulties and costs, the extraction of medicinally important secondary metabolites from cultivated plants or plants in the wild continues because of a lack of credible alternatives. Clearly, the development of alternative and complimentary methods to whole plant extraction for the production of clinically important secondary metabolites is an issue of considerable socioeconomic importance. These facts have generated great interest in the use of plant cell culture technologies for the production of inophyllums like pharmaceuticals and other plant derived secondary metabolites. Indeed, the plant cell culture technology is now sufficiently advanced to allow for large quantities of relatively homogeneous, undifferentiated cells to be produced. When compared to whole plant extraction, plant cell and tissue culture systems are complementary and may provide competitive metabolite production systems.

1.5.2. Objectives of the present study:

The present study "*In vitro* production of secondary metabolites from cultured cells/ tissues and molecular characterization of *Calophyllum inophyllum*" was taken up with the following objectives.

1. To develop and validate HPLC method for analysis of dipyranocoumarins in *in vitro* growing cultured cells / tissues and *in vivo* growing plant materials.
2. To study the chemo diversity and genomic diversity in *C. inophyllum.*
3. To establish micro propagation protocol for *C. inophyllum*.
4. To establish callus cultures using different media, hormones and explants for studying the *in vitro* expression pattern of dipyranocoumarins.
5. To establish cell suspension culture for increased biomass and dipyranocoumarins, expression.
6. To study the effects of different biotic and abiotic elicitors on *in vitro* expression of dipyranocoumarins.

CHAPTER 2. MATERIALS AND METHODS

INTRODUCTION

This chapter describes the materials and general techniques used in this study

2.1. Phytochemistry- Collection and extraction of seed material for chemical diversity studies, development and validation of HPLC method.

2.2. Plant tissue culture- *In vitro* micropropagation, induction of callus cultures, cell suspension cultures and their elicitation.

2.3. Molecular Characterization- Isolation and amplification of DNA.

The materials and methods, specific to particular experiment, are dealt in details in respective chapters.

2.1. PHYTOCHEMISTRY.

2.1.1. MATERIAL:

2.1.1.1. Glasswares:

Conical flasks (100ml, 250ml, 500ml, 1000ml capacity) for preparation and storing extracts, round bottom flasks (100ml. 250ml, 500ml, 1000ml capacity) for concentrating the extracts on rotary evaporator, measuring cylinders (25ml, 100ml, 250ml, 1000ml capacity) for preparing different solvent systems, test tubes (25x150mm) for storing extracts, all were procured from Borosil Glass Works Ltd (Mumbai, India). Beakers and funnels of variable sizes were also procured from Borosil Glass Works Ltd (Mumbai, India). HPLC autosampler vials (1.5 ml) were purchased from Perkin Elmer (Mumbai, India). For filtering the HPLC solvents, filtration unit (1 L capacity) was purchased from Supelco Pvt. Ltd. (India).

2.1.1.2. Chemicals:

Analytical grade solvents like chloroform from Merck Ltd, (Mumbai, India), acetone from Qualigens Fine Chemical Division (Mumbai, India), ethyl acetate and petroleum ether, (60- 80°C) from S. D. Fine Chem. Ltd. (Mumbai, India), were used for extraction and TLC analysis. HPLC grade ethyl acetate from Qualigens Fine Chemical Division (Mumbai, India), petroleum ether (60-80°C) from Spectrochem Pvt. Ltd. (Mumbai, India) were used for HPLC analysis. Authentic compounds isolated by repeated column chromatography, prepararive TLC and identified by taking different spectroscopic data like CNMR, HNMR, EIMS, IR, UV were provided by Division of Organic Chemistry, National Chemical Laboratory, Pune, India.

2.1.1.3. Equipements:

(i) Pulverizer:

For pulverizing the plant material, a local made pulverizer (Ichalkaranji, MS, India) was used. This was powered by two-horse power electric motor.

(ii) Rotary evaporator:

For standard distillations and evaporation of solvents from extracts, a rotary evaporator (Buchi, Germany) was used. This rotary evaporator was with stopcock for the fitting of evaporating flasks, vertical condenser, had temperature sensor and vaccum controller.

2.1.1.4. Plant Material:

Plant material of *Calophyllum inophyllum* was collected from different locations along the Western Ghats of India. For chemo diversity study, mature fruits of *C. inophyllum* were collected from 13 locations, each minimum 40 kms. apart from each other. The locations of collections were from five states of India viz. Maharashtra, Goa, Karnataka, Kerala and Tamil Nadu along the Western Ghats of India.

2.1.2. METHOD:

2.1.2.1. Extraction of plant material for chemo diversity study:

For chemodiversity studies, fruits collected from 13 locations were shade dried for two weeks. Shade dried fruits were then mechanically broken open to take out kernels. Kernels from these 13 locations were pulverized and soaked in acetone overnight. Next day acetone was removed and kernel were re-suspended in fresh acetone. This process was repeated three times and acetone was removed from combined acetone extract. These acetone extracts were then dissolved in HPLC mobile phase, filtered through micro filtration unit. Such prepared samples from 13 locations were used for HPLC analysis.

2.1.2.2. HPLC method development:

HPLC method for the analysis of bioactive compounds was developed on Perkin-Elmer's Series 200 HPLC system. This system was equipped with a quaternary gradient pump, an autosampler and diode array detector. Initially different HPLC columns with different bonded phases like C8, C18, RP18, Amino, and Phenyl were tried. Also different solvents such as methanol, acetonitrile, water, hexane, petroleum ether, ethyl acetate, in different combinations and

concentrations as a mobile phase in isocratic and gradient modes were tried for optimum separation of dipyranocoumarins under study.

2.1.2.3. HPLC method validation:

As a part of HPLC method validation, different validation parameters such as linearity, precision (intra day repeatability and inter day reproducibility), recoveries, limit of detection and limit of quantification were studied for each of the dipyranocoumarin under study. As a part of precision, intra day repeatability and inter day reproducibility were studied for retention factors, areas and peak height. Linearity was studied for each of the dipyranocoumarin to establish linear regression equation i.e. $y = mx + c$. For method validation, concentration range of 0.5 – 2.5 mg/ml of each of standard compound was used.

2.2. PLANT TISSUE CULTURE

2.2.1. MATERIALS

2.2.1.1. Glasswares and Plastic wares:

Test tubes (25x150mm), conical flasks (100ml, 250ml, 500ml, 1000ml capacity), pipettes (0.1,0.2,1,2,5,10 ml capacity) and measuring cylinders (25ml, 100ml, 250 ml, 1000ml capacity) of Borosil Glass Works Ltd. (Mumbai, India) were used for culturing the tissues and for preparation of media. Autoclavable screw cap bottles (100, 250 and 500ml) for storing stock solutions were procured from Qualigens Fine Chemicals, India. Plastic wares including sterile disposable plastic petriplates of 55 and 85 mm diameter were procured from Laxbro (Pune, India). Klin wraps, used for sealing the petriplates were locally purchased. Micropipette of different precision measurements (1000ml, 200ml, 100ml, 20ml, 10ml and 2ml) and microtips were procured from Gilson (India) and Tarson Products Pvt. Ltd. (Kolkata, India) respectively.

2.2.1.2. Chemicals:

Chemicals used for surface sterilization procedures were liquid detergent solution Labolene (Qualigens Fine Chemicals, India), Savlon (Johnson and Johnson Limited, Mumbai, India), fungicide Bavistin® (BASF, India) and Benomyl (E.I.D. Parry Ltd, Chennai, India), mercuric chloride (Qualigens Fine Chemicals, India), hydrogen peroxide (Merck Ltd, Mumbai, India). PVP used in sterilization procedure for adsorbing phenolic subtances from explants was procured from Sigma- Aldrich Chemicals (St.Louis, USA).

Inorganic salts and vitamins used for preparation of culture media and for other experiments were of AnalaR Grade procured from BDH (British Drug Houses Pvt. Ltd, Bombay), Hi-Media Laboratories Ltd. (Mumbai, India) and Qualigens Fine Chemicals Division, (Mumbai,

India). Sucrose was procured from Hi-Media (India) and Qualigens Fine Chemicals (Mumbai, India). Gelling agent Agar agar (bacteriological grade), used in the semisolid culture medium was procured from Hi-Media Laboratories Ltd (Mumbai, India). For isolating endophytic fungi from leaves of *Calophyllum inophyllum*, fungal medium Potato Dextrose Agar (PDA) was purchased from Hi-Media Laboratories Ltd (Mumbai, India) and surface sterilizing agents like ethanol and hydrogen peroxide (H_2O_2) were procured from Merck Ltd, Mumbai, India.

Growth regulators including 6-benzylaminopurine (BAP), N-phenyl-N'-1, 2,3-thidiazol-5-ylurea (Thidiazuron, TDZ), α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), indole-3-butyric acid (IBA), 4-amino-3,5,6-tricholopicollinic acid (Picloram) were obtained from Sigma -Aldrich Chemicals (St.Louis, USA).

2.2.1.3. Equipments:

The major equipments used include:

(i) pH meter:

pH is the negative logarithm of hydrogen ion concentration. The measurement of pH in pH meter is based on ion exchange in between hydrated layers formed on glass surface. Change in ion exchange results in emf or voltage difference causing current flow. The current intensity gives the value of pH. For adjusting the pH of the medium, digital pH meter from Global (Mumbai, India) was used.

(ii) Electronic Balance:

A manual top loading balance (Contech, Mumbai, India) was used for quick weighing and for analytical purposes. This is a single pan balance with capacity 100-200g, sensitivity 0.1mg operating on 230 V 50 H_2 AC mains. Precision of ± 0.005g, weighing range 0.1- 200g, digital read out was used for making stock solutions of growth regulators and for other fine weighing.

(iii) Autoclave:

The autoclave (NatSteel Equipment Pvt. Ltd., Mumbai, India) was used for sterilization of media, glassware, water, dissecting instruments etc. and for decontamination of contaminated cultures in culture vessels. It is based on application of steam under pressure. Autoclaving was carried out at 121°C temperature under 15lb/in^2 pressure. Except culture media, all other materials were autoclaved for one hour. The culture media were autoclaved for 20 min.

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(iv) Laminar airflow ultra clean unit (LAF):

For all aseptic manipulations and inoculations, Laminar Air Flow Units fabricated by Klenzoids or Microfilt, India were used. In laminar air flow unit, with the help of air pump, air is passed through HEPA filters of pore size, 0.22 micron. Due to positive pressure, the entry of any contaminant is restricted from the open side of the bench. The instrument is fitted with UV tubes in addition to the fluorescent tubes.

Apart from these, instruments like magnetic stirrer (Remi, India), steamer (Ultradent, India), temperature controlled oven (Pathak Electricals, India), light microscope (Carl-Zeiss Jena), camera (Nikon/Zeiss), membrane filter sterilizing unit (Laxbro, Pune) and Pipetman (Gilson/Tarson) were used. With the exception of pipetman, microscopes, camera and all other equipments used in the course of this study are fabricated by different companies in India.

2.2.1.4. Source of Explants:

For induction of callus cultures and micropropagation, mature fruits of *Calophyllum inophyllum* were collected from Harne village (17° 49' 53" N, 73° 05' 65" E; 2 m altitude), dist Ratnagiri, Maharashtra. Based on chemodiversity study, plants at this location were identified as elites and were used for micropropagation and induction of callus cultures.

2.2.2. METHODS:

2.2.2.1. Preparation of glassware and instruments:

Glasswares used in these studies were cleaned by boiling in saturated solution of sodium bicarbonate for 1 h. followed by washing under tap water. These were then immersed in 30% nitric acid solution for 30 min and were washed thoroughly with tap water. After rinsing with double distilled water these were allowed to dry on a draining rack.

Absorbent cotton (Safe Surgical Industries, Beawar, India) was used to plug tubes and flasks. All dissecting instruments like scalpels, foreceps, and blade holders were either wrapped singly or were put in closed aluminum cans for sterilization by autoclaving. Ordinary grade filter paper pieces of approximately 10x20cm were kept in stack alternately with brown paper pieces of similar size. These were packed in autoclavable plastic bags with 20-25 pieces in each bag and autoclaved. Aseptic handling of explants, their dissection and transfer were carried out on these autoclaved papers under aseptic conditions and disposed after use. Blue and yellow tips used for aseptic addition by micropipettes were arranged in cases meant for their size, wrapped with brown

paper and autoclaved. Sterilization of the glassware and instruments was carried out by autoclaving at 121°C for 1 h in 15 lbs/ $(inch)^2$.

2.2.2.2. Preparation of media:

Appropriate composition and preparation of of the medium plays important role in the success of a tissue culture protocol. Several basal formulations like Murashige's and skoog media, B5 Gamborg media, White's media, Woody plant media etc. are now available.

Concentrations of the macro and microelements, salts and organic constituents of the MS (Murashige and Skoog, 1962) and WPM (Lloyd and Mc.Cown, 1980) basal medium are listed in Table 2.1 and Table 2.2. Concentrated stock solutions of the media ingredients were prepared by dissolving weighed amounts of these salts as per Table 2.1 and 2.2 in double distilled water. Appropriate volumes of these concentrated stock solutions were mixed to prepare the media.

2.2.2.3. Preparation of growth regulators:

Growth regulators (GR) were prepared in concentrated stock solutions by adding few drops of solvent in the required amount of growth regulator to dissolve. After dissolution, the required volume was made with double distilled water to get required concentrations and stored in refrigerator in sterilized bottles. Table 2.3 describes the list of solvents and diluents, stock concentration of growth regulators used.

Table 2.1: Composition of macro and microelement salts and vitamins in Murashige and Skoog's (MS) basal medium

Ingredients	Amount (mg/L)	Stock solution
Macronutrients		**(20 X) in 500mL**
KNO_3	1900	19 g
NH_4NO_3	1650	16.5 g
$CaCl_2.2H_2O$	440	4.4 g
$MgSO_4.7H_2O$	370	3.7 g
KH_2PO_4	170	1.7 g
Micro-nutrients		**(100 X) in 100 mL**
$MnSO_4.4H_2O$	22.3	62 mg
$ZnSO_4.7H_2O$	8.6	223 mg
H_3BO_3	6.2	86 mg
KI	0.83	2.5 mg
$CuSO_4.5H_2O$	0.025	0.25 mg
$Na_2MoO_4.2H_2O$	0.25	8.3 mg
$CoCl_2.6H_2O$	0.025	0.25 mg
$FeSO_4.7H_2O$	27.8	278 mg
$Na_2EDTA.2H_2O$	37.3	373 mg
Vitamins		**(100 X) in 100 mL**
Myo-inositol	100	1 g
Thiamine-HCl	0.1	5 mg
Nicotinic acid	0.5	20 mg
Pyridoxine-HCl	0.5	1 mg
Glycine	2	5 mg

Table 2.2: Composition of macro and microelement salts and vitamins in WPM medium

Ingredients	Amount (mg/L)	Stock solution
Macronutrients		**(50 X) in 500mL**
NH_4NO_3	400	10.0 g
Ca $(NO_3).4H_2O$	556	13.9 g
$MgSO_4.7H_2O$	370	9.25 g
KH_2PO_4	170	4.25 g
$CaCl_2.2H_2O$	96	2.40 g
K_2SO_4	990	24.75 g
Micronutrients		**(50 X) in 500mL**
$MnSO_4.4H_2O$	29.43	0.7357 g
$ZnSO_4.7H_2O$	8.60	0.215 g
H_3BO_3	6.20	0.155 g
$CuSO_4.5H_2O$	0.25	0.00625 g
Na_2MoO_4	0.25	0.00625 g
Chelate		**(50 X) in 500mL**
$FeSO_4.7H_20$ $Na_2EDTA.2H_2O$	27.8 3.70	0.695 g 0.0925 g
Vitamins		**(50 X) in 500mL**
Nicotinic acid	0.5	0.0125 g
Thiamine HCL	1.0	0.025 g
Pyridoxine HCL	0.5	0.0125 g
Glycine	2.0	0.050 g
Inositol	100	2.50 g

Table 2.3: Preparation and Concentration of Growth Regulators

Growth Regulator	Molecular weight	Solvent	Diluent	Stock Concentration
BAP	225.3	1N NaOH	H_2O	10 mg/100mL
TDZ	220.2	DMSO	H_2O	10 mg/100mL
2,4-D	221	1N NaOH	H_2O	10 mg/100mL
Picloram	241.5	DMSO	H_2O	10 mg/100mL
NAA	186.2	1N NaOH	H_2O	10 mg/100mL
IBA	203.2	1N NaOH	H_2O	10 mg/100mL

For media preparation, calculated volumes from concentrated stock solutions were added to small volumes of double distilled water. Carbohydrate (Sucrose) was weighed and added in required quantity (1%, 2%, 3%, 4%, 5%, 6%) and allowed to dissolve. Volume was then made half of the final required volume of the medium and required volumes of hormone stocks were added to get desired concentrations in the medium. After addition of hormones, the final volumes were made up with double distilled water. Unless mentioned, pH of all the media was adjusted to 5.6-5.8 using 1N NaOH or 1N HCl after mixing all the constituents except the gelling agent. Gelling agent (agar agar) was then added and heated on water bath or steamed for the agar to melt. Molten medium was dispensed into sterile culture tubes (20ml of media), flasks (500ml of media) or bottles (80ml of media) after thorough mixing. Semisolid medium containing agar was used in most of the studies unless otherwise mentioned. All the culture media were autoclaved for 20 min. at 121°C and 15 lbs/(inch)^2. As and when required for culturing in petridishes, autoclaved media was poured aseptically in sterile petridishes before solidification, sealed, and stored.

2.2.2.4. Preparation of explants:

Mature air dried fruits were decoated mechanically to take out seeds from its hard, stony endocarp. Thses seeds were washed first with liquid detergent solution Labolene (0.1% v/v) followed by treatement with savlon (10% v/v) for 10 min. Seeds were then rinsed with double distilled water for 5-6 times and then pretreated with PVP insoluble (0.1 – 0.5% w/v) for 30 min. After thorough washing with double distilled water for 4-5 times, seeds were treated with fungicide Benomyl (0.5% w/v) or Bavistin 1% w/v) for 30 min. After fungicide treatement and thorough

washing with double distilled water, further treatement were given in laminar airflow cabinet. In LAF, seeds were transferred to a sterile bottle and surface sterilized with ethanol (70% v/v), for 15-20 S, washed with sterile distilled water 3- 4 times followed by $HgCl_2$ (0.1% w/v) and again thouroughly washed with sterile double distilled water for 5-6 times. For germination of whole seeds, seeds were presoaked in sterile double distilled water and/or gibberllic acid (GA_3; 0.058 μM).

2.2.2.5. Initiation of cultures for micro propagation and callus induction:

Callus cultures and seedlings for micropropagation were initiated from the surface sterilized seed explants. Callus cultures were also established from nodal/ internodal and leaf explants from *in vitro* grown seedlings. For callus induction, surface sterilized seeds and leaves of seedlings were cut into 4 – 6 pieces of size 3 x 3 cm and green stem of sterile seedlings were cut into circular slices or discs to get nodal/internodal explants prior to inoculations. All the handlings and dissections of explants were carried out on sterile filter papers. The instruments used for aseptic dissection or transfer of tissues were presterilized. During aseptic operations these were sterilized intermittently by dipping in rectified spirit and flaming. After transferring the tissues, the petriplates were sealed with klinwrap. All cultures were labeled appropriately prior to incubation.

2.2.2.6. Culture conditions:

All the cultures were incubated at 25 ± 2° C under cool white fluorescent light (16/8 h photoperiod, 35μmol m^{-2} s^{-1}; Philips, India). Cultures were maintained by sub culturing and transferring after every 45 days on their respective fresh medium containing same hormone combinations and concentrations. *In vitro* grown seedlings if required were also sub cultured after every 60 days on their respective fresh medium.

2.2.2.7. Statistical Procedures:

Statistical methods were used for comparison of treatment means during optimization of the parameters for micropropagation and induction of callus cultures. Completely Randomized Block Designs were used. The data for micropropagation was subjected to analysis of variance (ANOVA) and treatment means were compared (Panse and Sukhatme, 1967), while data for induction of callus cultures was subjected to two way ANOVA. The differences among the treatment means were tested using Duncan multiple range test (DMRT) at a 5% probability level ($P<0.05$), wherever applied. All statistical analyses were performed on software AgroBase 99, OriginPro 8 and Microsoft Excel.

2.2.2.8. Cell suspension culture:

Cell suspension cultures were established by inoculating known quantities of callus in WPM liquid medium (100 ml). Different experiments were carried out to study the effects of various hormones and medium ingredient manipulations. Hormone combinations and concentrations viz. Picloram (8.28 – 33.12 µM) along with BAP (8.88µM) constant, IBA (4.90 – 19.60µM) and IBA (4.90 – 19.60µM) along with BAP (4.44 µM) constant were studied for their effects on biomass and dipyranocoumarins productions.

Effect of different media components like nitrate (0- 5X), sulphate (0- 5X), vitamins (0- 5X), and sucrose were studied in cell suspension cultures. Total nitrate, (0, control, 1, 2, 3, 4 fold increase), total sulphate (0, control, 1, 2, 3, 4 fold increase), total vitamins (0, control, 1, 2, 3, 4 fold increase), $CaCl_2$ (0, 1, 2, 3, 4 mM) and Sucrose (0.5, 1, 2. 3, 4, 5, 6%,) were varied in cell suspension cultures to study their effect on biomass and dipyranocoumarins expression.

2.2.2.9. Elicitation:

(i) Chemicals:

For elicitation with abiotic elicitors, heavy metals like copper, cadmium and chromium were added in cell suspensions cultures. Salts of these heavy metals Viz. $CuSO_4$, $CdCl_2$ and $K_2Cr_2O_7$ were procured form, Qualigens Fine Chemicals, India, and added as sources of copper, cadmium and chromium respectively.

(ii) Medium preparation for Abiotic elicitation:

Elicitation media with abiotic elicitors were prepared by adding solutions of salts of heavy metals in WPM liquid medium. 0.5 M stock solutions of these heavy metals were prepared and used. To cover the broad range of concentration variation, all these heavy metals were added in range of 0. 0.1, 0.5, 1, 5, 10, and 20 mM concentrations.

(iii) Isolation and identification of endophytic fungi:

A leaf material of *Calophyllum inophyllum* was collected from NCL garden. Leaves were thoroughly washed with tap water, then treated with 10% savlon (v/v) for 10 min, and washed with sterile DDW. Next treatment was given in LAF cabinet where leaves were first treated with 70% ethanol for 2 min followed by washing with sterile DDW. Ethanol treatement was then followed by treatement with H_2O_2 (15%, v/v) for 15 min. Thereafter leaves were washed 4-5 times with sterile DDW and were inoculated on potato Dextrose agar (PDA) medium. Prior to inoculation, leaves were cut into small pieces and injured with sterile surgical blade. All cultures were incubated at room temperature (RT). Fungal mycelia grown on PDA were isolated and made

into pure cultures. Pure fungal cultures were identified microscopically at Mycology Division, Agharkar Research Institute, Pune.

(iv) Preparation of biotic elicitors:

Identified fungi were cultured in Potato Dextrose broth in larger volumes and incubated as static cultures at RT. After two weeks of incubation, flasks with cultures were autoclaved at 15 lb pressure for 15 min. Mycelial mats that floated at the surface of the medium were collected, washed thoroughly with sterile DDW and air dried. These mycelial biomass were then crushed to fine powder in liquid nitrogen. Dried cell powder of fungal mycelia was then added in liquid WPM medium as biotic elicitors. Remaining culture filtrate was filtered and centrifuged at 8000 rpm for 10 min to remove mycelial fragments and suspended particles. This culture filtrate was also added in cell suspension cultures as biotic elicitors.

2.3. MOLECULAR CHARACTERIZATION

2.3.1. MATERIALS:

2.3.1.1. Glasswares and Plasticwares:

Micropipettes of different precision measurements (1000, 200, 100, 20, 10 and 2µl) were procured from Gilson Medical Electronics, France. Micro tips, eppendorffs Tubes (0.2,0.5,1.5 and 2ml), PCR tubes with flat caps and microtip-boxes were procured from Axygen scientific Pvt. Ltd. (India), while centrifuge tubes (50ml) were purchased from Tarson products Pvt. Ltd, (Kolkata, India).

2.3.1.2. Reagents and Chemicals:

- Tris-HCl pH 8.0 (1M); EDTA pH 8.0 (0.5M); NaCl (5M); CTAB (20%); Chloroform:Iso-amylalchol (24:1 v/v); Polyvinyl pyrrolidone; β-mercaptoethanol; cold isopropanol and ethanol (70%)
- Extraction buffer: 100mM Tris-HCl (pH 8.0), 25mM EDTA, 1.5M NaCl, 2.5% CTAB, 0.2% β-mercaptoethanol (v/v) (added immediately before use) and 1% PVP (w/v) (added immediately before use).
- High salt TE buffer: 1M NaCl, 10mM Tris-HCl (pH 8.0) and 1mM EDTA.
- Agarose (Sigma- Aldrich Chemicals, St. Louis, USA)
- Electrophoresis buffer: Tris-borate-EDTA (1 X)
- Loading buffer: Bromophenol blue (0.25%) and glycerol (30%)
- Fluorescent dye: Ethidium bromide (10µg/mL, Sigma- Aldrich Chemicals, St. Louis, USA)

- ❖ Marker: Low range DNA ladder (3 Kb) (Bangalore Genei, India)
- ❖ Enzymes: RNAase A (10mg/mL) and Taq DNA Polymerase (Bangalore Genei, India)
- ❖ Buffers: *Taq* DNA Polymerase buffer with $MgCl_2$ (Bangalore Genei, India)
- ❖ Nucleotides: dNTPs (G, A, T, C) (Bangalore Genei, India)
- ❖ Primers: ISSR Primer Set #9 (801…900) obtained from UBC, British Columbia was used (Table 2.4)

2.3.1.3. Equipments:

(i) Milli-RO water system:

It is important to use deionized water for preparation of molecular reagents as the presence of minerals and salts may alter the final composition. Milli-RO water system (Millipore, USA) was used for obtaining deionized water. There are series of ion exchanging columns that makes tap water free of salts and minerals that are naturally present in tap water.

(ii) Horizontal electrophoresis unit (Tarson, India): The basic principle of electrophoresis is, charged ions or molecules migrate when placed in an electric field. The rate of migration of a substance depends on its net charge, size, shape and the applied current. This unit is used to separate amplified DNA fragments of different sizes. It consists of a power pack that supplies a stabilized current at controlled or required voltage and current output and electrophoresis unit, which contains the electrodes, buffer reservoirs and gel casting assembly.

(iii) UV Transilluminator:

This instrument is used for gel visualization under ultraviolet radiation. UV Transilluminator is fitted with UV lights that illuminates ethidium bromide stained gels.

(iv) Gel Documentation System (Biorad):

It is a powerful, flexible package including the hardware and the software for imaging and analyzing 1-D electrophoresis gels, dot blots arrays and colonies. The lane-based Band Analysis functions can be used in determination of molecular weights and other values.

(v) Water bath (Julabo):

It is used for maintaining the constant temperature in which, temperature setting, temperature indicator, and cooling effect are also available.

(vi) SpinWin (Tarson):

It is a mini centrifuge equipped with continuously variable electronic speed control, speed indicator, Amp meter, timer, dynamic break, zero starting switch and fuse safety device for 230 V 50 Hz AC mains.

(vii) SpectroPhotometer (Perkin & Elmer):

Hydrated and solvated DNA solutions are quantified spectrophotometrically by taking absorbance at wavelengths of 260 and 280nm and the ratio between them provide an estimate of the purity of the sample DNA ((Maniatis, 1982).

(viii) PCR Robocyler (Stratagene, USA):

It is microprocessor controlled for block laboratory instrument utilizing a robotic arm to quickly move from one temperature block to another based on user defined program. This system has four separate anodized aluminum temperature blocks (3-heating block &1 cold blocks) containing 96 precision cut wells that remain at set temperature, whereas other thermal cyclers have a single block that changes temperature during each cycle. In the Robocycler tubes are moved from one block to next by the robotic arm. The four-block design decreases cycling time by up to 30% and achieve a well to well temperature uniformity of ± 0.1 °C for amplification process.

2.3.1.4. Source of plant material:

For Genomic DNA isolation, mature leaf material was collected from different locations along the Western Ghats of India. Collection was done in the states of Maharashtra, Goa, Karnataka, and Kerala. Material was collected from locations that were atleast 40 km apart. Collected material was stored initially in air tight polyethylene bags. For long term storage, collected material was first frozen in liquid nitrogen and the stored in deep refrigerator at -70° C.

Table 2.4: List of ISSR Primers

UBC Primer Set #9 (Microsatellite)
3 nanomoles/tube

801	ATA TAT ATA TAT ATA TT	851	GTG TGT GTG TGT GTG TYG
802	ATA TAT ATA TAT ATA TG	852	TCT CTC TCT CTC TCT CRA
803	ATA TAT ATA TAT ATA TC	853	TCT CTC TCT CTC TCT CRT
804	TAT ATA TAT ATA TAT AA	854	TCT CTC TCT CTC TCT CRG
805	TAT ATA TAT ATA TAT AC	855	ACA CAC ACA CAC ACA CYT
806	TAT ATA TAT ATA TAT AG	856	ACA CAC ACA CAC ACA CYA
807	AGA GAG AGA GAG AGA GT	857	ACA CAC ACA CAC ACA CYG
808	AGA GAG AGA GAG AGA GC	858	TGT GTG TGT GTG TGT GRT
809	AGA GAG AGA GAG AGA GG	859	TGT GTG TGT GTG TGT GRC
810	GAG AGA GAG AGA GAG AT	860	TGT GTG TGT GTG TGT GRA
811	GAG AGA GAG AGA GAG AC	861	ACC ACC ACC ACC ACC ACC
812	GAG AGA GAG AGA GAG AA	862	AGC AGC AGC AGC AGC AGC
813	CTC TCT CTC TCT CTC TT	863	AGT AGT AGT AGT AGT AGT
814	CTC TCT CTC TCT CTC TA	864	ATG ATG ATG ATG ATG ATG
815	CTC TCT CTC TCT CTC TG	865	CCG CCG CCG CCG CCG CCG
816	CAC ACA CAC ACA CAC AT	866	CTC CTC CTC CTC CTC CTC
817	CAC ACA CAC ACA CAC AA	867	GGC GGC GGC GGC GGC GGC
818	CAC ACA CAC ACA CAC AG	868	GAA GAA GAA GAA GAA GAA
819	GTG TGT GTG TGT GTG TA	869	GTT GTT GTT GTT GTT GTT
820	GTG TGT GTG TGT GTG TC	870	TGC TGC TGC TGC TGC TGC
821	GTG TGT GTG TGT GTG TT	871	TAT TAT TAT TAT TAT TAT
822	TCT CTC TCT CTC TCT CA	872	GAT AGA TAG ATA GAT A
823	TCT CTC TCT CTC TCT CC	873	GAC AGA CAG ACA GAC A
824	TCT CTC TCT CTC TCT CG	874	CCC TCC CTC CCT CCC T
825	ACA CAC ACA CAC ACA CT	875	CTA GCT AGC TAG CTA G
826	ACA CAC ACA CAC ACA CC	876	GAT AGA TAG ACA GAC A
827	ACA CAC ACA CAC ACA CG	877	TGC ATG CAT GCA TGC A
828	TGT GTG TGT GTG TGT GA	878	GGA TGG ATG GAT GGA T
829	TGT GTG TGT GTG TGT GC	879	CTT CAC TTC ACT TCA
830	TGT GTG TGT GTG TGT GG	880	GGA GAG GAG AGG AGA
831	ATA TAT ATA TAT ATA TYA	881	GGG TGG GGT GGG GTG
832	ATA TAT ATA TAT ATA TYC	882	VBV ATA TAT ATA TAT AT
833	ATA TAT ATA TAT ATA TYG	883	BVB TAT ATA TAT ATA TA
834	AGA GAG AGA GAG AGA GYT	884	HBH AGA GAG AGA GAG AG
835	AGA GAG AGA GAG AGA GYC	885	BHB GAG AGA GAG AGA GA
836	AGA GAG AGA GAG AGA GYA	886	VDV CTC TCT CTC TCT CT
837	TAT ATA TAT ATA TAT ART	887	DVD TCT CTC TCT CTC TC
838	TAT ATA TAT ATA TAT ARC	888	BDB CAC ACA CAC ACA CA
839	TAT ATA TAT ATA TAT ARG	889	DBD ACA CAC ACA CAC AC
840	GAG AGA GAG AGA GAG AYT	890	VHV GTG TGT GTG TGT GT
841	GAG AGA GAG AGA GAG AYC	891	HVH TGT GTG TGT GTG TG
842	GAG AGA GAG AGA GAG AYG	892	TAG ATC TGA TAT CTG AAT TCC C
843	CTC TCT CTC TCT CTC TRA	893	NNN NNN NNN NNN NNN
844	CTC TCT CTC TCT CTC TRC	894	TGG TAG CTC TTG ATC ANN NNN
845	CTC TCT CTC TCT CTC TRG	895	AGA GTT GGT AGC TCT TGA TC
846	CAC ACA CAC ACA CAC ART	896	AGG TCG CGG CCG CNN NNN NAT G
847	CAC ACA CAC ACA CAC ARC	897	CCG ACT CGA GNN NNN NAT GTG G
848	CAC ACA CAC ACA CAC ARG	898	GAT CAA GCT TNN NNN NAT GTG G
849	GTG TGT GTG TGT GTG TYA	899	CAT GGT GTT GGT CAT TGT TCC A
850	GTG TGT GTG TGT GTG TYC	900	ACT TCC CCA CAG GTT AAC ACA

2.3.2. METHODS:

2.3.2.1. DNA isolation protocol:

The DNA isolation was carried out based on the Khanuja's isolation protocol (Khanuja et al., 1999). The protocol is as follows:

- Plant material (5 g) was finely ground in the liquid nitrogen using mortar
- To this, about 50 ml extraction buffer, preheated at 60^{o} C was added and properly mixed.
- This was then distributed in three centrifuge tubes (50 ml capacity), each containing about 20 ml.

- In each tube 100 mg PVP and 20 µl β-mercptoethanol was added and mixed by inversion to a slurry.
- Incubated at 60 ºC in a shaking waterbath (100 rpm) for 1-2 hrs.
- Added equal volume of choloform: isoamyl alchol (24:1) and mixed by inversion for 15 min.
- Spinned at 8000 rpm for 10 min at 25-30 ºC.
- Carefully transferred the upper clear aqueous layer to another 50 ml microfuge tube.
- Added 1/3 volume of 5M NaCl and mixed properly (do not vertex).
- Added 0.6 volume of cold Isopropanol and tubes were kept at RT for more than 1 h. After 1 h, careful mixing produced fibrous nuclei acid that were either scooped or transferred to 2ml microfuge tube and centrifuged. Alternatively, the samples were centrifuged at 10,000 rpm for 10 min at 25-30ºC after mixing of isopropanol.
- Discarded the supernatant and washed the pellet with 70% ethanol.
- Dried the pellet in a vacuum for 15 min and dissolved it in 500µl TNE buffer.
- Added 7 µl of RNAase A and incubated at 37ºC.
- Extracted with equal volume of choloroform: isoamyl alchol (24:1).
- Transferred the aqueous layer to a fresh 1.5ml microfuge tube and added equal volumes of isopropanol.
- Spinned at 10,000rpm for 10 min at 25-30ºC.
- Washed the pellet with 70% ethanol. Dried the pellet in a vacuum and dissolved in 200µl of sterile milliQ water and then stored at 4 ºC until required.
- DNA concentrations were determined either by running aliquots of DNA samples on a 0.8% agarose gel electrophoresis or by taking the absorbance at 260 nm. The ratio between 260 and 280nm provided an estimate of the purity of the sample DNA. DNA samples with a ratio of approximately 1.8 under spectrophotometer and producing an intact single band without smear on 0.8% Agarose gel electrophoresis were considered as good quality DNA.

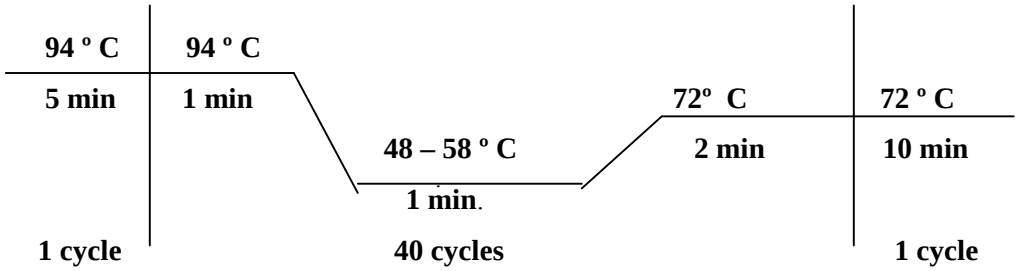
2.3.2.2. PCR protocol using ISSR primers:

- The PCR protocol described by Mullis (1986) was followed and carried out in a total volume of 25µl containing following components: 4µl of genomic DNA (80ng); 2.5µl of 10X *Taq* buffer without $MgCl_2$, 2.5 µl $MgCl_2$ (2.5mM); 5 µl dNTPs (0.2 mM); 0.6µl Taq Polymerase (0.6 U); 0.24 µM of Primer. PCR amplifications were performed on a Stratagene RoboCycler.
- Negative controls, containing all PCR components except DNA were also set up and run with each set of reactions.
- A low range DNA ladder (3 Kb) was used for band sizing.

- The ISSR products were loaded on 2.0 % agarose gel stained with ethidium bromide for electrophoresis in 1X TAE at a constant current 60mA, < 100V for 3 hrs. Visualization of gel was undertaken in Gel Documentation system.

2.3.2.4. PCR thermal cycling conditions:

The PCR conditions used n this study is given in following figure.



CHAPTER 3. DEVELOPMENT OF HPLC METHOD FOR ANALYSIS OF DIPYRANOCOUMARINS AND DIVERSITY STUDIES IN *CALOPHYLLUM INOPHYLLUM*.

3.1 INTRODUCTION:

3.1.1. Phytochemical importance of *C. inophyllum*

Plants belonging to genus *Calophyllum* (Guttiferae/ Clusiaceae) are known to be rich source of natural products, including xanthones (Somnathan and Sultanbawa, 1974; Kumar et al., 1982; Dharmaratne et al., 1986), steroids (Gunasekara and Sultanbawa, 1975), triterpenes (Dahanayake et al., 1974; Gunatilaka et al., 1984), coumarins (Stout et al., 1968; Gautier et al., 1972; Samaraweera et al., 1981) and benzopyrans (Stout et al., 1968). In India, genus *Calophyllum* is represented by eight species. Out of which these eight species, *Calophyllum inophyllum, Calophyllum apetalum, Calophyllum polyanthum* and *Calophyllum austroindicum* grow along the Western Ghats of India and remaining four species occur in Andaman and Nicobar island. Among these eight species, *Calophyllum austroindicum* (Bhosle et al., 2005), *Calophyllum inophyllum* (Stevens, 1998) *and Calophyllum polyanthum* are rare and endangered plants. *Calophyllum lanigerum* from which potential anti-HIV, NNRTIs (Non Nucleoside Reverse Transcriptase Inhibitors) calanolides were isolated and reported (Kashman et al., 1992) has not been reported so far from India. As an alternative to calanolide, another NNRTIs inophyllums isolated and reported from *Calophyllum inophyllum* (Patil et al., 1993) could be of immense help in anti retroviral therapies.

Chemistry of *C. inophyllum* has been extensively studied and several compounds belonging to different classes have been isolated (Table 3.1). Xanthones like caloxanthones A, B and D from root bark and caloxanthone E from root heartwood (Munekazu et al., 1994), jacareubin, 1-7 dihydroxyxanthone, 1,5,6-trihydroxyxanthone, 1,6-dihydroxy-5-methoxyxanthones (buchanaxanthone), 6 desoxyjacareubin from heartwood were isolated (Faik et al., 1971). Caloxanthones A, B, macluraxanthone and 1,5- dihydroxyxanthone were reported to have cytotoxic and antimicrobial activities (Yimdjo et al., 2004), while anti oxidant, radical scavenging activities of caloxanthone and macluraxanthone were also tested (Haraguchi et al., 2004). Among the triterpenes reported from *Calophyllum inophyllum* are friedelin, friedelan-3 *β-ol* (Kumar et al., 1976).

Table 3.1. Reports on phytochemical studies in *Calophyllum inophyllum*.

Material	Group of Compound	Compounds	Reference
Nuts.	4-phenylcoumarins	Calophyllolide (±) inophyllolide. Calophynic acid.	Polonski et al., 1957. 1958.
Leaves.	Triterpenes	Friedelin. Canophyllal. Canophyllol. Canophyllic acid.	Govindachari et al., 1967.
Leaves.	Piscicidal constituents.	Piscicidal constituents.	Kazuyoshi et al., 1968
Heartwood.	Xanthones.	6-desoxyjacaerubin. Jacareubin. 2-(3,3-dimethylallyl)-3,5,6-tetrahy-droxyxanthone.	Jackson et al., 1969
Heartwood.	Xanthones.	Jacareubin. 1,7-dihydroxyxanthone. 1,5,6-trihydroxyxanthone. 1,6-dihydroxy-5-methoxyxanthone (Buchanaxznthone). 6-desoxyjacaerubin. 2-(3,3-dimethylallyl)-3,5,6-tetra hydroxyxantone	Faik et al., 1971.
Timber.	Triterpene. Xanthones.	Friedelin. Fridelan-3β-ol. 6-(-3-methyl-2-butenyl)1,5dihydroxy-xanthone. 6-desoxyjacareubin. Sitosterol. 1,7-dihydroxy-3,6 dimethoxyxanthone.	Kumar et al., 1976.
Heartwood.	Xanthone.	2-(3-hydroxy-3 methylbutyl)1,3,5,6-tetrahydroxyxanthone. Jacareubin. 6-desoxyjacaerubin. 2-(3-methylbut-2-enyl)-1,3,5,6 tetrahydroxyxanthone. 2-(3-methylbut-2-enyl) 1,3,5-trihydroxyxanthone.	Goh et al., 1991

Continued Table 3.1

Material	Group of Compound	Compounds	Reference
Leaves/seeds.	Dipyranocoumarins.	Inophyllum A. Inophyllum B. Inophyllum C. Inophyllum D. Inophyllum E. Inophyllum P. Inophyllum G-1. Inophyllum G-2.	Patil et al., 1993.
Root bark Root Heart-wood.	Xanthone Xanthone	Caloxanthone D. Caloxanthone E. 1,3,8-trihydroxy-7-methoxy xanthone. 1,3,-dihydroxy-7,8-methoxy xanthone. 1,3,5-trihydroxy-2-methoxy xanthone 6-hydroxy-1,5-dimethoxy xanthone.	Munekazu et al., 1995
Root bark.	Xanthone.	Caloxanthone A. CaloxanthoneB. Macluraxanthone. 1,5-dihydroxyxanthone. (-) Epicatechin.	Munekazu et al., 1995
Leaves.	Benzodipyranone Derivatives.	(2S,3R)-2,3dihydro-5- dihydroxy-2,3,8,8-tetramethyl-6-(1phenylethenyl)4H,8H-benzo[1,2-b: 3,4-b'] dipyran-4-one. (2R,3R)-2,3-dihydro-5-hydroxy2,3,8,8-tetramethyl-6-(1phenylethenyl)-4H,8H-benzo [1,2-b: 3,4-b'] dipyran-4-one .	Khan *et al.,* 1996
Seeds.	Pyranocoumarins	Inocalophyllin A. Inocalophyllin B. Methyl ester of inocalophyllin A. Methyl ester of inocalophyllin B.	Shen et al., 2003
Root bark & Nuts.	Xanthones.	Caloxanthone A. Caloxanthone B. Macluraxanthone. 1,5-dihydroxyxanthone. Canophynic acid. Brasiliensic acid. Inophylloidic acid. Friedelan-3-one. Calaustrilin. Calophyllolide. Inophyllum C. Inophyllum E.	Yimdjo et al., 2004

Continued Table 3.1

Material	Group of Compound	Compounds	Reference
Stem Bark.	Coumarin. Xanthone. Triterpene.	Mucigerin. Cidraxanthone. Friedelin, stigmasterol.	Ee et al., 2004
Leaves.	Triterpenoids.	3,4-secofriedelan-3,28-dioic acid. 27-hydroxyacetate canophyllic acid. 3-oxo-27-hydroxyacetate friedelan-28-oic acid.	Laure et al., 2005

Properties like antiseptics, astringents, expectorants, diuretics and purgatives of ethanolic extracts of fresh leaves of *C. inophyllum* were attributed to the presence of pair of new epimers named as inophynone and isoinophynone along with some known constituents like friedelin, canophyllol and canophyllic acid (Muhammad, et al., 1999). Anti UV properties of *C. inophyllum* oil were investigated and were proposed it as a potential vehicle, free of toxicity with a natural UV filter action in ophthalmic formulations (Said et al., 2007). However, dipyranocoumarins are the most important group of bioactive molecules isolated from *C. inophyllum*. Eleven compounds of inophyllum class were isolated from *C. inophyllum* and were described together with the SAR (Structure- Activity Relationship) of these novel anti- HIV compounds (Patil et al., 1993).

C. inophyllum, also popularly known as "Indian Laurel", grows on sandy beaches near the coasts along the Western Ghats of India. This plant produces a good quality timber that can be used in boat and ship making and is cut fairly on large scale. Because of its large scale cutting for obtaining timber, this plant species has been included in IUCN's red list of threatened species (Stevens et al., 1998) and needs to be conserved. For the effective conservation of endangered species, knowledge of its habit and geographical conditions along with genetic variation available to a species are prerequisites, since its survival depends not only on habitat and geographical conditions but also on genetic variation available to a species (Dodd and Helenurm, 2002). As secondary metabolites play key role in the adaptation of the plants to the biotic and abiotic environments, intra specific, high secondary metabolite variation available can also contribute to survival of the species. Quantitative and qualitative variation in secondary metabolites has been found taxonomically significant for species recognition (Julkunen-Tiitto et al., 1996; Zhou et al., 2007).

3.1.2. Role of chemical diversity in secondary metabolite research.

In drug discovery, high throughput screening has become an important tool, which requires a large number of compounds to be effective. These cannot be supplied by traditional organic synthesis, so two other sources are used: combinatorial chemistry and chemo diversity from nature. It is currently estimated that there are at least 2,50,000 different plant species (Verpoorte, 1998) all of which coexist and interacts in ecosystem with different species of insects, fungi, algae and prokaryotes. All these species produce wide variety of secondary metabolites that are involved in interactions between organisms. Considering the number of organisms and infinite number of interactions possible, enormously wide varieties of secondary metabolites have evolved within organisms. Exploration of number of plant species studied and compounds isolated from them suggests that from all plant species at least a million different compounds are yet to be isolated. It is quite evident that nature provides an enormous potential for the discovery of new bioactive compounds. Because secondary metabolites represent features that can be expressed in terms of ecological, taxonomical and biochemical differentiation and diversity, it becomes most important to study the chemical diversity available in particular plant species. Quantitative and qualitative chemodiversity helps in proper selection of plants or locations of plants that would provide a strating material for initiating further work.

3.1.3. Techniques used in secondary metabolite analysis:

3.1.3.1. Thin Layer Chromatography (TLC):

TLC is a chromatographic method that offers the option of presenting the result as an image. TLC is the sole technique in which all the components of the sample are included in the chromatogram. Not only does the technique give visual results but also it excels in its simplicity and is low in cost. Parallel analysis of samples with high sample capacity is possible and the results can be obtained rapidly. The disadvantages of TLC are a lack of automation, the problems of reproducibility which sometimes occur, and the lack of accuracy in quantitation.

3.1.3.2. High Performance Thin Layer Chromatography (HPTLC):

HPTLC is ideally suited for the preliminary screening of plant extracts before HPLC analysis. In HPTLC, the plates are precoated with stationary phase with a typical mean particle size of 5 μm. The plates give better separations and reproducibility than normal precoated TLC plates (mean particle size 12 μm) and they also allow more sensitive detection. Shorter developing

distances are required. The separation power of HPTLC is still lower than that of HPLC and the latter is preferred for quantitative determination.

3.1.3.3. High Performance Liquid Chromatography (HPLC):

Until the advent of HPLC, quantitation of phytochemicals was inadequate and resolution of similar compounds was difficult with the phytochemical techniques like paper chromatography, TLC and HPTLC. A powerful technique capable to separate, identify, and quantitate the compounds present in any sample that can be dissolved in liquid was needed. HPLC fulfills these criteria and is now one of the most powerful techniques that could separate water soluble, thermally labile, non-volatile compounds with speed, precision and high resolution. For much more selectivity and precision, HPLC is coupled to different detectors (hyphenated technique) like mass spectrometry (LC/MS) and UV diode array (LC/UV).

(i) LC/UV:

HPLC coupled with UV photodiode array detection (LC/UV) allows the running of a chromatographic separation with simultaneous detection at different wavelengths. The UV spectra of natural products give useful information on the type of constituents. LC/ UV allows the recording of UV spectra of matching compounds, which can be performed automatically when screening for known constituents.

(ii) LC/MS:

This is the most sensitive analytical method. With the high power of mass separation of a MS detector, very good selectivities can be obtained. Moreover, this technique has the potential to yield information about the molecular weight as well as the structure of the analytes. For the analysis of natural products, the most commonly used LC/MS interfaces are atmospheric pressure chemical ionization (APCI) and electrospray (ESI). Each of these interfaces has its own characteristics and range of application but their combined use permits the analysis of small non polar natural products to very large polar molecules.

3.1.3.4. Gas Chromatography (GC):

GC is selective and suitable for small, volatile compounds. Gas chromatography provides excellent resolution but the restriction to volatile samples (less than 20% of organic compounds can be separated by gas chromatography) meant that derivatization is often necessary. Sensitive universal detection is provided by flame ionization (FID). GC is ideal for the analysis of

complex mixtures such as those found in essential oils. In one run, it is possible to separate hundreds of constituents and identify them.

3.1.3.5. Capillary electrophoresis (CE):

This is an analytical technique which provides efficiently high separation with short run time. Several modes of Capillary electrophoresis are available 1. Capillary zone electrophoresis (CZE). 2. Micellar electrokinetic chromatography (MEKC). 3. Capillary gel electrophoresis (CGE). 4. Capillary isoelectric focusing. 5. Capillary isotachophoresis. 6. Capillary electrochromatography (CEC). 7. Non- aqueous capillary electrophoresis. The simplest and most versatile CE mode is CZE, in which separation is based on differences in the charge- to- mass ratio and analyte migrates into discrete zones at different velocities.

3.1.3.6. Counter current chromatography (CCC):

Counter current chromatography also known as centrifugal partition chromatography is an all liquid separation technique, which relies on partition of the sample between two immiscible solvents. The relative proportion of solute passing into each of the two phases being determined by the respective partition coefficient. Because CCC is characterized by the absence of solid support, it has no irreversible adsorption, minimizes tailing and results in total recovery of samples. It also has low solvent consumption and low risk of sample decomposition.

3.1.4. Introduction to ISSR as molecular marker:

ISSR is a technique that uses repeat anchored primers to amplify DNA sequences between two inverted SSR (Zietkiewicz et al., 1994). ISSR markers do not require a prior knowledge of the SSR target sequence, furthermore, they are highly reproducible due to their primer length and to high stringency achieved by the annealing temperature. ISSR markers have been found to provide highly polymorphic fingerprints (Zietkiewicz et al., 1994). Recent ISSR studies of natural populations have demonstrated the hypervarible nature of these markers and their potential. The sequences with ISSR targets are abundant throughout the eukaryotic genome and are rapidly evolved. Consequently, ISSR may reveal a much higher number of polymorphic fragments from every primer than RAPD (Esselman et al., 1999). Compared with RAPD, a series of studies have indicated that ISSR could be able to produce more reliable and reproducible bands because of the higher annealing temperature and larger sequences of ISSR primers. (Tsumura et al. 1996). Therefore ISSR has proved to be useful in population genetic studies (Esselman et al.1999; Wolfe and Liston 1998; Zietkiewicz et al.1994).

This chapter envisages the development of HPLC method and its validation for dipyranocoumarins' analysis. Using the method developed, chemical diversity in *C. inophyllum* has been studied studies. As a part of chemical diversity study, dipyranocoumarins such as inophyllums A, B, C, D and calophyllolide were quantitatively analyzed with validated HPLC method in seeds collected from different locations. As a part of genetic diversity study, PCR based molecular markers, ISSRs were exploited to generate information on genetic diversity available in *C. inophyllum.* Subsequently correlation between genetic and chemical diversity in *C. inophyllum* has been studied.

3.2 EXPERIMENTAL PROTOCOLS:

3.2.1. Isolation of bioactive compounds:

From seeds of *C. inophyllum*, compounds were isolated by using different chromatographic techniques and characterized by taking spectroscopic data like C NMR, H NMR, UV, IR and EI MS. These compounds were identified by comparing the obtained data with data available in literature. This work was done at Division of Organic Chemistry, National Chemical Laboratory, Pune (Joshi et al., 2000).

3.2.2. Development of HPLC method:

The method for HPLC analysis of dipyranocoumarins was developed on Perkin- Elmer's Series 200 HPLC system. This system was equipped with a quaternary gradient pump, an autosampler and diode array detector. The HPLC method was developed with following conditions.

***HPLC Column*:** µ Porasil- Water's (Millford, MA, USA), 3.9 mm ID X 300 mm L; 10µm, irregular particles, stainless steel.

Mobile phase- Mixture of ethyl acetate and petroleum ether (25:75).

Run time- 20 minutes.

Flow rate - 1 ml/min.

Detection wavelength: 245 nm (UV).

Injection volume: 15 µl.

Quantification: External standard method.

3.2.3. Preparation of Standard solutions:

Standards of each of dipyranocoumarins (inophyllums A, B, C, D, P and calophyllolide) were provided by Division of Organic Chemistry, National Chemical Laboratory,

Pune, India. (Joshi et al., 2000). Stock solutions were prepared in optimized HPLC mobile phase i.e. mixture of HPLC grade ethyl acetate and petroleum ether, 60-80^0 C (25:75, v/v). Appropriate dilutions were made in mobile phase to obtain working solutions of 0.5 – 2.5 mg/ml dipyranocoumarins. These solutions were used for the study of linearity, repeatability, reproducibility and recovery studies.

3.2.4. Method validation:

3.2.4.1. Linearity:

For studying the linear relationship between peak areas and concentrations of each of the dipyaranocoumarins, linearity was tested at 245 nm over the concentration range of 0.5 – 2.5 mg/ml for each of dipyranocoumarins. 10 µl of each of the concentration range was injected. Measurment at all concentration levels were carried out in triplicates. Calibration curves were obtained by plotting the peak areas versus dipyranocoumarins' concentrations. The linearity equations were calculated by using linear regression analysis as $y= mx + c$

Where y= Area

m= Slope

c= Intercept

x= Concentration.

3.2.4.2. Precision:

To study the precision of the method, repeatability and reproducibility were measured for retention factors (times), areas and peak heights. For each of the dipyranocoumarins, 10 µl of 1.0 mg/ml working stock was injected. Intra- day repeatability of the retention times, peak areas and peak heights were determined as % RSD (Relative Standard Deviation) for three consecutive injections of each of the dipyranocoumarins. Reproducibility of the chromatographic data (retention times, areas and peak heights) of the dipyranocoumarins was determined in three consecutive days to obtain % RSD values for inter day reproducibility.

3.2.4.3. Recovery:

Seed samples used in recovery studied were first pulverized, extracted and the contents of the dipyranocoumarins were estimated with HPLC method. Stock solution of each of the dipyranocoumarins was added to such pulverized and pre estimated seed material to yield final concentrations of 0.5,1.0, 1.5, and 2.0 mg/g. These samples were allowed to settle for 1 h. prior to

extraction and were then processed according to extraction and sample preparation procedure described below. These samples were then analysed by HPLC method to test the recovery.

3.2.4.4. Limit of detection and quantification:

The limit of detection and limit of quantification was calculated at wavelength 245 nm. The limit of detection expressed as a concentration at a signal -to- noise ratio 3:1, was calculated on the basis of noise, which was evaluated by recording the detector response over the period approximately ten times the width of the peaks. The signal –to- noise ratio of 10:1 was used to determine the limit of quantification.

3.2.5. Collection of plant material for chemo diversity and genetic diversity studies:

Because of its wide scale cutting and endangered status, few plants were observed growing at several locations along the Western Ghats of India. For collection of plant material, states of Maharashtra, Goa, Karnataka, and Kerala were covered. For chemo diversity study, mature fruits from only 13 individual plants from 13 different locations, each more than 40 kms apart were collected in the months of May-July. For genetic diversity study (ISSR analyses), plant material in the form of leaves from the same 13 loactions along with 7 additional locations was collected. Plant material was authenticated by Botanical Survey of India, Pune Division. Details of the collection of plant material, locations and their geographical position have been given in Table 3.6.

3.2.6. Extraction and sample prepartion for HPLC analysis:

Mature fruits collected from 13 different locations were air-dried and seed kernels were taken out of its hard shell by mechanically breaking the fruits. Kernels were pulverized and known quantities were suspended in laboratory grade acetone. Acetone was filtered off and remaining pulverized kernel was resuspended in acetone. This process was repeated three times and acetone extracts of each sample were combined together. From combined acetone extracts, acetone was removed to dryness on rotary evaporator to get dark brown colored extracts of kernel. Known quantities of each extracts (1.0 mg) were dissolved in one ml HPLC mobile phase, centrifuged at 10000 rpm for 10 min and 15 µl of these samples were loaded for HPLC analysis.

3.2.7. HPLC analysis:

HPLC analyses were carried out on Perkin- Elmer's series 200 HPLC system equipped with quaternary gradient pump, an autosampler and diode array detector in isocratic mode using water's (Millford, MA, USA) µ Porasil (3.9 mm ID X 300 mm L; 10 µm) stainless steel HPLC

column. Petroleum ether and ethyl acetate (75:25) was used as mobile phase in a run time of 20 min with flow rate of 1 ml / min. 15 µl of each of mg / ml of stock solutions of inophyllums A, B, C. D and calophyllolide were injected and chromatograms were recorded at 245 nm to get the reference chromatograms of standards to evaluate the differences in the contents of dipyranocoumarins.

3.2.8. ISSR analysis of *Calophyllum inophyllum*:

3.2.8.1. DNA extraction:

Genomic DNAs, from leaf samples of 20 individual plants collected from different locations along the Western Ghats of India and stored initially in liquid nitrogen and then at -70º C were isolated using slightly modified cetyl trimethyl ammonium bromide (CTAB) method of Khanuja (Khanuja et al., 1999). DNA samples were solvated in sterile double distilled water and purities were checked by loading 0.8 % agarose gel. Their concentrations were estimated using a DNA ladder of known concentrations and spectrophotometrically at 260 and 280 nm. Working stocks of 20 ng / µl were prepared for further use in PCR amplifications. The details of DNA isolation and checking of purity of isolated DNAs are described in chapter 2 (General Materials and Methods).

3.2.8.2. ISSR PCR amplification:

After assessing the effects of different concentrations of $MgCl_2$, dNTPs and template genomic DNA, PCR reactions were carried out in 25 µl reaction volume that contained 2.5 µl of Taq buffer B (without $MgCl_2$), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.6 U of Taq polymerase, 0.24 µM of primers and 80ng of template DNA. Amplifications were performed with thermal cycling conditions that consisted of initial denaturation for 5 min. at 94º C, followed by 40 cycles of 1 min. at 94º C, 1 min. at 48 – 58º C (optimized for different primers), 2 min. at 72º C and 10 min. at 72º C for the final extension. Initially 100 ISSR primers (UBC, Primer set No 9) were screened and optimized for their optimal annealing temperatures on Stratagene's RoboCycler Gradient PCR machine with temperature gradient mode. The amplified ISSR fragments along with low range DNA ladder were then separated electrophoretically on 2.0 % agarose gel in 1.0 X TBE buffer at 100 V for 3 Hrs. Separated ISSR fragments were visualized by staining the gel with ethidium bromide and photographed under UV light. Amplifications with each primer were repeated twice to confirm its reproducibility.

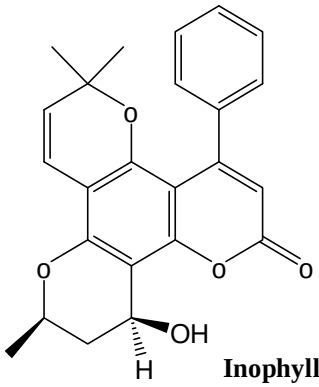
3.2.8.3. Data analysis:

For quantitative analysis of dipyranocoumarins, peak areas in sample chromatograms were compared with the reference chromatograms of the standard to evaluate the differences in the contents of dipyranocoumarins. TotalChrom Navigator software was used to process the chromatographic data and external standard method was used for quantification.

Amplified ISSR fragments were transformed into a binary character matrix by scoring 1 (presence) and 0 (absence). For statistical analysis, data only from intensely stained and clear bands were considered. For scoring of bands, lane based Band Analysis function of Biorad's Gel documentation system was used. To estimate the genetic relationship among the individuals, the Nei's genetic distance coefficient was used to calculate the pair wise band similarity and dendrogram was constructed by an unweighted pair group method of cluster analysis (UPGMA) using arithmetic average on NTSYS PC version 2.1. The principal component analysis (PCA) of data from 20 locations based on ISSR variations was performed using NTSYS PC version. Similarly, to reveal the correlation between chemical and genetic diversity, principal component analysis (PCA) was also carried out for 13 individuals based on variations in dipyranocoumarins' (inophyllums A, B, C, D, calophyllolide, and % acetone extract) content and ISSR variation of the same 13 locations.

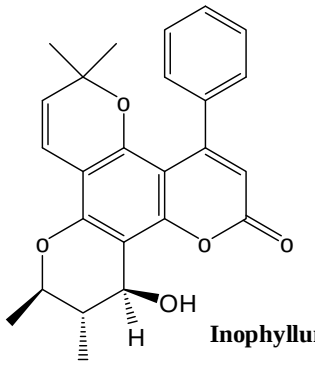
3.3 RESULTS AND DISCUSSION:

Using different chromatographic techniques, from seeds of *C. inophyllum*, six compounds were isolated at Division of Organic Chemistry, National Chemical Laboratory, Pune. These compounds were characterized by taking spectroscopic data like C NMR, H NMR, UV, IR and EI MS (Joshi *et al,* 2000). Isolated compounds were identified as inophyllum A (Structure1) inophyllum B (Structure 2), inophyllum C (Structure3), inophyllum D (Structure 4), inophyllum P (Structure 5) and calophyllolide (Structure 6). These compounds belonged to group dipyranocoumarins or 4-phenylcoumarins These compounds were made available by Division of Organic Chemistry, National Chemical Laboratory, Pune and were used as authentics for HPLC method development, chemo diversity studies and to study *in vitro* expression of these compounds in *in vitro* grown cells and tissues.



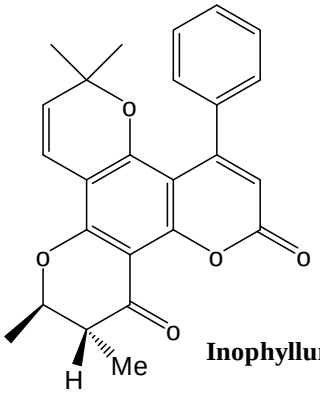
Inophyllum A

Structure 1. Inophyllum A



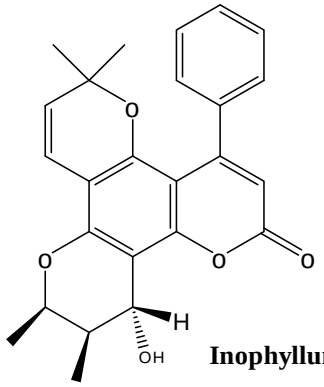
Inophyllum B

Structure 2. Inophyllum B



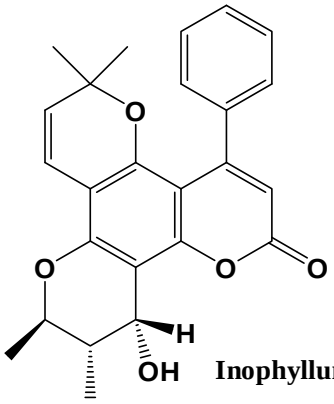
Inophyllum C

Structure 3. Inophyllum C



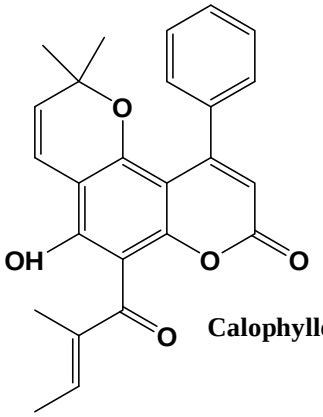
Inophyllum D

Structure 4. Inophyllum D



Inophyllum P

Structure 5. Inophyllum P



Calophyllolide

Structure 6. Calophyllolide

3.3.1. HPLC method development:

Because isolated dipyranocoumarins (inophyllums A, B, C, D, P and calophyllolide) were soluble in organic solvents, initially both, reverse phase and normal phase chromatography were tried for the HPLC separation of dipyranocoumarins. An attempts to develop reverse phase HPLC method with C18 (4.6 mm ID, 250 mm L, 5 µm), RP C18 (4.6 mm ID, 250

mm L, 5 µm) column with different solvents like methanol, acetonitrile and water in different combinations and concentrations as a mobile phase, either in isocratic or gradient were made. With these columns and mobile phases tried, dipyranocoumarins could not be separated. When HPLC column Cyclobond II Acylated was used with water and methanol (50:50) in an isocratic mode, all the dipyranocoumarins under study were eluted at the same retention time; therefore these conditions were also not used further.

There were no reports available on the method for HPLC analysis of the dipyranocoumarins. For the bioactive compounds like calanolide A, HPLC method was reported for quantification of calanolide A in rat, dog and human plasma. In this method, synthetic intermediate (±)- 12- oxocalanolide A was used as internal standard (Xu et al., 2000). Because calanolides and inophyllums are related compounds and belong to coumarin group of natural products, same method was also tried for separation of inophyllums. An attempt to separate inophyllums with this method also failed. Since the endeavors to optimize reverse phase HPLC separation with C18, RP18 and Cyclobond were not satisfactory, as a suitable alternative to reverse phase HPLC, normal phase HPLC was tried with different HPLC column and solvent mixtures as mobile phase. An idea was taken from the previous TLC experiments where all the dipyranocoumarins were separated on TLC using mixture of ethyl acetate and petroleum ether as a mobile phase. This indicated that unmodified silica or bare silica rather than modified silica in HPLC column can separate the dipyranocoumarins. Initially several HPLC columns, solvents and their mixtures as mobile phase were tried for optimization of normal phase HPLC.

Best base line separation of dipyranocoumarins was achieved when method was developed with Water's µPorasil HPLC column. µPorasil with 30 mm ID, 300 mm L., irregular particles of size 10µm were the optimum required specifications of the column. This column has bare silica or unmodified silica as a stationary phase. 10µm particles, irregular particles were the most essential specifications required for best base line separation. The other optimum separation conditions were mixture of ethyl acetate and petroleum ether (60-80° C) (25:75) as mobile phase, flow rate, 1.0 ml/ min., run time, 20 minutes., and detection wavelength 245 nm. No other solvents and their mixtures as mobile phase resulted in as good base line separation of dipyranocoumarins as did ethyl acetate and petroleum ether in isocratic mode. When gradient mode of elution (where % ethyl acetate was linearly increased up to 60 %) was used, peaks either merged or their separation was minimized. Run time of 20 minutes was sufficient since all the standard dipyranocoumarins were eluted before 20 minutes of rum time. The corresponding UV absorption maxima for each of the standard dipyranocoumarins were recorded within the range of 200-400 nm. It was established that 245nm was the maximum absorption wavelength for all the dipyranocoumarins. Chromatograms were best recorded at 245 nm since all the dipyranocoumarins

under study have absorbance maxima 245 nm. This optimized normal phase HPLC method separated all six dipyranocoumarins under study in a single isocratic run of 20 minute.

Upon loading 15µl of each of 1mg/ml solution of standard samples of dipyranocoumarins, inophyllum A, RT 13.30 ± 0.04 min (Fig. 3.1); inophyllum B, RT 9.17 ± 0.09 min **(**Fig. 3.2); inophyllum C, RT 8.06 ± 0.03 min (Fig. 3.3); inophyllum D, RT 8.93 ± 0.04 min (Fig. 3.4) inophyllum P, RT 7.35 ± 0.04 min (Fig. 3.5) and calophyllolide, RT 5.00 ± 0.08 min (Fig. 3.6) were eluted.

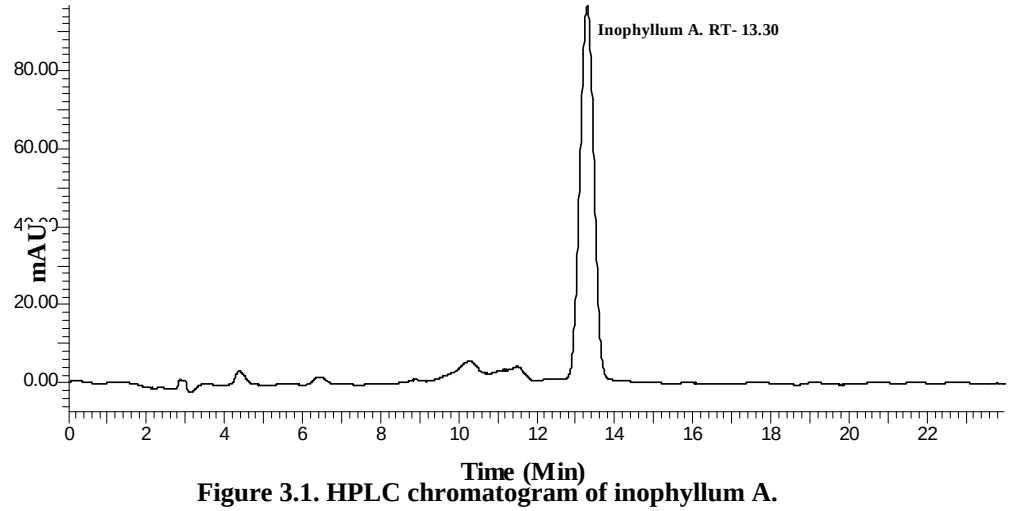


Figure 3.1. HPLC chromatogram of inophyllum A.

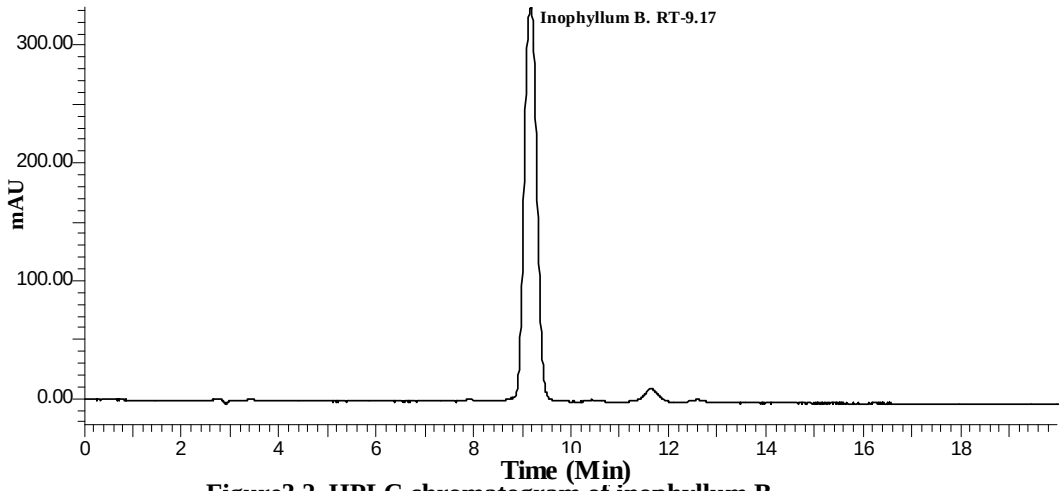


Figure3.2. HPLC chromatogram of inophyllum B.

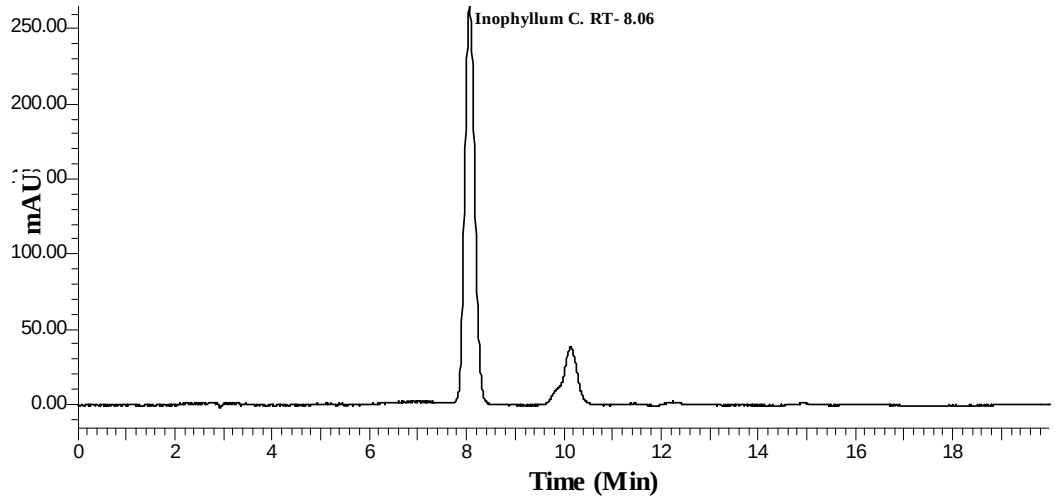


Figure 3.3. HPLC chromatogram of inophyllum C.

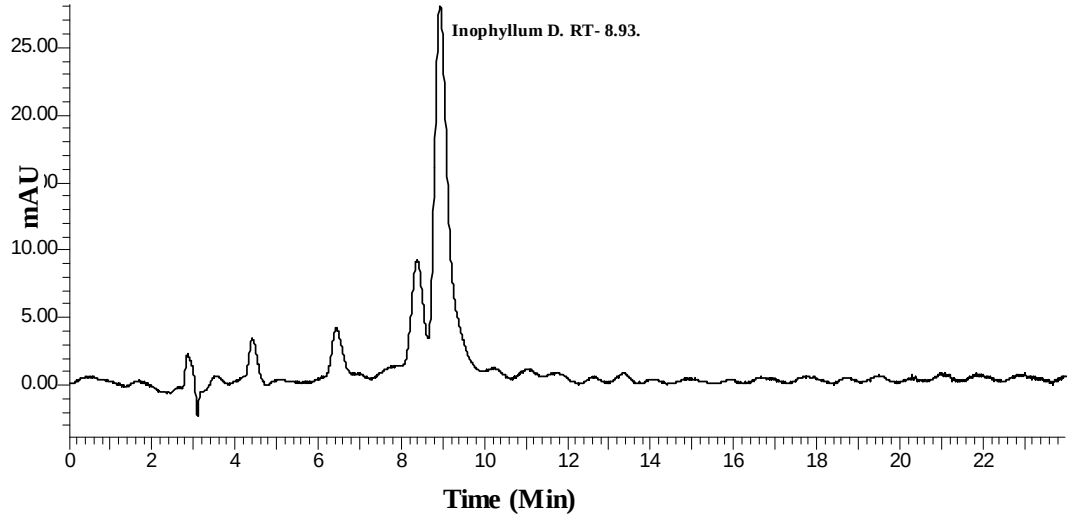


Figure 3.4. HPLC chromatogram of inophyllum D.

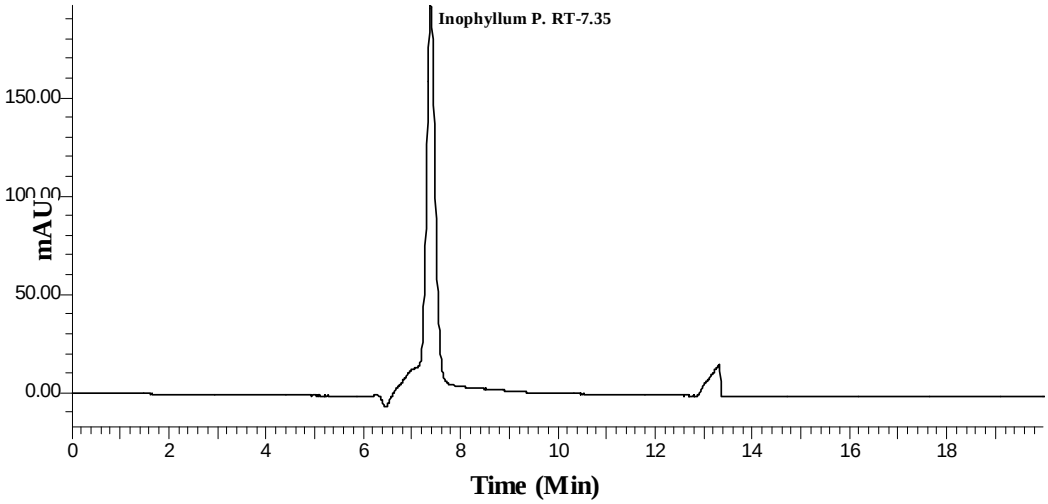


Figure 3.5. HPLC chromatogram of inophyllum P.

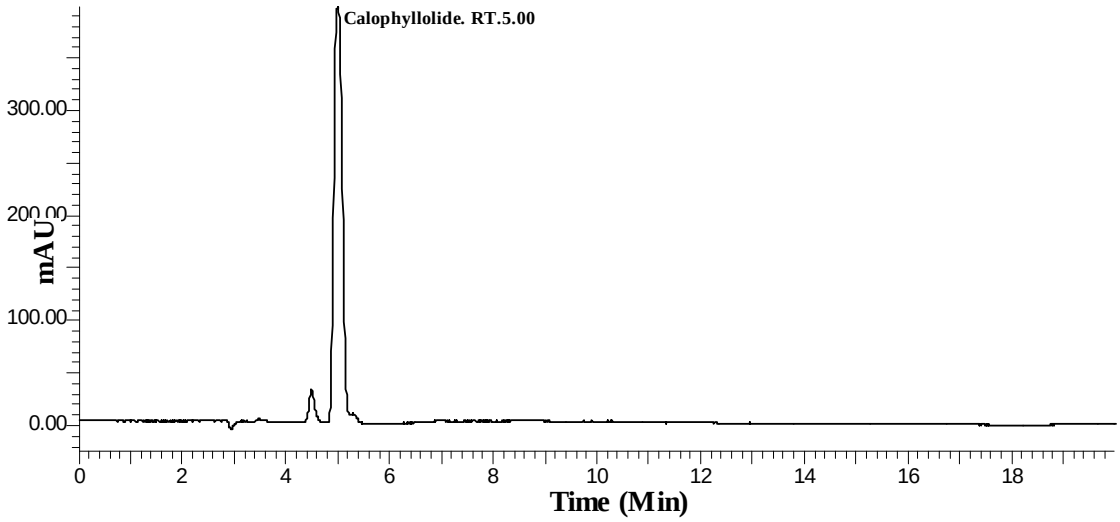


Figure 3.6. HPLC chromatogram of inophyllum calophyllolide.

3.3.2. HPLC method validation:

During the method development, the efficiency and power of resolution of µPorasil column was checked by measuring theoretical plates which were > 8000 for µPorasil whereas tailing factor was <2 and resolution was >2.

3.3.2.1. Linearity:

Since the initial experiments suggested that concentrations of the dipyaranocoumarins per ml test samples prepared from *in vivo* growing plants and *in vitro* growing plant material was expected to be in µg range, for linearity of each of the dipyranocoumarins, concentration range of 0.5 – 2.5 mg/ml was selected. In this method, linearity was established in a triplicate injections. For each dipyranocoumarins, 0.5 – 2.5 mg/ml concentration range employed in the construction of calibration curve was found to be linear. The correlation coefficients were greater than 0.99 for all dipyranocoumarins. The graphs for linear regression curve (Fig 3.7 to 3.12), the data for linear equations and correlation coefficients have been given in Table 3.2. The representative linear equations and correlation coefficients for inophyllum A: $y = 146235x + 2816$; $R^2 = 0.9991$, inophyllum B: $y = 152032x – 7960$; $R^2 = 0.999$, inophyllum C: $y = 65953x + 17018$; $R^2 = 0.9993$, inophyllum D: $y = 35688x + 2271$; $R^2 = 0.9998$, inophyllum P: $y = 154246x – 9534$; $R^2 = 0.9997$ and calophyllolide: $y = 6957.1x + 647$; $R^2 = 0.999$ were obtained.

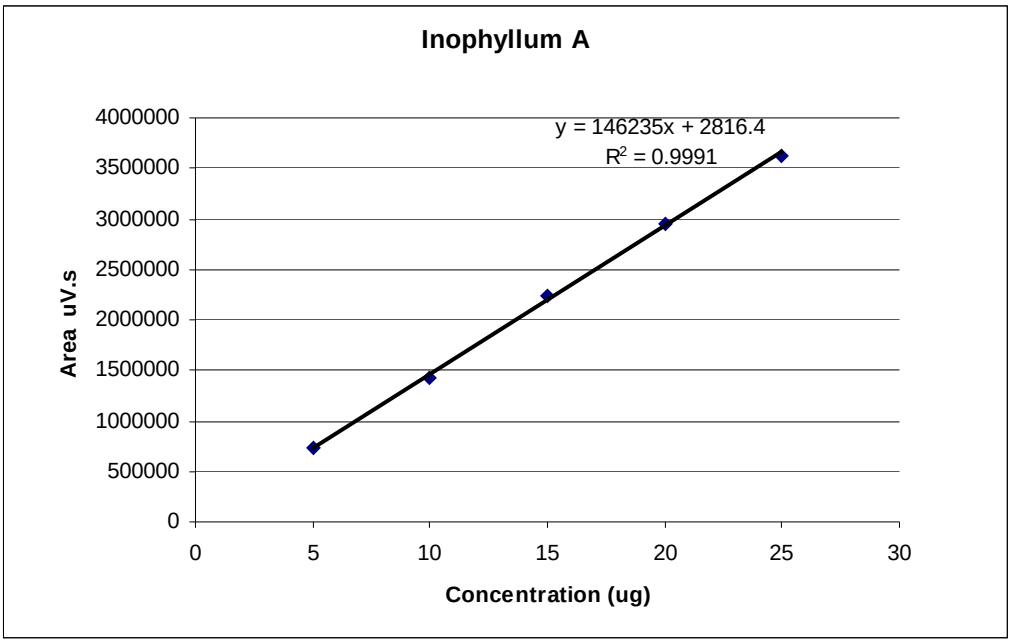


Figure 3.7. Linearity graph and correlation coefficient for inophyllum A

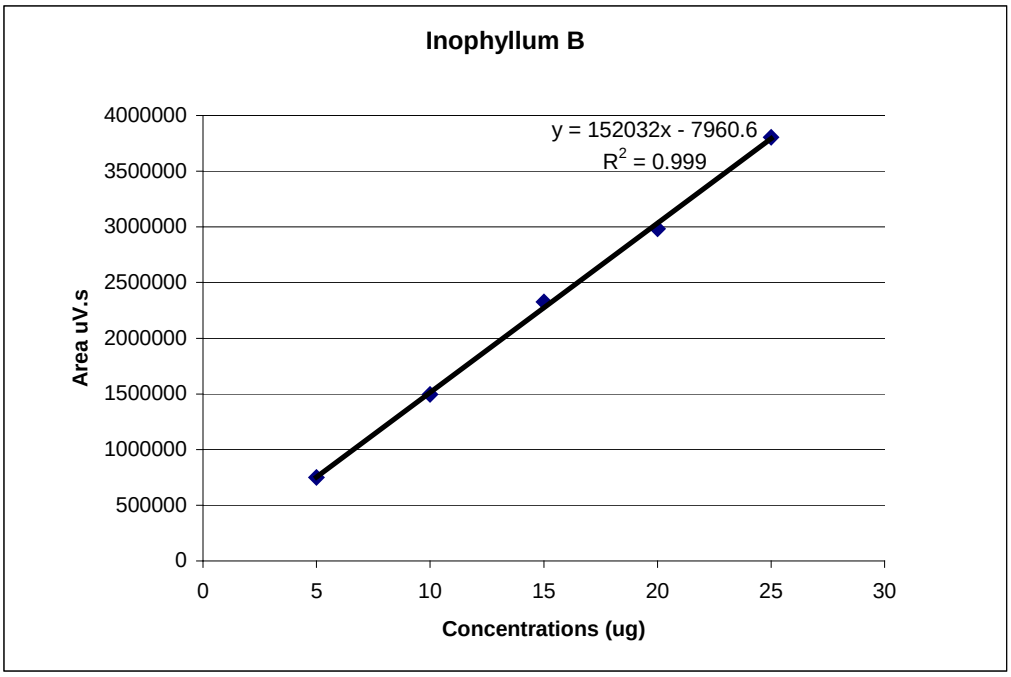


Figure 3.8. Linearity graph and correlation coefficient for inophyllum B

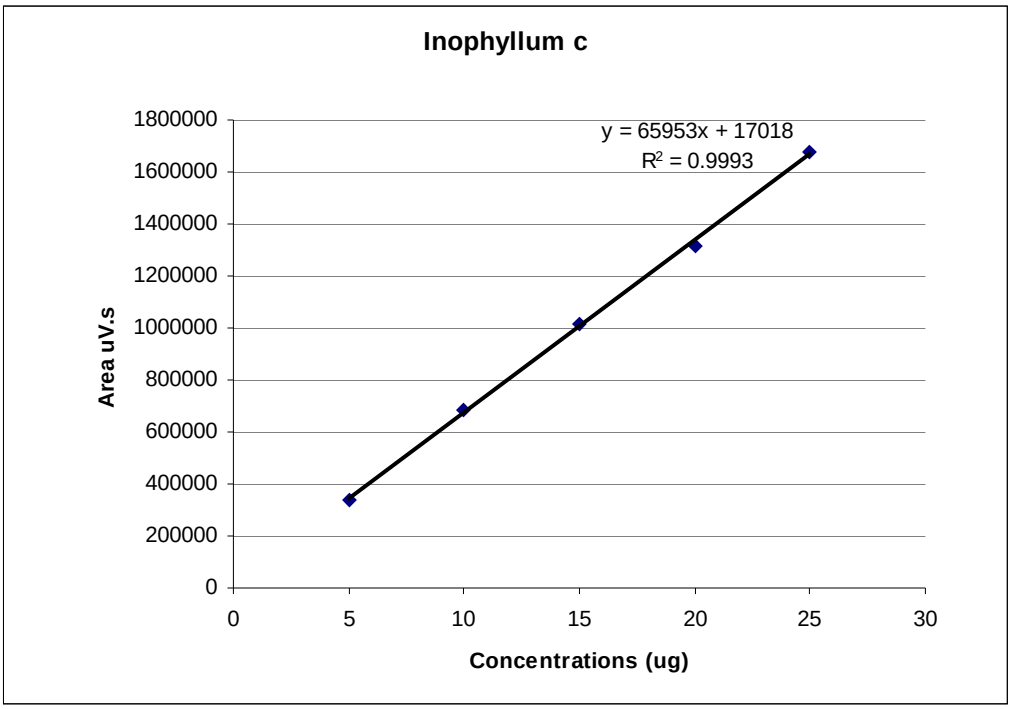


Figure 3.9. Linearity graph and correlation coefficient for inophyllum C.

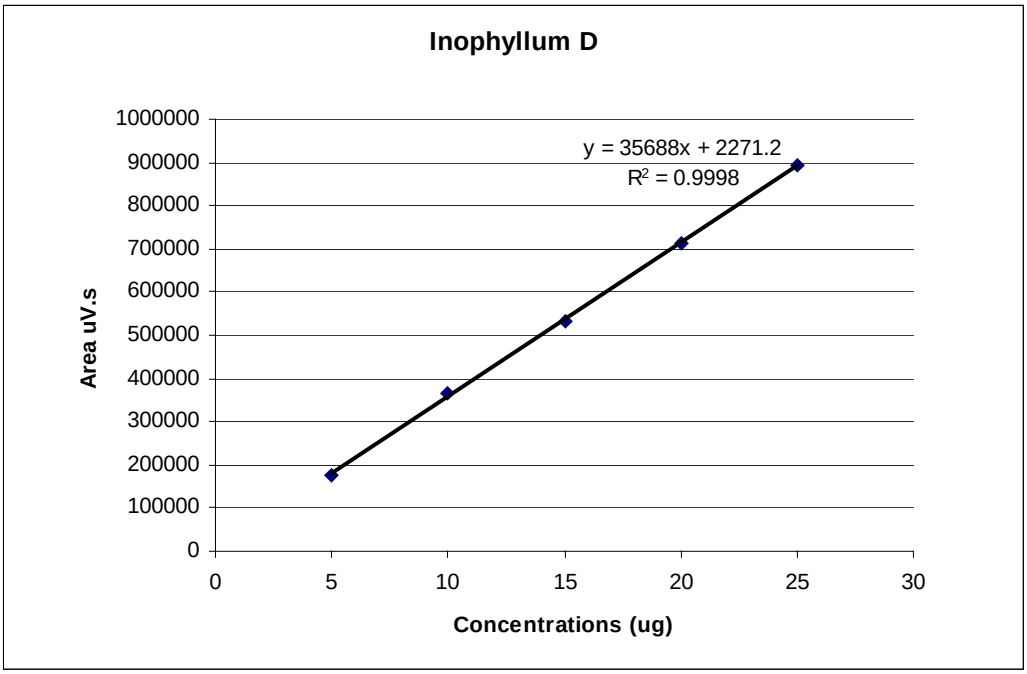


Figure 3.10. Linearity graph and correlation coefficient for inophyllum D

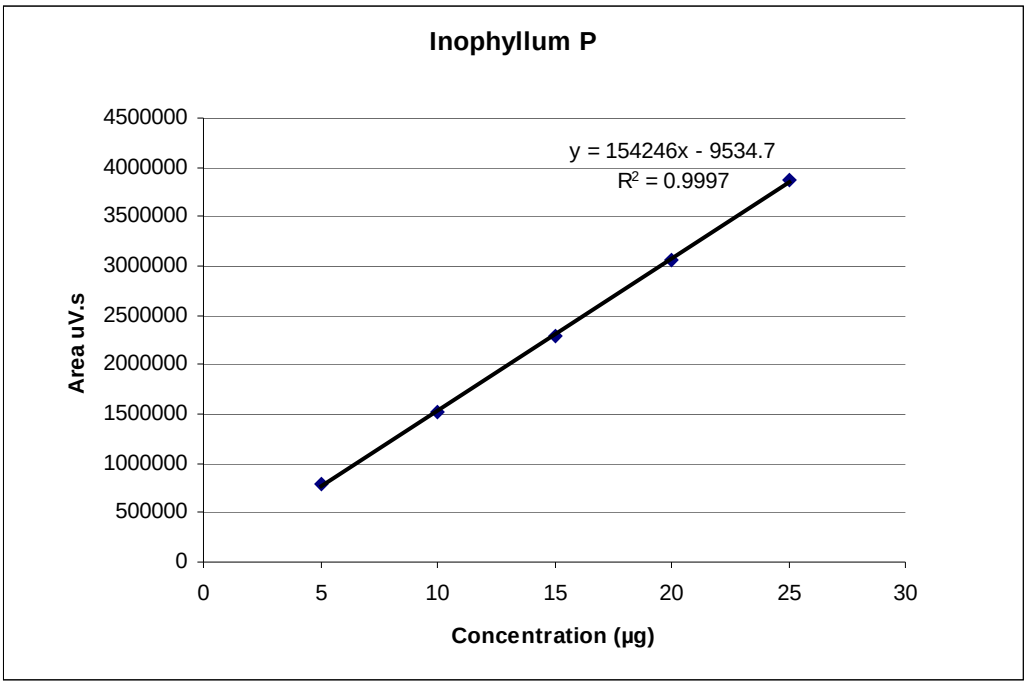


Figure 3.11. Linearity graph and correlation coefficient for inophyllum P

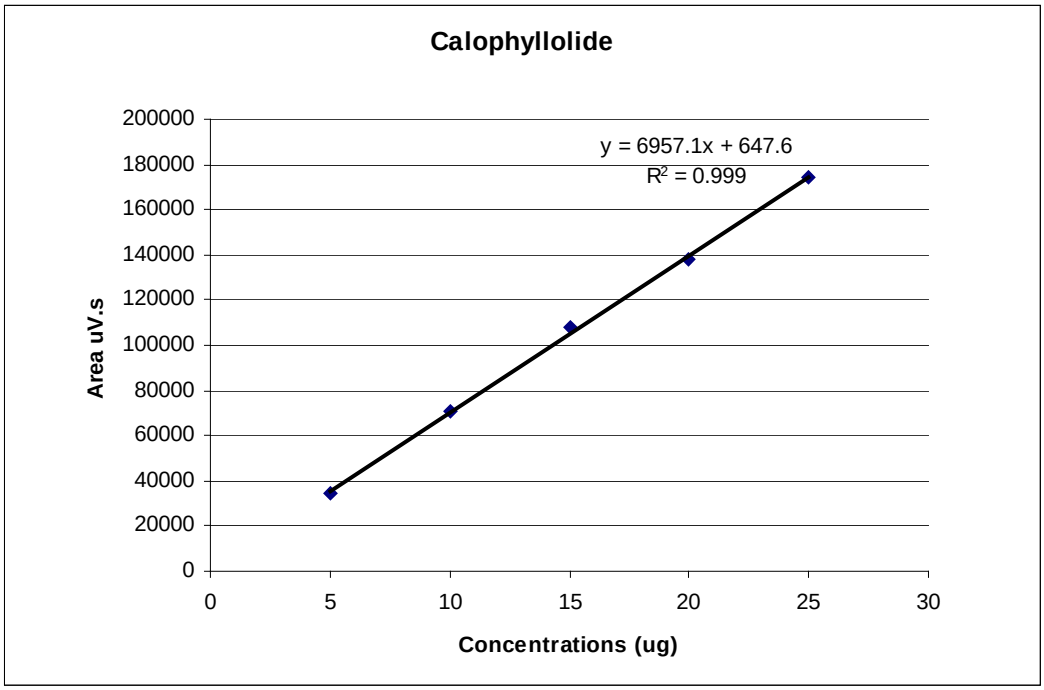


Figure 3.12. Linearity graph and correlation coefficient for calophyllolide

Table 3.2. Standard linearity data of dipyranocoumarins.

Dipyrnocoumarin/ µg injected	Mean Peak Area	Slope (m)	Intercept (c)	R^2
Inophyllum A				
5	70960			
10	1422435			
15	2235164	146235	2816	0.9991
20	2951249			
25	3632154			
Inophyllum B				
5	749304			
10	1496258			
15	2326518	152032	7960	0.999
20	2984575			
25	3805948			
Inophyllum C				
5	341495			
10	682992			
15	1017782	65953	17018	0.9993
20	1314997			
25	1674326			
Inophyllum D				
5	177834			
10	365559			
15	534414	35688	2271	0.9998
20	714678			
25	895476			
Inophyllum P				
5	785811			
10	1516581			
15	2285423	154246	9534	0.9997
20	3065423			
25	3867542			
Calophyllolide				
5	34122			
10	70882			
15	107590	6957	647	0.999
20	137872			
25	174554			

Table 3.3. Repeatability and reproducibility data for retention times, areas and heights.

Inophyllum A	Day I	Day II	Day III	% RSD Inter day	% RSD Intra day
R.T.	13.26 ± 0.07	13.29 ± 0.04	13.28 ±0.02	0.041	0.06
Area	1440342±59388	1426127±50363	1380215±42032	0.73	1.01
Height	18619±802	15945±1159	15417±725	3.43	1.56
Inophyllum B					
R.T.	9.22±0.03	9.25±0.03	9.19±0.02	0.12	0.11
Area	1510343±24263	1573484±54407	1556641±38107	0.70	1.15
Height	30590±937	30030±633	32170±1279	1.19	0.70
Inophyllum C					
R.T.	8.02±0.05	8.03±0.04	8.02±0.10	0.02	0.22
Area	682397±26160	658911±20730	657817±28206	0.69	1.27
Height	52386±1705	50520±1830	49913±862	0.84	1.08
Inophyllum D					
R.T.	8.97±0.02	8.94±0.03	8.97±0.05	0.07	0.19
Area	347875±11154	361529±16591	320383±14693	2.03	1.52
Height	25879±782	27439±1094	24681±1026	1.77	1.38
Inophyllum P					
R.T.	7.31±0.02	7.35±0.03	7.35±0.02	0.09	0.20
Area	1538561±42198	1564491±42926	1557274±31712	0.28	1.09
Height	184397±4463	186205±4493	184721±4361	0.17	0.41
Calophyllolide					
R.T.	4.94±0.03	4.95±0.03	4.92±0.02	0.11	0.20
Area	72109±2367	70784±1672	71084±1365	0.32	1.09
Height	18143±225	18049±206	18023±106	0.11	0.41

The values shown are mean ± SD (n=3); RSD- Relative Standard Deviation
R. T.- Retention Time, (min); Area- µV.s; Height- µV.

3.3.2.2. Precision:

Precision of HPLC method was determined as intra-day repeatability and inter-day reproducibility for retention factors (retention times), peak areas and peak heights. The repeatability was estimated for three determinations at 1.0 mg/ml of each of dipyranocoumarins whereas inter-day reproducibility was estimated for three determinations on three consecutive days. The % RSD values for retention times, peak areas and peak heights for each of dipyranocoumarins have been given in Table 3.3.

Inter-day and intra day RSD values of retention times for inophyllum A, 0.04 % and 0.06%; inophyllum B, 0.12% and 0.11%; inophyllum C, 0.02% and 0.22 %; inophyllum D, 0.07% and 0.19%; inophyllum P, 0.09% and 0.20%; calophyllolide, 0.11% and 0.20% respectively were obtained (Table 3.3). Inter-day and intra day RSD values of peak areas for inophyllum A, 0.73% and 1.01%; inophyllum B, 0.70% and 1.15%; inophyllum C, 0.69% and 1.27%; inophyllum D, 2.03% and 1.52%; inophyllum P, 0.28% and 1.09%; calophyllolide, 0.32% and 1.09% respectively were obtained (Table 3.3), whereas, inter-day and intra day RSD values of peak heights for inophyllum A, 3.43% and 1.56%; inophyllum B, 1.19% and 0.70%; inophyllum C, 0.84% and 1.08%; inophyllum D, 1.77% and 1.38%; inophyllum P, 0.17% and 0.41%; calophyllolide, 0.11% and 0.41% were noted respectively

Overall, the intra day % RSD values obtained were <0.22%, for retention factors, <1.52% for peak areas and <1.56 for peak height were %; whereas inter-day RSD values obtained were <0.12% for retention factors, <2.03% for peak areas and 3.43% for peak height (Figure 3.3).

3.3.2.3. Recovery:

Method accuracy was calculated by spiking the predetermined samples with standard stock solutions to get samples with spiked concentration range 0.5-2.0 mg/g. Three injections of each preparations were made and the average percentage analyte recovered in the spiked solutions were calculated. At the spiked concentration range 0.5-2.0 mg/g, recoveries were in the ranges of 97.86-99.94 % for inophyllum A; 96.06- 100.16 % for inophyllum B; 95.36 – 100.18 % for inophyllum C, 97.86 – 100.3 % for inophyllum D, 93.4 – 96.5% for inophyllum P and 96.30 – 100.33 % for calophyllolide (Table 3.4).

Table 3.4. Recoveries of dipranocoumarins.

Final concentration (mg/g)	Recoveries (percentage ± RSD)					
	Inophyllum A	Inophyllum B	Inophyllum C	Inophyllum D	Inophyllum P	Calophyllolide
0.5	97.86±0.51	100.01±0.19	96.10±0.36	97.86±0.56	95.2±0.90	96.30±0.75
1.0	98.36±0.42	97.80±0.29	100.18±0.09	100.20±0.36	96.5±0.80	99.96±0.56
1.5	99.94±0.21	100.16±0.11	95.36±0.86	98.10±0.63	93.4±0.60	100.33±0.18
2.0	98.93±0.77	96.06±0.5	96.81±0.64	100.3±0.13	94.2±0.9	98.73±0.17

The values shown are mean ± SD (n=3); RSD- Relative Standard Deviation

3.3.2.4. Limit of detection (LOD) and quantification (LOQ):

The LOD was taken as the lowest absolute concentration of analyte in the sample that could be detected, and LOQ was taken as the lowest absolute concentration of analyte in the sample that could be quantified. The LODs were 0.036 µg/ml for inophyllum A; 0.075 µg/ml for inophyllum B; 0.0086 µg/ml for inophyllum C; 0.0065 µg/ml for inophyllum D; 0.056 µg/ml for inophyllum P and 0.045µg/ml for calophyllolide. The LOQs were 0.1 µg/ml for inophyllums A and B; 0.03 µg/ml for inophyllum C; 0.02 µg/ml for inophyllum D; 0.18 µg/ml for inophyllum P and 0.1 µg/ml for calophyllolide (Table 3.5).

Table. 3.5. Limit of detections and quantification of dipyranocoumarins

Compound	LOD	LOQ
Inophyllum A	0.036 µg/ml	0.1 µg/ml
Inophyllum B	0.075 µg/ml	0.1 µg/ml
Inophyllum C	0.0086 µg/ml	0.03 µg/ml
Inophyllum D	0.0065 µg/ml	0.02 µg/ml
Inophyllum P	0.056 µg/ml	0.18 µg/ml
Calophyllolide	0.045µg/ml	0.1 µg/ml

These results obtained for method validation parameters suggested that the method is suitable for both qualitative and quantitative analysis of dipyranocoumarins like inophyllums A, B, C, D, P, and calophyllolide.

3.3.3. Chemical diversity

3.3.3.1. Extraction:

As *C. inophyllum* produces good quality timber, Farmers and Fishermen cut this plant on large scale because of which very few and isolated plants were observed growing at several locations along the Western Ghats of India. For diversity study, we could collect plant material from only 20 locations (Table 3.6).Out of these, 12 locations were from Maharashtra state, 4 from Karnataka state, 3 from Kerala state and one from Goa state. Initially for the extraction of seed kernel, several solvents like acetone, methanol, chloroform, dichloromethane were tried. Out of these solvents, acetone resulted in less complexity and best extraction of the dipyranocoumarins from the kernels for HPLC analysis. HPLC analysis of seeds extracted with acetone resulted in best baseline separation of dipyranocoumarins under study (Fig. 3.13), whereas rest of the solvents tried resulted in much complex pattern and minimum separation on HPLC analyses (Fig 3.14, 3.15, 3.16).

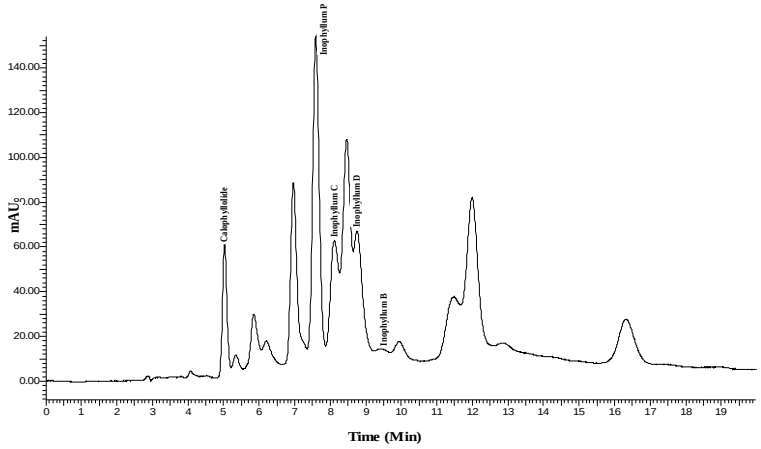


Figure 3.13. HPLC profile of seed extract extracted with acetone.

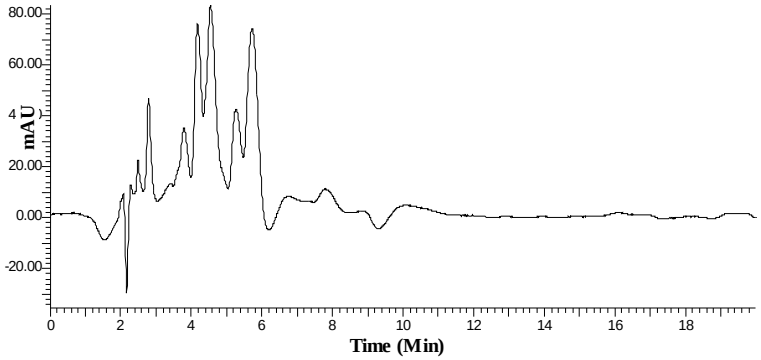


Figure 3.14 HPLC profile of seed extract extracted with methanol

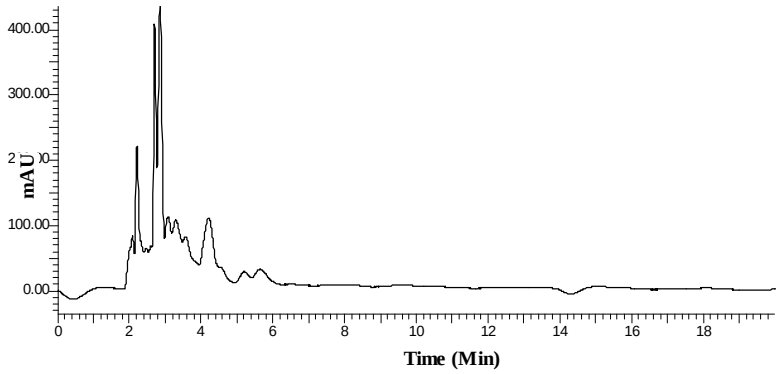


Figure. 3.15 HPLC profile of seed extract extracted with chloroform.

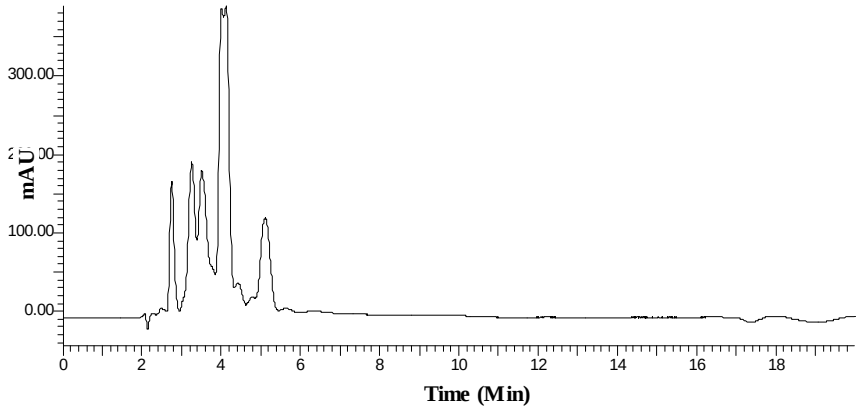


Figure 3.16 HPLC profile of seed extract extracted with dichloromethame.

In the present study, HPLC method developed and validated with Water's μ Porasil column and ethyl acetate and petroleum ether (25:75) as a mobile phase was found to be most suitable for separation and analysis of dipyranocoumarins in seed kernel and was used.

3.3.3.2. Content of dipyranocoumarins in seed kernel:

Quantitative chromatographic analysis of acetone extracts of seed kernels collected from 13 different locations showed abundant variation in yields of acetone extracts, contents of individual dipyranocoumarins and total dipyranocoumarins. HPLC profiles of the seeds collected form different locations revealed that individual dipyranocoumarins were present in all the seeds collected from almost all the locations**.** Only in locations Allapuzza 1 and 2, inophyllums A and B were absent and rest of the dipyranocoumarins were present either in negligible or trace quantities.

Table 3.6. Accession code, Location, Latitude, Longitude, Altitude and material collected.

ACC Code	Collection Site/ Location	Latitude	Longitude	Elevation MSL	Material Collected
A	Kihim	18º 43' .307 N	72º 51' .924 E	00	Friuts, Leaves
B	Agardanda	18º 17' .260 N	75º 59' .386 E	01	Friuts, Leaves
C	Harne	17º 49' .630 N	73º 05' .650 E	01	Friuts, Leaves
D	Anjarle	17º 45' . 357 N	73º 07' .330 E	02	Friuts, Leaves
E	Ladghar	17º 43' .589 N	73º 09' .265 E	03	Friuts, Leaves
F	Burcondi	17º 42l' .911 N	73º 08' .650 E	00	Friuts, Leaves
G	Kalavali	16º 97' .040 N	73º 28' .039 E	01	Friuts, Leaves
H	Vijaydurga	16º 3l' .991 N	73º 19' .994 E	04	Friuts, Leaves
I	Cuncolim	15º 10' .018 N	74º 00' .602 E	09	Friuts, Leaves
J	Karwar	14º 46' .145 N	74º 11' .399 E	11	Friuts, Leaves
K	Cheppada	09º 13' .898 N	76º 28' .380 E	32	Friuts, Leaves
L	Allapuzza 1	09º 28' .982 N	76º 19' .389 E	07	Friuts, Leaves
M	Allapuzza 2	09º 36' .612 N	76º 21' .851 E	10	Friuts, Leaves
N	Bangalore	12º 57' .039 N	77º 35' 014 E	921	Leaves
O	Dabhol	18º 10' .237 N	72º 49' .378 E	02	Leaves
P	Malvan	15º 52' .079 N	73º 29'. 487 E	05	Leaves
Q	NCL Pune	15º 20' .036 N	76º 27' .437E	415	Leaves
R	Panhala	16º 48' .049 N	74º 06' .093 E	923	Leaves
S	Ratnagiri	17º 09' .513 N	73º 16' .206 E	02	Leaves
T	Thiruvanathpuram	08º 23' .579 N	76º 58' .471 E	13	Leaves

Highest inophyllum B, 15.55 mg ⁄ 100 g seed, and 8.780 mg ⁄ 100 g seed were estimated in seeds from locations Kalavali and Harne respectively (Table 3.7). These two locations were also estimated with highest total dipyranocoumarins (23.02 and 20.19 mg ⁄ 100 g seed) and yield of acetone extracts (30.35 and 38.93 g ⁄ 100 g seed) (Table 3.7). Although, location Harne was estimated with 8.780 mg inophyllum B ⁄ 100 g seed, which is less than 15.55 mg inophyllum B ⁄ 100 g seed estimated in location Kalavali, location Harne was also estimated with highests of other dipyranocoumarins (inophyllum A, 5,49 mg ⁄ 100 g seed**,** inophyllum C, 3. 510 mg ⁄ 100 g seed, inophyllum D, 0.907 mg ⁄ 100 g seed and calophyllolide 1.510 mg ⁄ 100 g seed) and yield of acetone extract (38.93 g ⁄ 100 g seed) (Table 3.7). Because location Harne and Kalavali were estimated with highest dipyranocoumarins, total dipyranocoumarins and yield of acetone extract, we concluded these two locations as locations of elite plants.

Overall in all 13 locations, inophyllum A varied in the range of 0 – 5.49 mg ⁄ 100 g seed, inophyllum B in the range of 0 – 15.55 mg ⁄ 100 g seed, inophyllum C in the range of trace – 3.51 mg ⁄ 100 g seed, inophyllum D in the range of trace – 0.907 mg ⁄ 100 g seed and calophyllolide 0.000019 – 1.510 mg ⁄ 100 g seed; whereas total dipyranocoumarins content yield of acetone extract varied in the ranges of 0.00106 – 23.02 mg ⁄ 100 g seed and 2.60 – 38.93 g ⁄ 100 g seed respectively (Table 3.7).

Table 3.7. Variation in dipyranocoumarins contents in seeds of *Calophyllum inophyllum* collected from different locations along the Western Ghats of India.

Acc code	g % acetone extract	Inophyllum A	Inophyllum B	Inophyllum C	Inophyllum D	Calophyllolide	Total dipyranocoumarin
	14.13±2.15	0.101±0.05	1.540±0.62	0.260±0.08	0.017±0.006	0.142±0.07	2.06
	02.60±0.52	0.073±0.009	0.022±0.003	0.036±0.02	0.009±0.004	0.016±0.006	0.156
	38.93±9.21	5.490±2.54	8.780±2.36	3.510±1.52	0.907±0.25	1.510±0.85	20.19
	07.54±3.41	0.104±0.06	0.034±0.0013	0.056±0.022	0.044±0.018	0.026±0.011	0.264
	04.68±1.51	0.071±0.008	0.004±0.001	0.526±0.14	0.670±0.23	1.370±0.62	2.641
	17.25±10.32	0.368±0.056	0.128±0.09	0.209±0.10	0.166±0.068	0.099±0.032	0.97
	30.35±12.54	3.940±0.98	15.55±6.32	2.980±1.65	$1.5 X10^{-4}$±$5.2 X 10^{-5}$	0.559±0.32	23.02
	10.29±4.22	0.00089±$2.1 X10^{-5}$	$9.7 X 10^{-5}$±$3.1 X 10^{-5}$	$1.99 X10^{-5}$±$8.7 X 10^{-6}$	$3.61 X 10^{-5}$±$1.1 X 10^{-5}$	$1.96X 10^{-5}$ ± $1.2X 10^{-5}$	0.00106
	15.00±6.30	0.218±0.064	3.500±1.15	0.269±0.11	trace	0.478±0.19	4.465
	15.39±5.91	$4.9 X10^{-4}$± $2.51 X10^{-5}$	$1.9 X 10^{-5}$±$4.6 X 10^{-6}$	0.437±0.19	0.152±0.08	0.120±0.06	0.709
	14.54±4.31	0.060±0.008	0.236±0.08	0.034±0.14	0.094±0.022	0.325±0.12	0.749
	23.10±14.54	nil	nil	0.456±0.21	0.125±0.03	0.130±0.11	0.711
	13.48±8.20	trace	6.28 X 10-$^{-5}$±1.1X 10^{-5}	$5.31 X 10^{-5}$±4.6 X 10	$2.54 X10^{-5}$±1.0 X 10	0.054±0.045	0.0541

Values in g % for acetone extract (g per 100 g seed kernel), and mg% for dipyranocoumarins mg per 100 g seed kernel). Values are mean of three replicates. Acc. Codes as per Table 3.6.

McKee et al., (1998) conducted the chemotaxonomic study of extracts of tropical species of genus *Calophyllum.* The study indicated that there were several distinctive coumarins chemotaxonomic markers distinguishing species of genus *Calophyllum*. In *C. inophyllum,* quantitative HPLC analyses of dipyranocoumarins and chemical fingerprinting can have chemotaxonomic significance. Li et al., (2007) studied the similar type of chemical fingerprinting of alkaloids in *Corydalis saxicola*. Five alkaloids were simultaneously determined with reverse phase HPLC in different plant parts of wild and cultivated *C. saxicola* to investigate difference between chemical profiles of wild and cultivated *C. saxicola* populations.

3.3.4. Genetic diversity

3.3.4.1. DNA extraction:

For the isolation of genomic DNAs from leaf samples of *C. inophyllum*, initially several protocols like Doyle and Doyle method (1987), Murray and Thompson's method (1980), Lodhi's CTAB method (1994), Khanuja's method (1999) were tried. Difficulties were encountered from the stages of cell lysis to DNA separation in case of procedures described by Doyle and Doyle (1987), Murray and Thompson (1980) and Lodhi (1994). Low DNA yield, poor quality with contaminations of RNA and proteins were the problems encountered with these protocols. Problems were also encountered because of high phenolic contents in *C. inophyllum*. This was taken care by using PVP in extraction buffer. Moreover yield and quality of DNA depended on condition and storage period for storing leaf samples. Fresh leaf samples yielded DNAs with good quantity and quality. Samples stored at –70º C for more than two years yielded minimum quantities of DNA. Khanuja's DNA extraction protocol (1999) yielded better quality and quantity of DNA. Only modification in this protocol was the use of TNE buffer (Tris, NaCl, EDTA), instead of high salt TE buffers (described in Khanuja's DNA extraction protocol) for dissolving DNA pellets before RNAse A tratement. This modification resulted in further increase in quality and quantity of DNAs. Out of 20 samples of DNA extracted, five DNA samples were used for optimization of PCR conditions, screening primers and optimization of annealing temperatures of primers.

3.3.4.2. PCR amplification and ISSR analysis:

Various parameters like template DNA concentration (20, 40, 60, 80, 100 ng / reaction) and *Taq* polymerase (0.2, 0.4, 0.6, 0.8, 1.0 U/ reaction), $MgCl_2$ (1.0. 1.5, 2.0, 2.5, 3.0mM/ reaction), dNTP's (0.1, 0.2, 0.3, 0.4 mM/ reaction) were tested to optimize PCR conditions. These parameters were tested in five randomly chosen DNA samples using ISSR primer. For clear and reproducible amplification and banding pattern, 2.5 µl of 10X *Taq* buffer B (without $MgCl_2$), 2.5

mM $MgCl_2$, 0.2 mM dNTPs, 0.6 U of *Taq* polymerase, 0.24 µM of primers and 80ng of template DNA were the optimum concentrations per 25 µl reaction volume. For PCR reaction, parameters like extension (synthesizing/ polymerization) temperature and time were optimized.

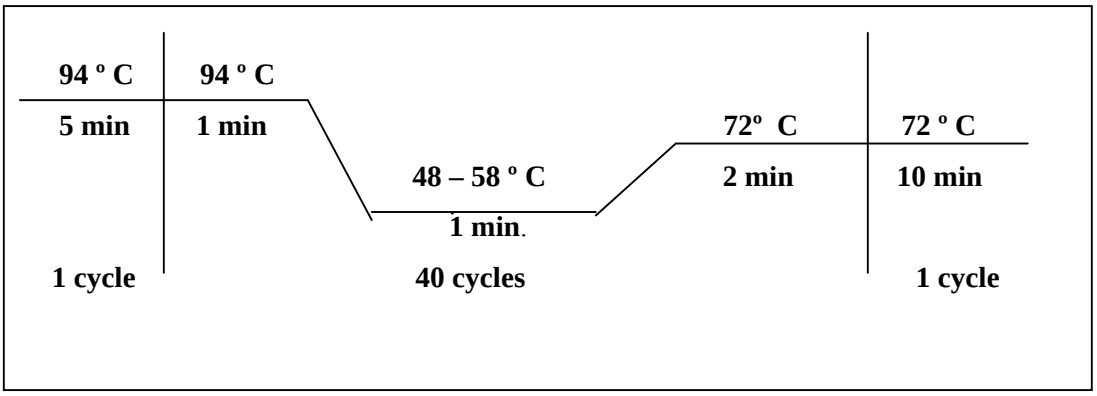


Figure 3.17 PCR thermal cycling conditions

Best DNA extension was resulted when synthesis temperature was set at 72º C for 10 min. The rapid and fast optimization of annealing temperatures for each of the ISSR primers was facilitated by Stratagene's RoboCycler Gradient PCR machine. With this machine range of the temperature was selected and used to study the effect of different annealing temperatures on amplification. Thermal cycling conditions that consisted of initial denaturation for 5 min. at 94º C, followed by 40 cycles of 1 min. at 94º C, 1 min. at 48 – 58º C (optimized for different primers), 2 min. at 72º C and 10 min. at 72º C for the final extension resulted in clear and reproducible banding patterns. PCR thermal cycling conditions have been given in Figure 3.17.

Out of 100 ISSR primers screened initially, genomic DNA was amplified by 26 ISSR primers, 8 of which did not yield reproducible bands and 5 primers did not show any discernible polymorphism. Remaining 13 primers generated 139 bands ranging in a size from 290 – 4190 bp, with an average of 10.69 bands per primer. Of these bands 41 % i. e. 54 in total were polymorphic (Table 3.8). With all the 13 primers used to distinguish between samples from 20 locations, PPBs ranged from 20 – 66.66%. Of all the primers, UBC 844 generated highest (66.66%) PPB. ISSR successfully identified 38.68 % polymorphism. The results of ISSR in *C. inophyllum* suggested that di nucleotide repeat motifs occur at high frequencies while tri, tetra and penta nucleotide appear less frequent along the *C. inophyllum* genome. Agarose gel electrophoretic patterns generated with each of 13 primers with genomic DNAs of 20 leaf samples from 20 locations have been given in figures 3.18 to 3.30.

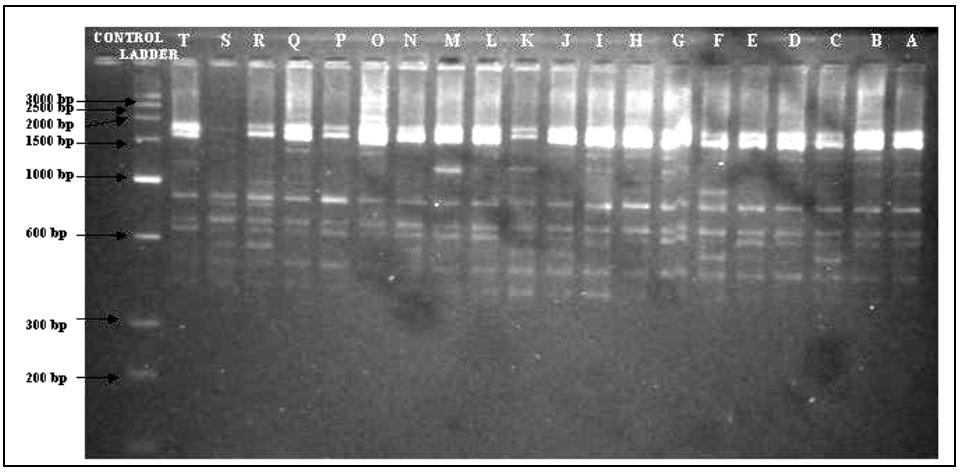


Fig. 3.18 Agarose gel electrophoretic pattern of amplification products obtained with 20 trees using the primer 807 at annealing temperature 50º C. Lane A-T: Trees in order.

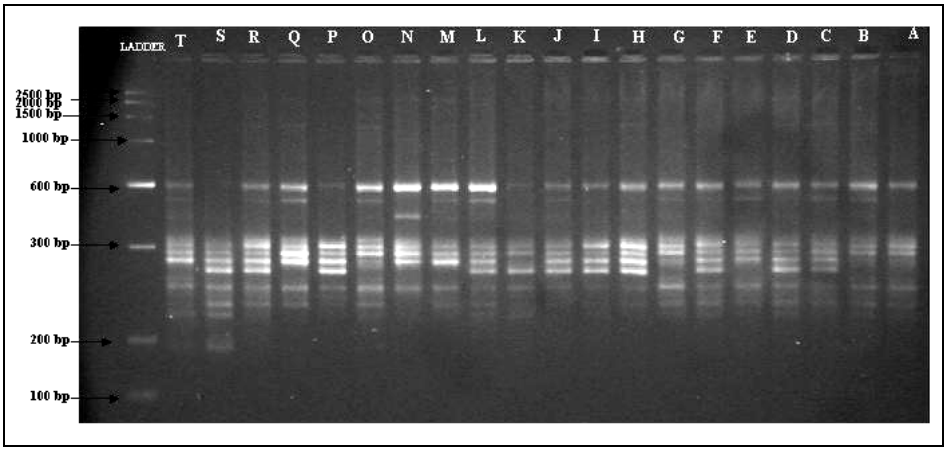


Fig. 3.19 Agarose gel electrophoretic pattern of amplification products obtained with 20 trees using the primer 812 at annealing temperature 51º C. Lane A-T: Trees in order.

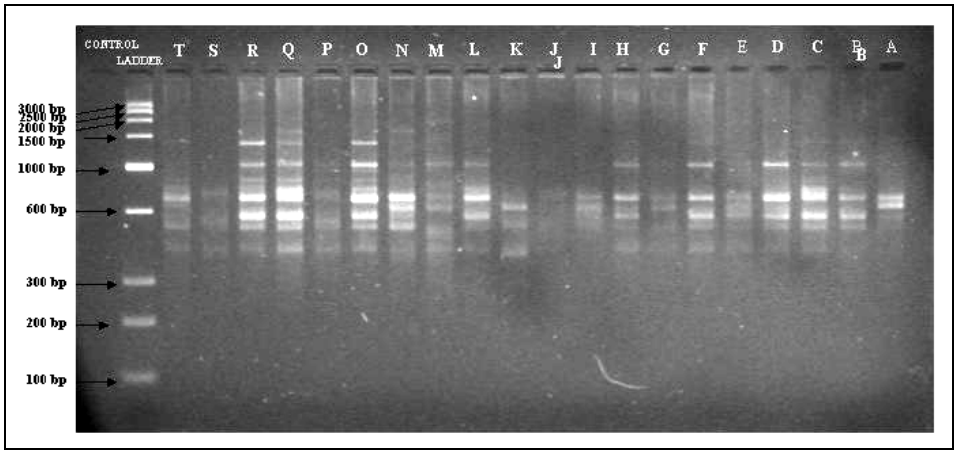


Fig. 3.20 Agarose gel electrophoretic pattern of amplification products obtained with 20 trees using the primer 817 at annealing temperature 50º C. Lane A-T: Trees in order.

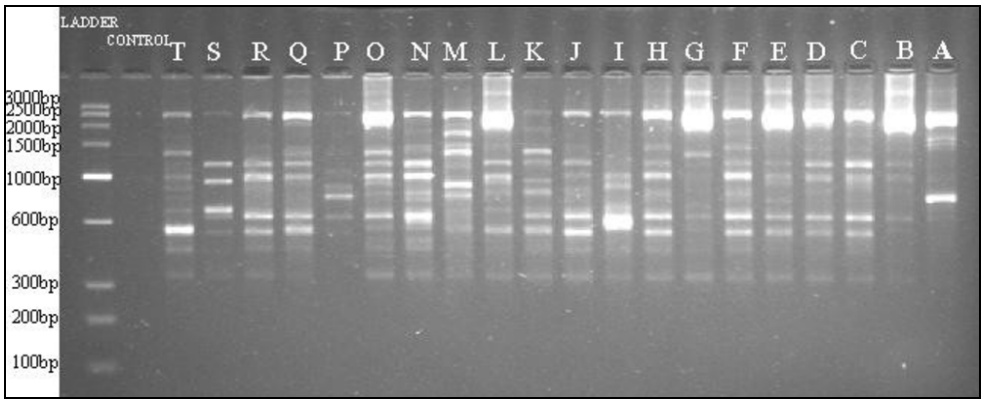


Fig 3.21 Agarose gel electrophoretic pattern of amplification products obtained with 20 trees using the primer 826 at annealing temperature 56º C. Lane A-T: Trees in order

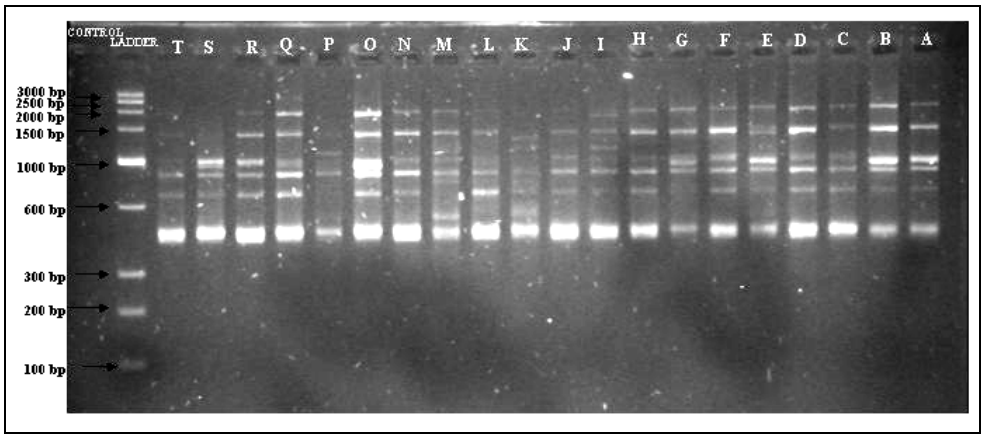


Fig. 3.22 Agarose gel electrophoretic pattern of amplification products obtained with 20 trees using the primer 824 at annealing temperature 52º C. Lane A-T: Trees in order

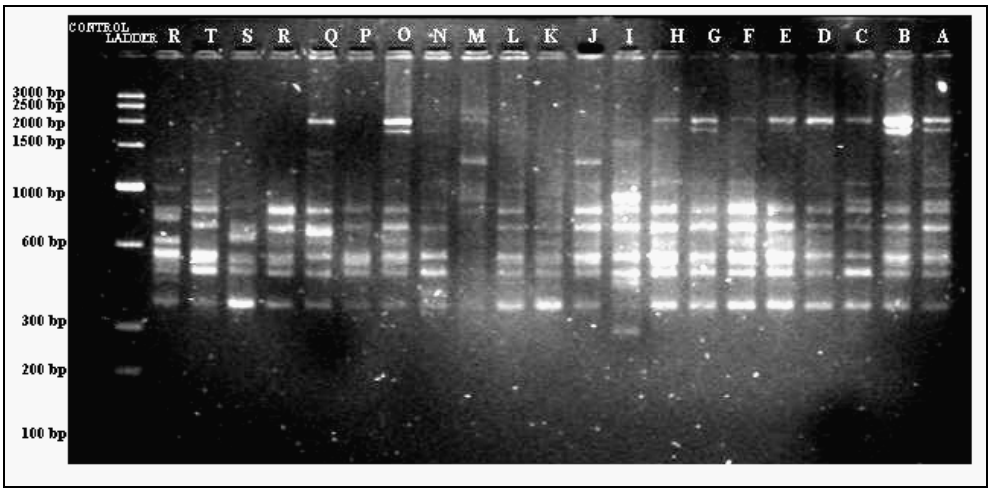


Fig. 3.23 Agarose gel electrophoretic pattern of amplification products obtained with 20 trees using the primer 830 at annealing temperature 56º C. Lane A-T: Trees in order

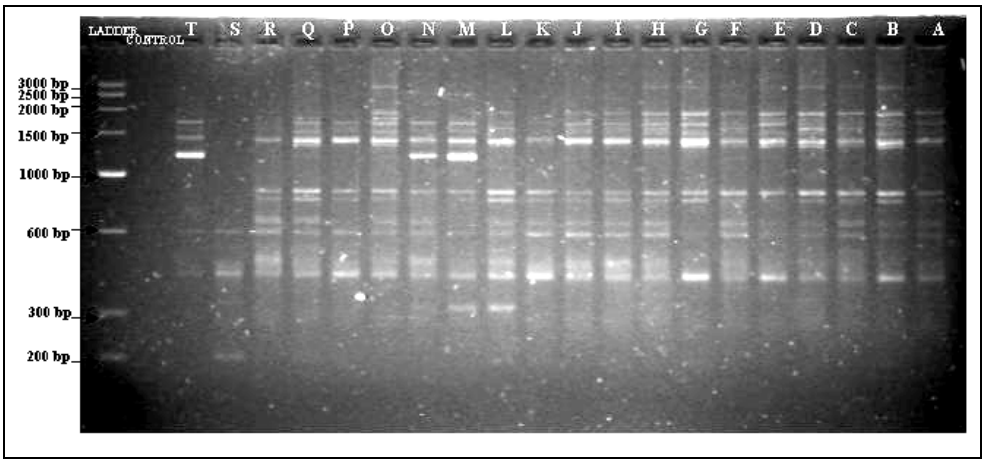


Fig. 3.24 Agarose gel electrophoretic pattern of amplification products obtained with 20 trees using the primer 836 at annealing temperature 53º C. Lane A-T: Trees in order

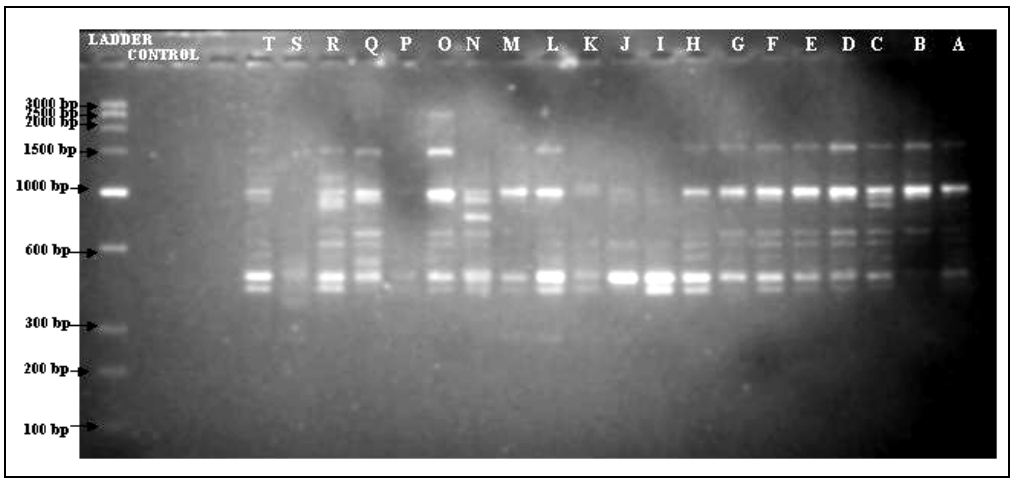


Fig.3.25. Agarose gel electrophoretic pattern of amplification products obtained with 20 trees using the primer 841 at annealing temperature 48º C. Lane A-T: Trees in order

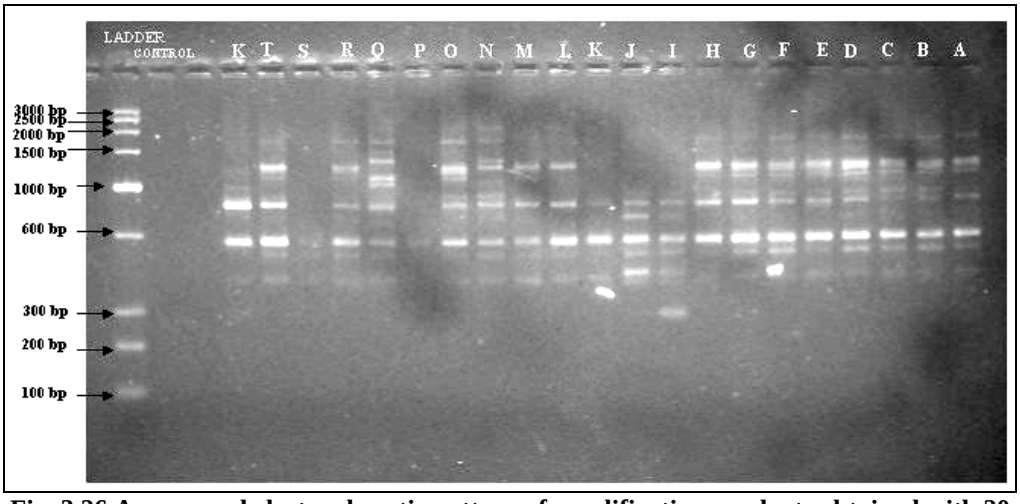


Fig. 3.26 Agarose gel electrophoretic pattern of amplification products obtained with 20 trees using the primer 842 at annealing temperature 48º C. Lane A-T: Trees in order

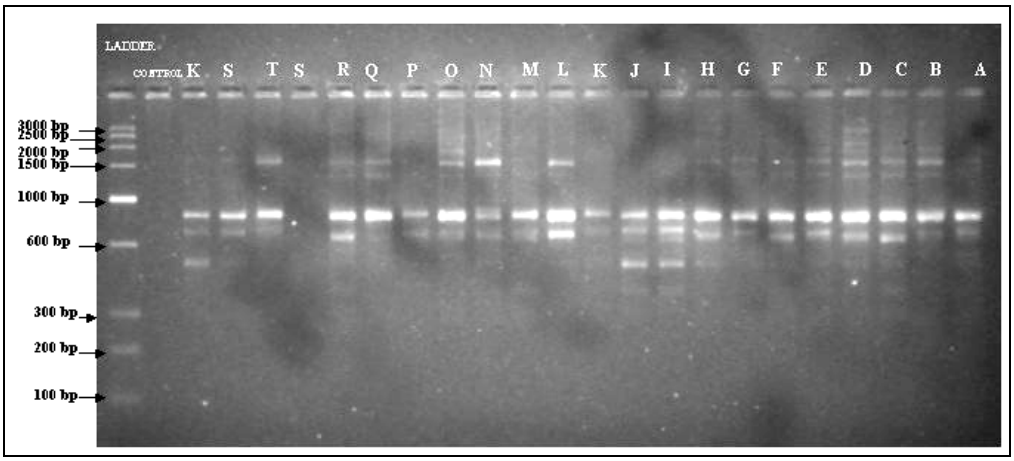


Fig. 3.27 Agarose gel electrophoretic pattern of amplification products obtained with 20 trees using the primer 844 at annealing temperature 48º C. Lane A-T: Trees in order.

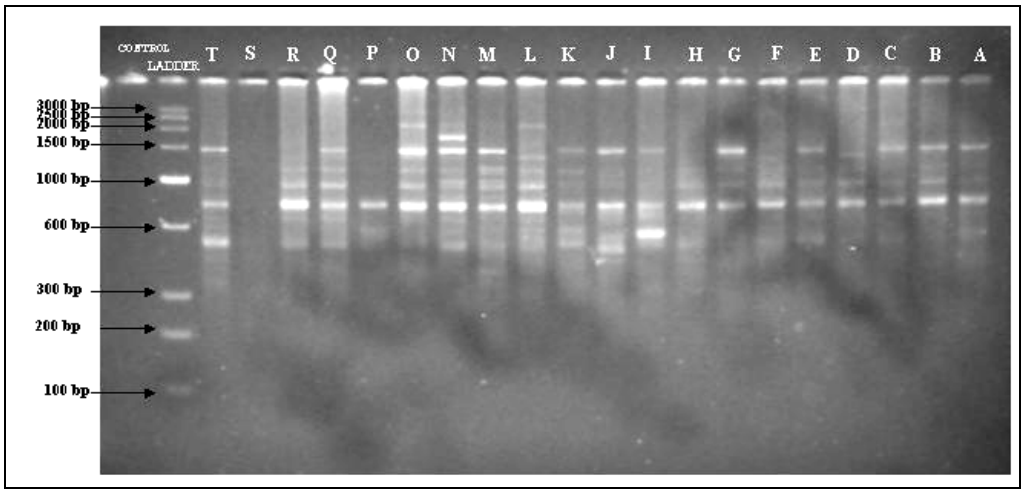


Fig. 3.28 Agarose gel electrophoretic pattern of amplification products obtained with 20 trees using the primer 845 at annealing temperature 52º C. Lane A-T: Trees in order

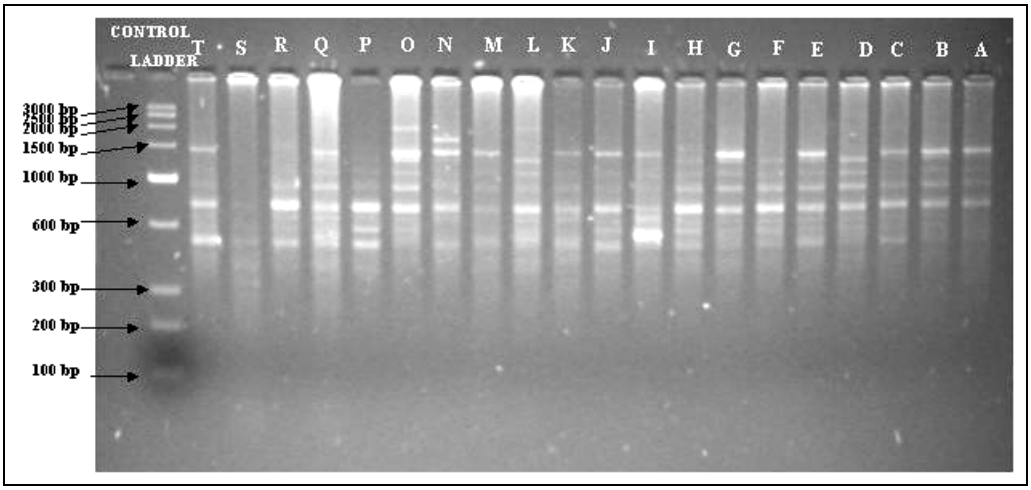


Fig.3.29 Agarose gel electrophoretic pattern of amplification products obtained with 20 trees using the primer 847 at annealing temperature 52º C. Lane A-T: Trees in order

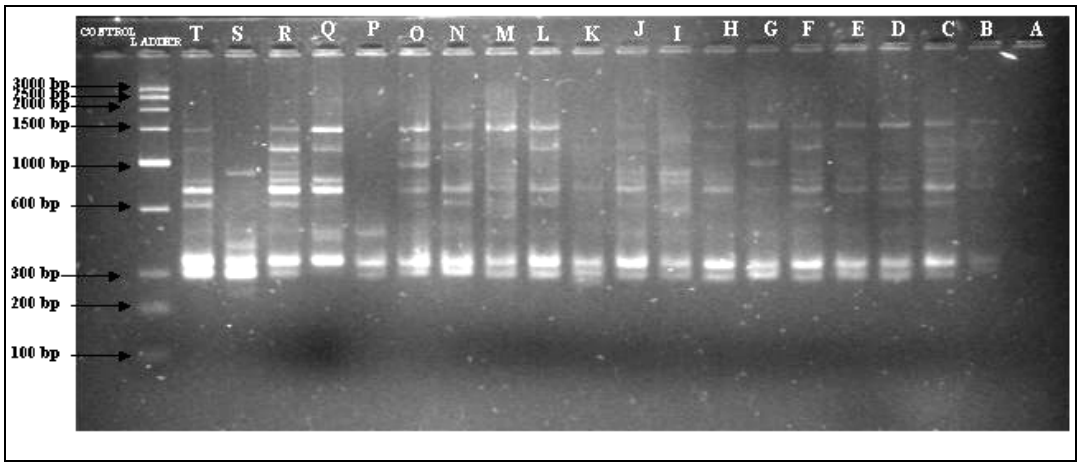


Fig.3..30 Agarose gel electrophoretic pattern of amplification products obtained with 20 trees using the primer 857 at annealing temperature 55º C. Lane A-T: Trees in order.

Table 3.8. ISSR primers, annealing temperatures optimized, No of bands scored, No of polymorphic bands scored and percent polymorphism per primer.

ISSR Primer	Primer sequence	Annealing temp. ^{0} C	No. of bands Scored	No. of polymorphic bands scored	Band size range (bp)	PPB
UBC 807	AGA GAG AGA GAG AGA GT	50	11	5	390 - 1700	45.45
UBC 812	GAG AGA GAG AGA GAG AA	51	09	2	370 – 1000	22.22
UBC 817	CAC ACA CAC ACA CAC AA	58	09	4	760 – 2150	44.44
UBC 824	TCT CTC TCT CTC TCT CG	52	08	2	460 – 1950	25.00
UBC 826	ACA CAC ACA CAC ACA CC	56	16	7	340 – 4190	43.75
UBC 830	TGT GTG TGT GTG TGT GG	56	12	7	290 – 2050	58.33
UBC 836	AGA GAG AGA GAG AGA GYA	53	13	3	310 – 2910	23.07
UBC 841	GAG AGA GAG AGA GAG AYC	48	12	4	420 – 2440	33.33
UBC 842	GAG AGA GAG AGA GAG AYG	48	11	3	300 – 2060	27.27
UBC 844	CTC TCT CTC TCT CTC TRC	48	09	6	540 – 2690	66.66
UBC 845	CTC TCT CTC TCT CTC TRG	52	09	3	480 – 1950	33.33
UBC 847	CAC ACA CAC ACA CAC ARC	52	10	2	450 – 2200	20.00
UBC 857	ACA CAC ACA CAC ACA CYG	55	10	6	310 - 1470	60.00

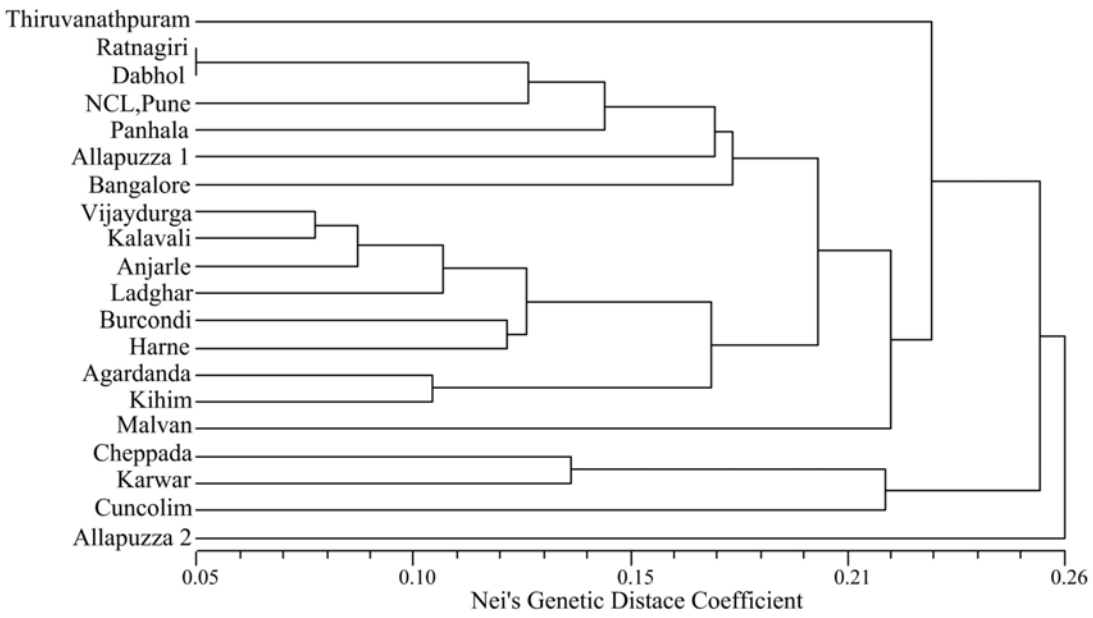


Fig. 3.31. UPGMA Dendrogram based on Nei's genetic distance coefficient from ISSR data of 20 *Calophyllum inophyllum* locations along the Western Ghats of India.

ISSR data of 20 locations was used to construct UPGMA dendrogram based on Nei's genetic distance coefficient. Same data was also used for principal component analysis. UPGMA dendrogram based on Nei's genetic distance coefficient showed that Nei's genetic distance coefficient ranged between 0.05 – 0.26 (Fig 3.31). UPGMA dendrogram (Fig. 3.31) and principle component analysis (Fig. 3.32) based on ISSR data generated from 13 primers clustered 20 locations into three distinct clusters or groups. Group Ist comprised of locations Kihim, Agardanda, Harne, Anjarle, Ladghar, Burcondi, Kalavali and Vijaydurga, group IInd comprised of locations Dabhol, NCL (Pune), Panhala, Ratnagiri and Thiruvananthpuram while group IIInd comprised of Cuncolim, Karwar, Cheppada, Allapuzza 2 and Malvan (Fig. 3.31, 3.32). Locations clustered in a group Ist are clearly coastal region locations i.e. these locations lie at not more than 4 m from MSL (Table 3.6), whereas the locations from group IInd are relatively high altitude locations. Four out of six locations lie at high altitude among which the plants at the locations Bangalore, NCL (Pune) and Panhala are planted and introduced. Locations of group IIIrd (Cuncolim, Karwar, Cheppada, Allapuzza 2 and Malvan) are from altitudes in the range of 5 – 32 m from mean sea level (Table 3.6). This trend of clustering based on ISSR markers clearly indicated the correlation between geographical position i.e. elevation and genetic differentiation (variation).

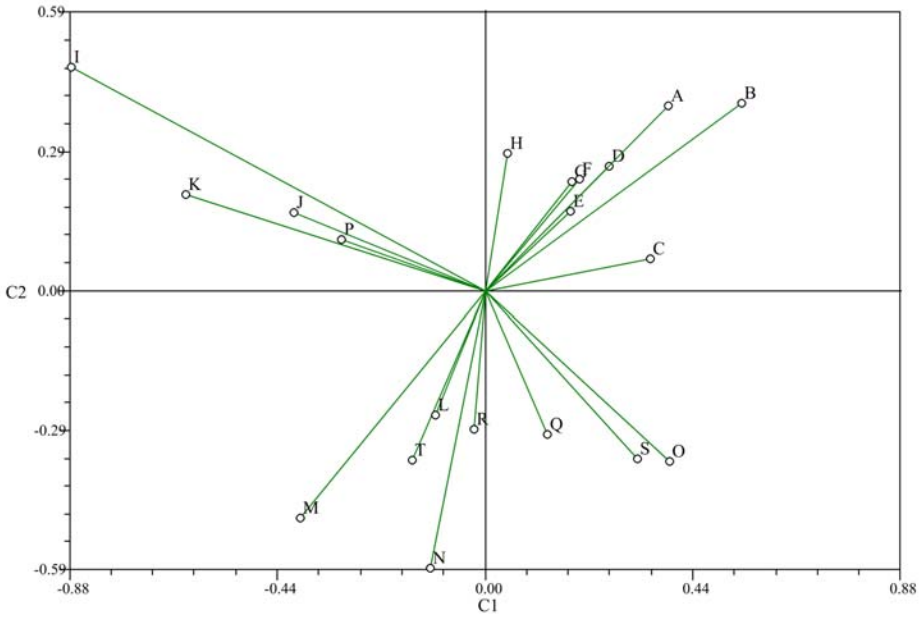


Fig 3.32. Principal Component Analysis based on ISSR data of 20 locations generated from 13 ISSR primers. Accession code as per Table 4.1.

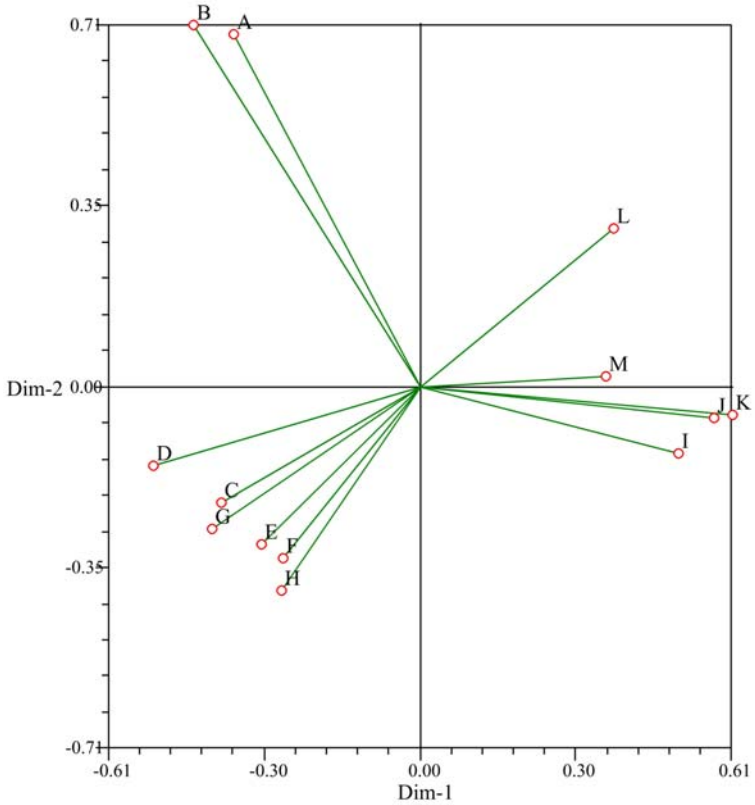


Fig 3.33. Principal Component Analysis based on variation in dipyanocoumarins' and ISSR data of 13 locations generated from 13 ISSR primers. Accession code as per Table 4.1.

3.3.4.3. Principal Component Analysis (PCA):

Data based on dipyranocoumarins variation (inophyllums A, B, C, D, calophyllolide, total dipyranocoumarins and yield of acetone extract) in 13 locations along with ISSR variation in same 13 locations was further used for principal component analysis that provided two-dimensional graphical presentations of correlation between ISSR variation and dipyranocoumarins variation (Fig. 3.33). The clustering based on PCA was quite comparable to the groupings obtained from UPGMA dendrogram. PCA also clustered 13 locations into three groups (Fig. 3.33). Group Ist (Kihim and Agardanda) and group IInd (Harne, Anjarle, Ladghar, Burcondi, Kalavali, Vijaydurga) are low lying coastal region locations having altitude less than 4 m MSL, whereas as group III rd (Cuncolim, Karwar, Cheppada, Allapuzza 1, Allapuzza 2) are the locations lying at the elevation in between 7 – 32 m MSL. Locations Harne and Kalavali which were estimated with highest dipyranocoumarins and concluded as elites were clustered together in group IInd indicating the significant correlation of chemical diversity (dipyranocoumarins content) with genetic variation and ecological factors (Fig 3.33 and Table 3.7). Zhou et al, (2007) also reported similar correlation of content of dauricine with genetic distance and ecological factors in *Menispermum dauricum* DC, while Hu et al (2007) also found close association between genetic variation pattern revealed by molecular markers and chemical constituents in the fruits of *Vitex rotundifolia*. Tori et al., (2008) studied the intra specific diversity in *Liularia vellerea* by chemical and genetic approaches. These reports and present study suggested that chemical (dipyranocoumarins) and genetic (ISSR) approaches could be used to study intra specific diversity in *C. inophyllum.*

3.3.4.4. Conservation implications:

Thus ISSR markers can be employed to study the genetic variation in *C. inophyllum.* Quantitative chemical diversity of dipyranocoumarins along with genetic diversity can be helpful in identifying the elite plants in terms of highest content of medicinally important dipyranocoumarins. Based on this study we identified Harne and Kalavali as a location of elite plants. As a part of our project for *in vitro* conservation of Guttiferrae plants, we colleted plant material from these locations and developed the micropropagation protocol for *C. inophyllum* (Chapter 5). The plant material from the same elite location was also used to develop protocol for callus cultures to study the pattern of anti HIV dipyranocoumarins expression in *in vitro* grown callus cultures (Chapter 6).

3.4 CONCLUSION:

Normal phase HPLC method developed with µ Porasil column and ethyl acetate and petroleum ether, as a mobile phase was best suited for best base line separation and quantifications. As per the results of HPLC method validation (like linearity, precision, recovery, LOD, and LOQ), method was acceptable. This validated method was then used for determinations of dipyarnocoumarins in seed. Chemical diversity study revealed that seeds from locations Harne and Kalavali were estimated with highest dipyranocoumarins, total dipyranocoumarins and yield of acetone extracts. Based on these results, it was concluded that plants from these two locations were elite in terms of highest dipyranocoumarins, total dipyranocoumarins and yield of acetone extracts. Plants from these locations were collected for further *in vitro* experiments.

Khanuja's DNA extraction protocol (1999) yielded good quality and quantities of DNAs from *C. inophyllum* leaves. PCR conditions optimized were the optimum for reproducible and clear amplifications. ISSR successfully identified the variations (38.68%) among the 20 trees. Clustering in dendrogram and PCA based on ISSR variation grouped 20 locations in three groups and indicated correlation between geographical position and genetic differentiation. PCA based on ISSR and chemical diversity of dipyranocoumarins in 13 locations also grouped 13 locations in three groups. This grouping indicated the correlation of chemical variation (dipyranocoumarins' content) with genetic variation and ecological factors.

CHAPTER 4. *IN VITRO* PROPAGATION OF *CALOPHYLLUM INOPHYLLUM*.

4.1 INTRODUCTION

Micropropagation is the practice of rapidly multiplying desired plant material to produce a large number of progeny plants, using modern plant tissue culture methods. Micropropagation is popular and alternative mean of plant vegetative propagation in which in relatively short time and space, a large number of plant can be produced starting from single individual. Micropropagation is used to multiply novel plants, such as those that are medicinally impotant or have been genetically modified or are endangered or threatened plant species. Micropropagation is the true-to-type propagation of a selected genotype using *in vitro* culture techniques. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative propagation. The technique can broadly be divided in four stages:

4.1.1. Stage I - Establishment of culture:

The purpose of stage-I is to initiate axenic culture. This stage begins with the excision of meristem tissue from an identified stock plant and its culturing into appropriate nutrient medium. A rapidly proliferating culture is established under optimum levels of light and temperature.

Because woody plants harbor lot of microflora and contain high levels of phenolic compounds, microbial contamination and interference of phenolic exudates are major constraints in establishing sterile cultures of woody tissues. Contamination in tissue culture can originate from two sources, through carry over of microorganisms on the surface and/or in the tissues of explants or through faulty procedures and improper handling in the laboratory. The explant is treated with anti microbial chemicals to remove contaminating organisms. Use of fungicides and antibiotics limits the microbial infection to some extent. Use of antioxidants like ascorbic acid, PVP and charcoal helps in eliminating the interfering phenolic exudates.

For most micropropagation work, the initial explant must be physiologically competent to survive the initial culture conditions and elicit the appropriate response. In general, the developmental stage of the explant is an important factor. Younger tissues, such as terminal or axillary shoot buds are more responsive than older and mature tissues of the same stem. The initial explant of choice is generally an apical or axillary bud.

For development of micropropagation protocol, success is largely determined by age of stock plant, physiological age of the explant and its developmental stage, as well as its size. Developmental stage of an explant is an important factor for initiation of cultures for propagation. Younger the tissue, better the *in vitro* response. Explants from mature plants are reported to be highly recalcitrant *in vitro* and pose problem in the establishment of cultures due to high degree of contamination in mature tissues. Juvenile explants such as cotyledons, hypocotyls, epicotyls,

embryo axis or buds from seedlings are more responsive in culture than the tissues like bud explants or leaves from mature trees (Ahuja, 1993). Semi mature seed derived explants like cotyledon, embryo axis are juvenile in nature, and, can also be used for micropropagation. Juvenile explants have been extensively employed for clonal propagation of woody plants. Frequency of sprouting is always higher in the buds taken from juvenile stage plants than from mature plant during its vegetative phase. Propagation system using juvenile tissues provides a better understanding on the requirement of the plant for its growth and maturity. Micropropagation from juvenile explants is useful for differentiation studies. Propagation using juvenile tissues can serve as a model system for standardization of protocol with mature tissues (Pierik, 1987). Successful micropropagation of various woody plant species are reviewed extensively and reported (Trigiano et al., 1992; Parrott et al., 1992). In forest tree species, the success has been largely restricted to seedling materials (Rodriguez and Vendrame, 2003).

In many tree species for establishment of propagation protocol seedling derived explants are used. In this method, seeds are germinated *in vitro*. After the seedling attain appropriate size, the nodal explants and the cotyledon nodes are excised from seedlings and are used for *in vitro* propagation. Factors that influence the establishment of culture like seed coat, microbial interference, growth regulators, organic supplements, sucrose concentration, culture vessel, culture conditions for seedling of appropriate size are studied during optimization.

In vitro protocols, using seedling explants, have been employed for rapid propagation and manipulation of woody plants, such as *Albizzia* (Gharyal and Maheshwari, 1981); *Dalbergia latifolia* (Sudhadevi and Nataraja, 1987a,b); *Swartzia madagascariensis* (Berger and Schaffner, 1995); *Acacia tortilis* subsp. *raddiana* (Nandwani, 1995); *Mimosa tenuiflora* (Villarreal and Rojas, 1996); *Acacia mearnsii* (Beck et al., 1998a); *Dalbergia sissoo* (Pradhan et al., 1998); *Bauhinia vahlii* (Bhatt and Dhar, 2000, Upreti and Dhar, 1996); *Leucaena leucocephala* (Saffi and Borthakur, 2002); *Cercis yunnanensis* (Cheong, 2003); *Ceropegia candelaebrum* (Beena et al., 2003); *Acacia mangium* (Monteuuis, 2004); *Sesbania drummondii* (Cheepala et al., 2004); *Peltophorum pterocarpum* (Uddin et al., 2005); *Pterocarpus marsupium* (Anis et al., 2005; Chand and Singh, 2004);

In general, the ingredients of the culture medium, exogenous supply of cytokinins and auxins, their combinations and concentrations in this stage are determined by the kind of response desired e.g. axillary shoot formation or adventitious shoot formation or callusing etc., Exogenous supply of cytokinins like BAP, TDZ, kinetin in media are preferred for axillary shoot formation whereas auxins like NAA, 2, 4-D are desired for callus induction. Carbohydrate as carbon source is one of the important component of the plant tissue culture medium. Sucrose is the most commonly used carbon source in the media. Most of the earlier studies strongly suggested the use

of sucrose as carbon source. Depending on the mineral requirements of different plant species, several media compositions have been formulated. The most commonly used basal medium is Murashige and Skoog's medium, whereas Woody Plant Medium is widely preferred for woody plant species. Details of these media composition are described in chapter 2.

4.1.2. Stage II - Proliferation of shoots in culture:

In this stage, shoots are proliferated in culture medium. For proliferation, culture medium is supplied with auxins and cytokinins. Combinations and concentrations of auxins and cytokinins in culture medium are needed to be optimized. Proliferated shoots then serve as the source of shoots for subsequent propagation as well as the material that is required to maintain the stock. Multiplication of shoots is achieved by sub culturing the shoot cultures at regular intervals in appropriate medium. Variant plants may arise from the cultures maintained *in vitro* for a long period. For the purpose of micropropagation, usage of shoots from the cultures maintained for several passages and having higher subculture numbers is avoided to ensure genetic fidelity.

4.1.3. Stage III – Rooting:

In this stage, roots are induced to the shoots obtained from multiplication media. Shoots are first separated and then shifted to conditions that favor root initiation and shoot elongation. Rooting in shoots can be induced either *in vitro* or *ex vitro*. For rooting *in vitro*, shoots are cultured either in growth regulator free medium or subjected to pulse treatment of auxin prior to transferring to an auxin free medium for root induction. For *ex vitro* rooting, shoots are excised as small cuttings (micro cuttings), treated with commercial rooting mixture and planted in soil or potting mixture. These are then placed in a high humidity chamber for rooting. Rooting *ex vitro* has several advantages (Debergh and Read, 1991):

- It is easier to stick a cutting in soil than to plant a rooted plantlet.
- Labor-intensive *in vitro* operations of single shoots are avoided.
- The root system produced *ex vitro* simultaneously establishes in soil.
- Possibility of damaging the roots while transferring the plant to soil is avoided. These damages cause root or stem diseases.
- For difficult-to-root plants it is easier and cheaper to create appropriate conditions for ex *vitro* rooting.

4.1.4. Stage IV – Hardening of tissue culture raised plants:

Plantlets developed within culture vessel under aseptic condition grow on a medium that contains ample sugar and nutrients to allow for heterotrophic growth and in an atmosphere with

high relative humidity, low levels of light and controlled temperature. All these controlled parameters contribute to a phenotype that cannot survive the environmental conditions when directly placed in green house or field. Because *in vitro* raised plants grow in controlled physical conditions like temperature, humidity and light, before their out planting at final location, they are needed to be hardened. Hardening involves transfer of plantlets from aseptic condition to the green house and ultimately to the final location (environment). Thus it is necessary to gradually acclimatize the plantlets to ensure survival until they develop new leaves that are more adapted to the ambient conditions under which plants are normally grown. High relative humidity has to be maintained during hardening process to protect the plants from desiccation and enable them to initiate new roots and shoots.

In the present study, the experiments were conducted with the juvenile explants from *in vitro* grown sterile seedlings of *Calophyllum inophyllum* to optimize protocol for micro propagation. In this chapter, results on the effect of WPM and MS basal medium on seed germination, effect of BAP and TDZ on multiple shoot induction and elongation and effect of IBA and BAP on *in vitro* root induction are represented and discussed. The optimized protocol then can be extended for large scale micropropagation of *Calophyllum inophyllum* and *in vitro* conservation.

4.2 EXPERIMENTAL PROTOCOL:

4.2.1. Optimization of sterilization protocol:

For effective sterilization and to control fungal contamination in cultures, fungicides like Bavistin (0.1% w/v) and Benomyl (0.5 w/v) were used. Seeds were first treated with Bavistin or Benomyl followed by thorough washing with sterile distilled water (SDW) for 4-5 times. To optimize the concentrations of Bavistin or Benomyl, their concentrations were varied in the range 0.1 – 1.0 %. Treatment time for these fungicides were also optimized by varying treatment times in the range 10 – 60 min. Treatment with fungicides was then followed by treatment with sterilizing agents $HgCl_2$ or NaOCl or and H_2O_2 in different concentrations. After fungicide treatment, explants were treated separately with different surface sterilizing agents such as $HgCl_2$, NaOCl and H_2O_2 for different time period with different concentrations. Treatment time for each of the sterilizing agent tried was varied from 10 to 50 min and concentration range for $HgCl_2$ (0.05 – 4.0 %, w/v), NaOCl (2- 10 % v/v) and H_2O_2 (2- 10 % v/v) were used. Experiments were set with 50 seeds per treatment and each experiment was repeated three times.

4.2.2. Establishment of sterile seedling cultures:

For *in vitro* germination, the mature fruits of *Calophyllum inophyllum* Linn. (Fig1:b) were collected from Harne village (17º 49′63″ N, 73º 05′65″ E; 2 m altitude) near Dapoli, Ratnagiri along the coast from the state of Maharashtra, in the fruiting season during second week of March. Mature fruits were decoated mechanically to remove the hard, stony endocarp and the seeds were surface sterilized using the procedure described in Chapter 2. Surface sterilized seeds were presoaked in SDW and / or Gibberellic acid solution (GA_3; 0.058 µM) and / or heated at 35°C and then presoaked in GA_3 solution (0.058 µM), for 24 h prior to inoculation. For germination, four types of media were tested, viz: hormone free Woody Plant Medium (WPM) and / or supplemented with 6-Benzyl amino purine (BAP; 2.22 µM) and half strength and full strength Murashige and Skoog's (MS) medium without any growth regulators. All the media were supplemented with sucrose (2%, w/v) as a carbon source and the pH was adjusted to 5.6–5.8 with 0.1 N NaOH solution. All the media were gelled with 0.80% agar (w/v). The growth regulators were incorporated in media prior to autoclaving. The media were autoclaved at 1.05 kg cm^{-2} and 121° C for 20 min before use. One seed was inoculated per glass culture bottle with 50 seeds per treatment. The cultures were incubated at 25±1°C under cool white fluorescent light (16/8-h photoperiod, 35 µmol m^{-2} s^{-1}; Philips, India). The frequency of seed germination was scored after 25 days based on 5 replications for each experiment.

4.2.3. Multiple shoot induction:

The *in vitro* germinated seedlings were removed from the culture bottles under sterile conditions in a laminar airflow chamber. Well germinated seedlings (>5.0 cm) were decapitated and used for multiplication. Decapitation (of shoot and root) was done using sterile surgical blade. The decapitated seedlings (one per culture bottle) were then inoculated on hormone free WPM and / or WPM supplemented with BAP (2.22, 4.40, 8.90, 13.30, 22.19, 44.00 µM) and Thidiazuron (TDZ; 0.91, 2.27, 4.54 µM). The culture conditions were similar to germination experiment. The numbers of multiple shoots induced were scored after every 20 days of interval from the day of inoculation to a period of 60 days. The explants on all the combinations of media were subcultured twice during this period at 20 days of intervals.

4.2.4. Elongation of micropropagated shoots and induction of roots:

For elongation, the multiple shoots induced on lower concentrations of BAP and TDZ were transferred on WPM supplemented with BAP (2.22 – 44.00 µM) and TDZ (0.91-4.54 µM). The stunted shoots induced on higher concentrations of BAP (13.30 – 44.00 µM) and some from TDZ (0.91 – 4.54 µM) were transferred onto half strength WPM without any growth regulators.

The numbers of multiple shoots induced were scored after every 20 days of interval from the day of inoculation to a period of 60 days. Well-elongated, developed shoots were selected for induction of roots. Shoots more than 4.0 cm size were excised and shifted to half and full strength hormone free WPM or supplemented with Indole-3-butyric acid (IBA; 2.46 –24.60 µM) alone or in combination with 2.22 µM BAP for root induction. For all the treatments, minimum of 50 shoots with 3 replicates were maintained.

4.2.5. Acclimatization and planting of rooted shoots:

The rooted shoots were acclimatized by planting in sterilized mixture of soil, cocoa peat and sand (1 : 2 : 1) under greenhouse conditions with 70 – 80 % humidity and 26 ±2°C temperature for four weeks and later were transferred to earthen pots for further growth and development in the nursery. These micropropagated plants were planted after 3–5 months in the institute's campus in an attempt for their *ex situ* conservation. Pits of size 50 cm were made at the plantation sites, which were filled with garden soil, and farmyard manure (1: 1). Plants were carefully transplanted to the pits from the earthen pots.

4.3. RESULTS AND DISCUSSION:

4.3.1. Effect of sterilizing agents on sterility:

Many of the difficulties encountered during seed germination of *Calophyllum inophyllum* were common to woody plants in general. Major constraint encountered was extent of fungal and bacterial contamination. *C. inophyllum* is rich in phenolic compounds because of which exudation of phenolics associated with culture initiation was also a prevalent difficulty. In initial experiments to optimize sterilization protocol, the different fungicides such as Bavistin, Benomyl and surface sterilants like NaOCl, $HgCl_2$ and H_2O_2 in different concentrations with different treatment times were tried. Treatment of seeds with 0.1% Bavistin for 30 min. or 0.5 % Benomyl for 30 min was found to be most effective in controlling fungal contamination. Compared to NaOCl and H_2O_2,, treatment with 0.1% $HgCl_2$ for 30 min resulted in 49.5 % sterility (Table 4.1). Although, treatment with higher concentrations of H_2O_2 (10%) for 20 min. established maximum 38% sterile cultures which later turned brown because of which they did not germinate at all. Treatment with 0.1% $HgCl_2$ for 30 min was best suited for establishing sterile cultures and did not cause browning of treated seeds. These surface sterilizing agents were similarly evaluated for surface sterilization of sugarcane buds to eliminate bacterial contamination and treatment with 0.1% mercury chloride resulted in 56.3% sterility (Moutia and Dookun, 1999). Depending on the atmospheric humidity and intensity of mansoon rains, concentration of $HgCl_2$ and treatment time slightly varied for the

material collected in such condition. When atmospheric humidity in air was high in the month of July, seeds collected during this period were treated with slightly increased concentration and treatment time was also slightly increased to get maximum sterility. Since *C. inophyllum* is rich in phenolic compounds, secretion of phenolics during treatments was also creating the problems during sterilization. Pretreatment of seeds with PVP insoluble (0.1 – 0.5% w/v) for 30 min. adsorbed the phenolic compounds from the seeds.

Table: 4.1. Effect of $HgCl_2$, NaOCl and H_2O_2 on mean % sterility.

%$HgCl_2$ (w/v)	0.05				0.1				0.2				0.3				0.4			
Treatment Time (Min)	10	20	30	40	10	20	30	40	10	20	30	40	10	20	30	40	10	20	30	40
%Sterility	12.5	17.5	14.0	13.8	05.0	23.0	49.5	38.0	03.0	20.0	19.5	19.0	09.0	05.0	11.0	23.0	07.5	13.0	12.0	22.0
%NaOCl (v/v)	2.0				4.0				6.0				8.0				10.0			
Treatment Time (Min)	10	20	30	40	10	20	30	40	10	20	30	40	10	20	30	40	10	20	30	40
%Sterility	04.0	16.5	13.5	08.5	08.0	09.0	23.5	24.0	06.5	23.0	16.5	15.5	24.0	21.5	17.5	26.0	19.0	21.5	27.0	31.0
%H_2O_2 (v/v)	2.0				4.0				6.0				8.0				10.0			
Treatment Time (Min)	10	20	30	40	10	20	30	40	10	20	30	40	10	20	30	40	10	20	30	40
%Sterility	04.0	06.0	11.0	17.5	14.0	28.0	23.0	09.0	07.0	14.5	19.0	21.5	12.0	18.5	24.5	29.0	41.5	38.0	36.0	37.0

All values are mean of three replicate

4.3.2. Effect of basal medium on seed germination:

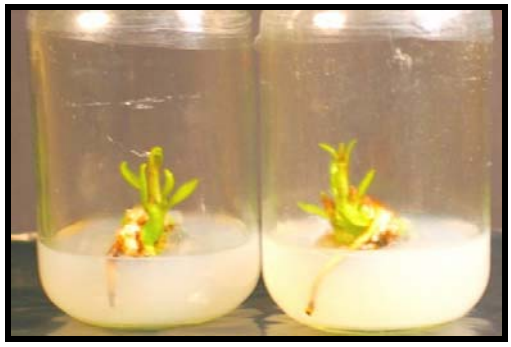


Fig 4.1. Seeds germination on MS Full and MS Half.

Fig 4.2 Seed germination on WPM with BAP (2.22 µM).

Fig 4.3 Seed germination on WPM after 24 hrs presoaking and incubation for 30 days.

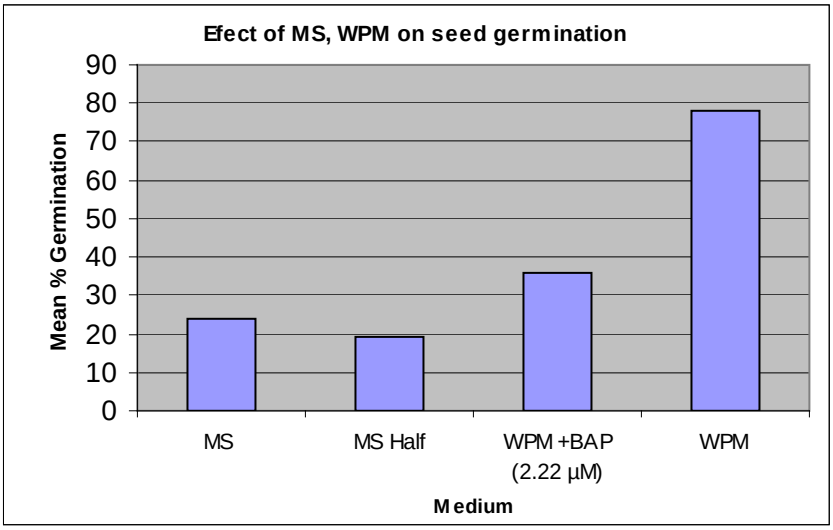


Fig. 4.4. Effect of MS and WPM on seed germination.

For germination, maturity of the seeds was a critical factor. Well grown, fully matured seeds germinated in both, WPM and MS medium, whereas immature seeds did not germinate at all. Germination of seeds was observed after 10–15 days of incubation on all media combinations. During germination, seed first swelled and the cotyledons slightly got separated apart to expose its embryo. Germinating embryo initially appeared creamish pink or brown colored and the turned green colored on attaining height of more than two cm. Without phytoharmone, seed germination on MS basal (MS full and MS half) medium was poor (19-24 %) (Fig. 4.4) and slow compared to WPM basal medium, with/ or without hormone, which showed good and faster germination (36–78%) (Fig. 4.1 and 4.4). Presoaking facilitated leaching of phenolics from the seeds, prevented browning and caused swelling of seeds, thereby hastening the germination process by three weeks, compared to the seeds which were not presoaked prior to inoculation (used as control). The best germination 78% (significant at $P = 0.01$) was observed when seeds were soaked for 24 hrs prior to inoculation on WPM basal medium without any growth hormones (Fig. 4.4*)*. However, with increase in presoaking time to 36 or 48 h, there was no further increase in the germination percentage. Moreover, it resulted in contamination of cultures. Therefore, for seedling establishment 24 hrs presoaked seeds were used. On an average, all seeds germinated within 10–15 days. However, growth was slow and it took about 25–30 days to develop into seedlings of 5–8 cm size).

4.3.3. Multiplication:

Fig. 4.5 Multiplication on high concentration of BAP (44 .0 µM)

F ig. 4.6 Multiplication on low concentration of TDZ (0.91 µM)

Table 4.2. Influence of BAP and TDZ on multiple shoot induction from the decapitated seedling explant.

Medium WPM		Average No. of multiple shoots after incubation (Days)		
BAP (μM)	TDZ (μM)	20	40 (S1)	60 (S2)
0	0	1.2	2.3	4.8
2.22	---	3.6	9.7	12.0*
4.40	---	1.3	5.1	8.1
8.90	---	2.0	4.9	6.4
13.30	---	3.5	6.3	9.8
22.19	---	3.8	8.8	12.7*
44.00	---	6.9	11.6	13.3*
---	0.91	6.9	15.0	20.9**
---	2.27	4.4	10.1	15.0**
---	4.54	3.6	6.6	13.1*

LSD (p = 0.05) = 6.97; LSD (p = 0.01) = 9.24
*, ** Significant at 5% and 1% level, respectively; S1, S2: Subcultures one & two.

The induction of multiple shoots was observed 10–15 days after inoculation. More multiple shoots were induced on 2.22, 22.19, and 44.00 μM BAP compared to other concentrations (4.40, 8.90 and 13.30 μM) (Table 4.2). Low concentration of TDZ (0.91 μM) induced equal number of shoots as that of higher concentration of BAP (44.0 μM) after 20 days of incubation (Table 4.2). The same trend of response continued for 60 days. After 60 days of incubation, the maximum number of multiple shoots observed was 20.9 per explant with TDZ (0.91 μM; Fig. 4.6 and Table 4.2). All concentrations of BAP induced multiple shoots from 6.4 to 13.3 per explant. ANOVA showed that BAP (2.22, 22.19 and 44.00 μM) and TDZ (4.54 μM) were significant at 5% level, while TDZ (0.91 and 2.27 μM) was found to be significant at 1% level. On the basis of statistical analysis, it appears that TDZ is significantly better than BAP for multiple shoot induction, since it induced greater number of shoots at much lower concentrations. Though more multiple shoots were induced at high concentrations of BAP, the number of elongated shoots of size greater than 4.0 cm was significantly higher at lower concentrations of BAP (2.22 and 4.40 μM). Generally, longer shoots are produced at lower BAP concentrations, whereas more shoots are induced with higher BAP concentration (Ye et al, 2002). However, TDZ even at very low concentration (0.91 μM) induced more multiple shoots. Also, the proportion of elongated shoots of size >4.0 cm was significantly higher (4.9–7.2 per explant) compared to BAP (Table 4.3). The possible reason is auxin and cytokinin- like activity of TDZ (Visser et al., 1992).

4.3.4. Elongation:

Table 4.3: Average number of elongated shoots induced on different hormone concentrations.

Medium WPM		Average No. of elongated shoots (>4.0 cm) after incubation (Days)		
BAP (µM)	TDZ (µM)	20	40 S1	60 S2
0	0	0.4	1.2	2.2
2.22	---	1.8	6.1	8.7**
4.40	---	0.8	5.1	6.2*
8.90	---	0.6	1.5	4.9
13.30	---	1.0	2.1	3.7
22.19	---	0.7	2.0	2.7
44.00	---	1.3	1.9	2.3
---	0.91	3.1	6.3	7.2*
---	2.27	2.1	5.6	6.4
---	4.54	2.7	3.8	4.9

LSD (p = 0.05) = 3.77; LSD (p = 0.01) = 5.00
*, ** Significant at 5% and 1% level, respectively; S1, S2: Subcultures one & two.

Fig. 4.7 Stunted multiple Shoots induced on BAP and TDZ

Fig. 4.8 Elongated multiple Shoots after 60 days inoculation

Maximum number of elongated shoots, 8.7 per explant (significant at $P = 0.01$), was noticed on WPM supplemented with BAP (2.22 µM) (Table 4.3). Transfer of the stunted shoots induced on BAP/TDZ (Fig. 4.7) to half strength WPM without any growth hormones showed considerable elongation in about 30–40 days (Fig. 4.8). Statistically, elongation on WPM with BAP (2.22 µM) is significant at 1 % level (P=0.01); whereas elongation on WPM with BAP (4.40 µM) and TDZ (0.91µM) was significant at 5% level (P=0.05) (Table 4.3.).

4.3.5. Rooting:

Table 4.4: *In-vitro* root induction in shoots of size >4.0 cm.

Medium	Hormones		Mean % Rooting	No of plantlets obtained	% Acclimataization
	IBA (µM)	BAP(µM)			
WPM half	0	0	14 (21.59)	10	71.40
	2.46	2.22	40 (39.230**	32	80.00
	4.90	2.22	34 (35.26) **	26	76.47
	9.80	2.22	27 (31.30) **	22	78.57
	14.70	2.22	23 (28.65) **	20	83.33
	24.60	2.22	18 (24.57) *	14	77.77
WPM	0	---	16 (23.04)	12	75.00
	2.46	---	52 (45.76) **	42	80.76
	4.90	---	41 (39.42) **	36	85.71
	9.80	---	29 (32.15) **	22	73.33
	14.70	---	43 (40.59) **	34	77.27
	24.60	---	41 (39.61) **	30	71.42

LSD (p = 0.05) = 2.85; LSD (p = 0.01) = 3.87,
*, ** Significant at 5% and 1% level, respectively.
Figures in parenthesis are angular transformation values of percentage of response.

Fig 4.9 a & b. Well rooted plantlets prior to transfer to potting mixture

Combination of half WPM supplemented with IBA (2.46–24.60 µM) had effect on root induction (Table 4.4). Shoots induced on BAP-containing medium induced rooting in 8–12 days, with 1–5 roots per explant (Fig. 4.9 a and b). However, shoots induced on TDZ rooted only after two passages of one-month duration each on half strength WPM supplemented with IBA (2.46–24.60 µM) alone or in combination with BAP (2.22 µM). The high carry-over effect of TDZ

has been well documented in the literature (Meyer et al, 1998; Lu, 1993). In all the combinations, rooting was observed with 14–52% frequency (Table 4.4). Maximum rooting (52%) was observed on WPM supplemented with IBA (2.46 µM) alone. On the basis of ANOVA, it was observed that full strength WPM and/or WPM supplemented with IBA (2.46– 24.60 µM) was significantly superior to half strength WPM and/or half WPM supplemented with IBA (2.46– 24.60 µM) and BAP (2.22 µM).

4.3.6. Acclimatization and potting:

For acclimatization, rooted shoots of about 2 cm responded well in green house conditions whereas shoots with roots less than 2 cm responded poorly. After trying several ratios of sterile soil, cocoa peat and sand, sterile mixture of soil, cocoa peat and sand (1:2:1) was found to be optimum and supported the further growth of the shoots (Fig. 4.10 a and b). % survival ranged between 71.40 – 85.71% (Table 4.4). Factors like basal medium on which rooting was induced (Half strength WPM and full strength WPM), hormones (IBA and BAP) supplemented for root induction and % rooting had no effect on survival (Table 4.4). Incubation for minimum four weeks in green house condition was essential before shoots can be transferred to earthen pots for further growth in nursery (Fig. 4.11). Transfer before four weeks resulted in poor growth or death of plantlet because of shock. At the final plantation site, pits which were filled with garden soil and farm yard manure (1:1) were best suited for successful plantation and further growth.

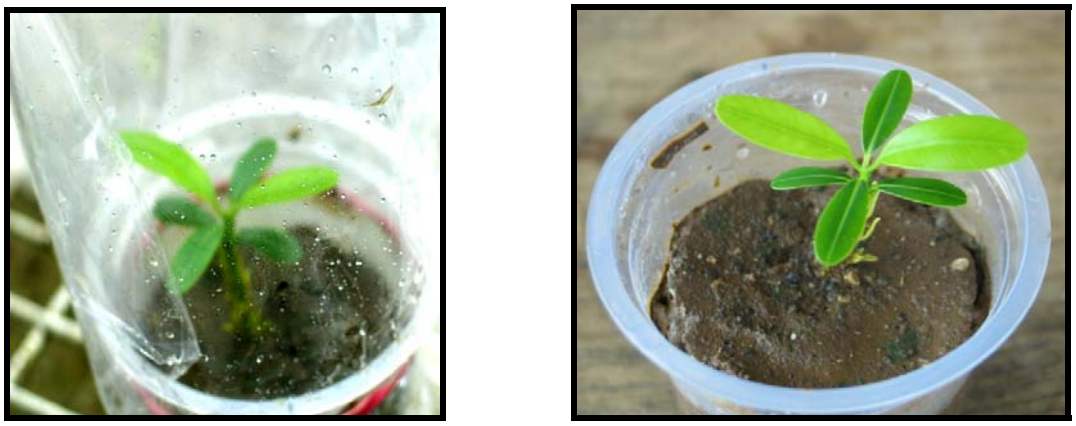


Fig. 4.10. a & b. Acclimatized plants 3 weeks after transfer to green house

Fig. 4.11 *In vitro* propagated plantlets 4 months after transfer to pots.

4.4 CONCLUSIONS:

These experiments were conducted with an objective to generate information on *in vitro* propagation of *C. inophyllum* with establishment of sterile shoot culture, to understand nutritional requirement for *in vitro* proliferation for multiple shoots and their elongation, *in vitro* induction of rooting, rooting behavior, acclimatization and survival in soil etc. Having known various difficulties in using mature tissue and advantages in using immature tissue, seedling derived meristematic tissues were tested. During germination of seeds, we encountered serious limitation due to microbial contamination from seed borne microbes. In order to control microbial contamination, several experiments were conducted by using fungicides Bavistin, Benomyl and surface sterilizing agents $HgCl_2$, NaOCl, and H_2O_2 in different concentrations and treatment time. Bavistin (0.1%, w/v) or Benomyl (0.5%, w/v) for 30 min. was found best to control fungal contamination; whereas among the $HgCl_2$, NaOCl, and H_2O_2 used as surface sterilents, $HgCl_2$, (0.1% w/v) for 30 min. effectively controlled the microbial contamination.

For seed germination, among MS full strength, MS half strength, WPM and WPM with BAP (2.22µM) media used, WPM and WPM with BAP showed good and fast germination. Presoaking in SDW and/ or GA_3 (0.058µM) hastened the germination process. WPM supplemented with BAP (2.22- 44.0µM) and TDZ (0.91- 4.54 µM) induced the multiple shoots from decapitated seedling explants. Higher concentrations of BAP (44.0µM) or lower concentration of TDZ were optimum for multiple shoot induction and induced almost equal number of multiple shoots. Statistically TDZ was significantly better than BAP for multiple shoot induction. WPM with BAP (2.22 µM) was optimum for elongation of multiple shoots whereas stunted shoots induced on WPM with BAP and TDZ elongated well on half strength WPM without any growth regulators.

For rooting, shoots of more than 4.0 cm responded well. Half strength and /or full strength WPM supplemented with IBA (2.46-24.60 µM) alone or in combination with BAP (2.22

μM) was found to be best. Rooted shoots were acclimatized best in greenhouse condition when planted in a sterile mixture of soil, cocoa peat and sand in proportion 1:2:1 which was best suited. Only minimum 3-5 months old and acclimatized plantlets were able to survive in a field conditions when planted in garden soil and farm yard manure (1:1).

There is only one report on *in vitro* multiplication/ micro propagation in *Calophyllum* species. Nair and Seeni (Nair and Seeni, 2003) reported *in vitro* multiplication of *C. apetalum* Willd. using mature tree explants on MS medium supplemented with BAP (8.8 μM). The multiplication ratio was 1:2/3. Although *C. inophyllum* produces large number of fruits, the species being littoral, the fruits get washed away. Also as most of the mature trees are cut down for timber that is used in boat making. With the present work on *in vitro* propagation of *C. inophyllum* Linn. a tree species with immense medicinal importance (especially in AIDS chemotherapy), efforts can be made for the *ex situ* conservation of this threatened medicinal tree. The above protocol can be used for mass propagation of *C. inophyllum*, since the success rate of vegetative propagation is low and is usually not practiced.

This work has been reported in the following publication

Thengane, S. R., Bhosle, S. V., Deodhar, S. R., **Pawar, K. D.,** and Kulkarni, D. K., 2006. Micropropagation of Indian Laurel *(Calophyllum inophyllum*), a source of anti- HIV compounds. Current Science. 90, 1393-1397.

CHAPTER 5. ESTABLISHMENT OF CALLUS CULTURES IN *CALOPHYLLUM INOPHYLLUM*.

5.1. INTRODUCTION:

Plants have the ability to produce secondary metabolites that are of importance in the healthcare, food, flavour and cosmetics industries. Many pharmaceuticals are the secondary metabolites of plants origin. Presently, these and many other natural products are obtained by extracting and isolating solely from massive quantities of whole plant parts. Often the source plants are either wild or cultivated in tropical or subtropical, geographically remote areas which are subject to political instability, drought, disease and changing land use patterns and other environmental factors. In addition, the long cultivation periods, especially in tree species between planting and extraction make selection of high-yielding plants difficult, thus resulting in high cost of drugs. Cultivation periods may range from several months to decades. In spite of these difficulties and costs, the extraction of medicinally important secondary metabolites from cultivated plants or plants in the wild continue because of lack of credible alternatives. Clearly, the development of alternative and complimentary methods to whole plant extraction for the production of clinically important secondary metabolites is an issue of considerable socioeconomic importance. These facts have generated great interest in the use of plant cell culture technologies for the production of pharmaceuticals and other plant derived secondary metabolites. Indeed, the plant cell culture technology is now sufficiently advanced to allow large quantities of relatively homogeneous, undifferentiated cells to be produced. As compared to whole plant extraction, extraction from plant cells and tissues are less cumbersome and may provide competitive metabolite production systems.

5.1.1. Identification of elite plants:

In plants, secondary metabolites are formed in very minute quantities. More over seconadry metabolites are synthesized in plants under certain specific stress conditions and represents features that can be expressed in terms of ecological, taxonomical and biochemical differentiation and diversity. For raising *in vitro* cultures for the production of secondary metabolites, initial plant material to be used as explants needs to be elite in terms of maximum initial level of secondary metabolite of interest. For identifying the elite plants, chemodiversity studies are conducted wherein metabolites of interest are isolated, identified, and suitable chromatographic techniques is developed for their estimation. Once suitable chromatographic technique is developed, plant metaerial is collected from different locations, extracted and analyzed quantitatively. Based on such quatitative chemodiversity studies, plants or locations of the plants with maximum initial content of secondary metabolites of interest can be identified and used for raising *in vitro* cultures.

5.1.2. Callus cultures:

As an alternative and complementary method to whole plant extraction for the production of medicinally important secondary metabolites, several techniques of tissue culture like callus culture (cell culture), shoot culture, root culture and cell suspension culture can be utilized. Among these cultures, callus cultures are most attractive because they can be initiated by using small part of the parent plant as explant. Callus is a mass of undifferentiated cells and consists of somatic undifferentiated cells formed from explants. Callus mass is not necessarily genetically homogeneous because callus is often made from structural tissue, not individual cells. A callus cell culture is usually established on soldified media, much in the same manner as bacteria are grown. Morphologically callus may have different shapes, texture and colors. Callus masses can be hard, compact, or friable. Callus masses also can be white, yellowish or creamish, brown colored in appearance. For establishing callus cultures, different tissue culture basal media are tried and most appropriate and suitable basal medium is often selected. To support the fast and healthy induction and growth of callus mass, basal medium consists of macronutrients, micronutrients, vitamins, chelate, mostly sucrose as a carbon source and agar agar as a gelling agents. Induction of callus and its further growth is due to rapid cell division which is a function of endogenous level of auxins and cytokinins in an initial explant. Callus induction can be accelarated by altering the endogenous level by exogenous supply of auxins and cytokinins. Phytohormones are supplied exogenously by incorporating them into basal medium in different combinations and concentrations. For efficient callus induction, types of basal medium, types of auxins and cytokinins, their combinations and concentration are optimized. Initial plant meterial or explants also determine the success of callus induction. Almost all parts of the plant like seed, cotyledon, leaf, petals, node, internode contain meristematic tissues and can be used as a initial material or explants. For a higher plant like *C. inophyllum*, establishing a sterile culture becomes difficult because of explant borne, endogenous contaminations. Effective sterilization protocol to control microbial contaminations is neded to be optimized. Method for sterilization involves treatment of explants with different fungicide and sterilizing agents in different concentrations for different duration.

5.1.3. Estimaion of callus cultures for secondary metabolites:

When callus cultures are established for the study of *in vitro* expression of secondary metabolites, effects of different factors like basal medium, hormones, medium components, explant origin and type on callus induction frequency and expression pattern of

secondary metabolites are studied. Expression pattern of secondary metabolites in callus cultures is studied by analysing callus cultures with suitable chromatogarphic techniques like TLC, HPTLC, HPLC, GC etc. HPLC is the most appropriate and widely used technique for analysis of secondary metabolites. HPLC is a separation process which is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Components that are held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase. HPLC is a sophisticated technique which can separate and quantitate solutes in nano quantities. The success of HPLC analysis is determined by extraction of callus samples and sample preparation method. For extraction, different solvents, their volume required for extraction, steps involved in extraction are optimized to get maximum recovery and reproducibility. Development of HPLC method involves optimization with most suitable HPLC column, solvents and their composition in mobile phase, run time, detection mode, injection volume and elution mode. Based on HPLC analysis, hyper producing callus lines can be screened and used for further experiments to enhance secondary metabolite expression. These callus lines can be used in cell suspension cultures and elicitation experiments.

This chapter describes the results on experiments conducted for optimization of callus cultures and describes the effects of explant's types and different hormone combinations and concentrations on callus induction. Results on HPLC analysis of the callus cultures and expression pattern of dipyranocoumarins in those callus cultures are also discussed.

5.2 EXPERIMENTAL PROTOCOLS:

5.2.1. Source and collection of plant material:

For identification of elite plants in terms of higher initial contents of dipyranocoumarins, chemodiversity study was conducted. In chemo diversity of *C. inophyllum,* fruits were collected from different locations along the Western Ghats of India, extracted and analyzed with quantitative HPLC method developed. Based on this study, locations of plants with maximum initial contents of inophyllums were identified as elite plants. Plants from location Harne, (17° 49' 63" N, 73° 05'65" E; 2 m altitude) near Dapoli, Dist- Ratnagiri, Maharashtra, were identified as elites and were used for *in vitro* germination and callus induction. Details of the chemodiversity study have been discussed in Chapter 3. The fruits were collected in May to July, the fruiting season for *C. inophyllum*.

5.2.2. Induction of Callus Cultures:

After collection, fruits were air dried for 2 weeks, and then decoated mechanically to take out the seeds from its hard shell. Seeds were surface sterilized following the method given in Chapter 2. For germination, surface sterilized whole seeds were inoculated on hormones free, agar solidified WPM basal medium with 2 % sucrose in larger culture vessels to raise sterile seedlings.

Fig. 5.1. Bunch of mature fruits of *C. inophyllum*

Fig.5.2. Surface sterilized seeds of *C.inophyllum*.

For the callus induction from seeds, prior to inoculation, mature, surface sterilized seeds (Fig. 5.2) were cut into four to six pieces to get 3X3 cm size initial explants (Fig. 5.3). For induction of callus cultures from leaf and nodal/ internodal explants, two- three months old, well grown sterile seedlings were used as a source of explants. Seedlings of 5-6 cm in height with 5-6 leaves were preferred (Fig. 5.4).

Fig.5.3. Seed explant inoculated on WPM+Hormones

Fig. 5.4.Three month old sterile seedling of *C. inophyllum*.

Leaves from sterile seedlings were cut into 3X3 cm pieces and were injured with sterile blade (Fig. 5.6), while green stems of *in vitro* grown seedlings were cut into circular slices or discs before placing onto a media in Petri dishes (Fig. 5.5).

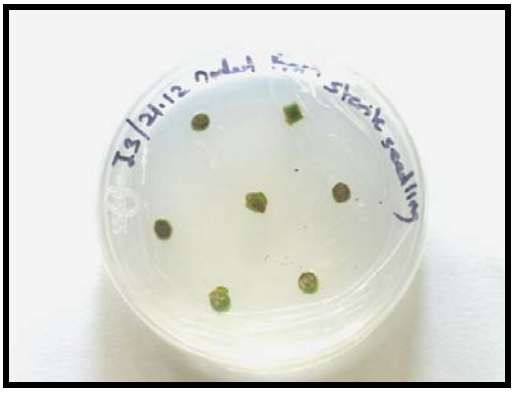


Fig.5.5. Nodal/internodal explants inoculated on WPM+hormone

Fig.5.6. Leaf explants inoculated on WPM+hormone

All three types of explants were inoculated on agar solidified (0.7 %, w/v) WPM basal medium with 2 % sucrose and incorporated with IBA, BAP, NAA, Picloram, 2,4-D and Kinetin in combinations and concentrations given below:

IBA - 4.90 – 19.60 µM. (A1-A4)

IBA - 4.90 – 19.60 µM + BAP - 4.44 µM. (A5-A8)

NAA -10.74 – 42.96 µM. (B1-B4)

NAA- 10.74 – 42.96 µM + BAP- 8.88 µM. (B5-B8)

Picloram - 8.28 – 33.12 µM + BAP-8.88 µM. (C1-C4)

2,4-D - 4.52 – 18.09 µM. (D1-D4)

2,4-D - 4.52 – 18.09 µM + BAP- 4.44 µM. (D5-D8)

2,4-D- 4.52 – 18.09 µM + Kinetin- 4.65 µM. (D9-D12)

BAP- 4.44 – 17.85 µM + Kinetin- 4.65 µM. (E1-E4)

Kinetin - 4.65 – 18.60 µM + BAP - 4.44 µM. (E5-E8)

All the cultures were incubated at 25± 2°C under cool white fluorescent light (16/8 photoperiod, 35 µmol m^{-2} s^{-1}; Philips, India). Cultures were maintained by sub culturing and transferring after every 45 days on their respective fresh medium containing same hormone combinations and concentrations.

5.2.3. Extraction of callus masses and sample preparation for HPLC analysis:

Two month old callus masses induced from seed, leaf and nodal/ internodal explants were used for extraction and HPLC analysis. 1 g (FW) of callus masses were crushed and

suspended in acetone (4 ml /g callus) for six hours. Yellowish, brown colored acetone extracts were filtered off and callus masses were resuspended in fresh acetone for four more times. From combined acetone extracts, acetone was evaporated under reduced pressure in rotary evaporator to yield yellowish oily extracts. As a part of preliminary experiments to optimize extraction protocol, acetone extract of callus was washed with petroleum ether (60-80 ^{0}C) and found to contain negligible quantities of fatty material. Hence defatting acetone extracts for the removal of non polar compounds was not included further in sample preparation for quantitative analysis by HPLC. These acetone extracts were then partitioned in a mixture of chloroform and water (1:1). Chloroform soluble portions were separated and evaporated to dryness under reduced pressure and stored at room temperature till further use. These chloroform extracts were re- dissolved in 1 ml HPLC mobile phase (25% ethyl acetate in petroleum ether, v/v). For HPLC analyses, 15µl of these extracts were injected.

5.2.4. HPLC Analysis:

The HPLC analyses were done in isocratic mode on Perkin Elmer's series 200 HPLC system equipped with a quaternary gradient pump, an autosampler and a diode array detector using Water's (Milford, MA, USA) µPorasil (3.9 mm ID × 300 mm L; 10µm) stainless- steel HPLC column. Ethyl acetate and petroleum ether, 60-80 ^{0}C (25:75) was used as mobile phase in a run time of 20 minutes with flow rate of 1 ml /min. and chromatograms were recorded at 245 nm. The injection volume was 15µl for callus extracts and each of 1 mg /ml solution of standard samples of inophyllums B, P, C, A, D and calophyllolide in mobile phase. Peak areas in sample chromatograms of seed, nodal/ internodal and leaf callus extracts were compared with the reference chromatograms of standards to evaluate the differences in the expression of dipyranocoumarins. TotalChrom Navigator software was used to process the chromatographic data. External standard method was used for quantification and two way ANOVA was performed on software AgroBase 99, Microsoft Excel and OrginPro 8.

5.3. RESULTS AND DISCUSSIONS:

5.3.1. Effect of hormones on callus induction:

Among the different basal media like M.S (Murashige and Skoogs, 1962), Gamborgs B5 (1968), White's medium (1963), WPM (Woody Plant Medium, Lloyd and Mc.Cown, 1980) used initially to test the response of explants, WPM medium was found to be most suitable for callus induction and seed germination. Method for seed germination was optimized and effects of WPM and MS on seed germination have been discussed in Chapter 4. For

callus induction the media were supplemented with various combinations and concentrations of IBA, NAA, Picloram, 2, 4- D, BAP, and kinetin. Out of these hormones, only combinations of IBA, NAA, Picloram and BAP in different concentrations induced the calluses (Table 5.1); while combinations of 2, 4- D, kinetin and BAP either did not induce callus or induced negligible quantities of calluses (Table 5.2). Also responses from these hormone combinations were not reproducible.

Fig. 5.7. Seed explants slightly tore apart.

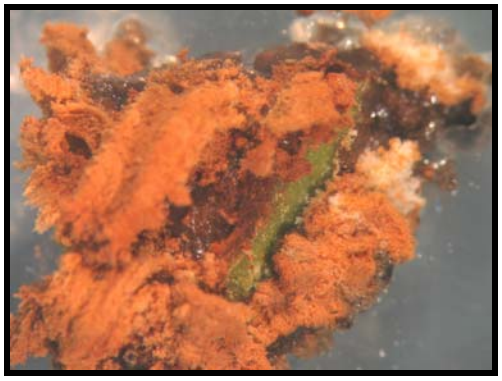


Fig. 5.8.Two month old brown, friable callus from seed explant.

Only well grown, fully matured seeds induced callus whereas immature seeds did not induce callus, rather they either turned black or brown after 10-15 days of inoculation. Size of the seed explants determined the success of callus induction. Explants of size less than 3X3 cm size showed poor induction of the callus from seed explants. Immediately after inoculations, fluorescent yellow colored latex oozed out of the cut surfaces of the seed explants. After 10 days of inoculation, swelling of the seed explants was observed while few explants slightly tore apart (Fig. 5.7). Induction of callus from seed explants was observed after 45 days of incubation. Callus masses induced from seed explants were initially white, friable and irregular in appearance (Fig. 5.7) and turned dark brown colored on subsequent sub culturing and transferring (Fig. 5.8). After two subcultures or transfers, seed callus became more friable, brown, necrolytic and ceased further growth.

Fig. 5.9 White friable Callus induced from cut edges of leaf explant.

Leaf and nodal/ internodal explants from fully grown, well matured seedlings were best suited as a source of explants and responded well (Fig. 5.4). Well grown, dark green colored leaves and nodal/ internodal explants induced the callus (Fig. 5.5 and 5.6); while uppermost young leaves of seedlings which were thin and brown colored did not induce callus.

Fig. 5.10 White friable callus induced from leaf explant

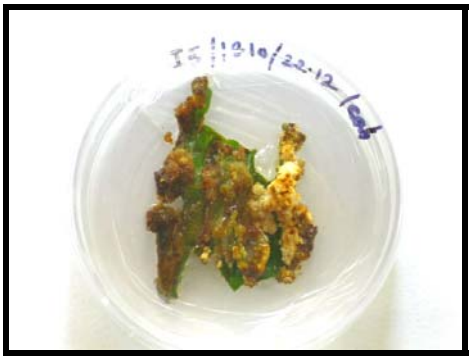


Fig 5.11. Brown, nodular, compact callus induced from leaf explant.

Induction of callus from leaf was also determined by initial size of explant. Leaf explants of size 3x3 cms or more responded well whereas explants of size less than 3x3 cms responded poorly. Like seed explants, leaf explants also required extensive injury prior to inoculation and slightly oozed out fluorescent yellow colored latex from the injured portion of the explants. On incubation for 15 days after inoculation, leaf explants swelled up at the cut edges and then started forming callus (Fig. 5.9).

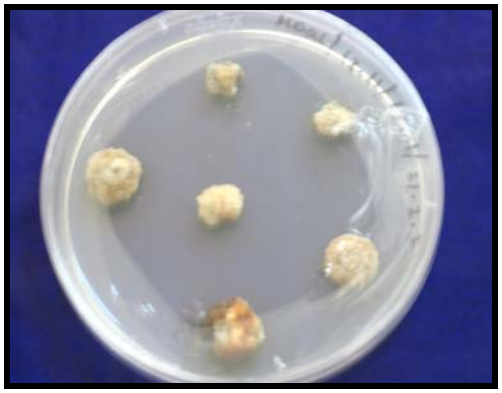


Fig. 5.12. White, friable callus induced from internodal explant

Fig. 5.13 Brown, nodular, compact callus Nodal/ induced from nodal/ internodal explant.

Unlike seed and leaf explants, callus induction from nodal/ internodal explants was determined by size and shape of explants. Circular slices or discs of green stem of sterile seedlings

(Fig. 5.5) were best suited for callus induction when compared to other shapes and larger pieces of nodal/ internodal explants. After 15 days of inoculation, upper surface of the nodal/ internodal explants became white, granular or friable indicating the beginning of the callus formation (Fig. 5.12). Extensive injury with sterile blade to these explants prior to inoculation was most critical factors. Injury to the explants exposed the maximum number of cells to the medium and hastened the induction process. Unlike seed explants, leaf and nodal/ internodal explants induced callus formation after 30 days of inoculation. Calluses induced from nodal/ internodal and leaf explants were rarely white and friable, (Fig. 5.12 and 5.10) mostly dark brown, nodular, compact (Fig. 5.13 and 5.11) and remained same throughout their sub culturing and transferring. Induction of callus started from all over the upper surface of nodal/ internodal discs (Fig. 5.12 and 5.13).

2, 4-D in the range 4.52 – 18.09 µM alone as well as along with BAP 4.44 µM constant and kinetin 4.65 µM constant did not induce callus from seed explants. Only 2,4-D in the range 4.52 – 18.09 µM along with BAP 4.44 µM constant induced very minute and negligible quantities of calluses from nodal/ internodal and leaf explants (Table 5.2). Also this response was not reproducible because of which, these negligible quantities were not harvested for HPLC analyses. Moreover, all three types of explants turned extensively brown and black when inoculated on medium incorporated with 2 4-D, BAP and kinetin in different combinations and concentrations. 2 4-D, BAP and kinetin for the induction of callus have been widely used and reported (Shcroder and Bohm, 1984; Baumert et al., 1992; Andrijani, et al., 1999; Rajasekaran et al., 1991; Barthe et al., 1999). But in *C. inophyllum,* combinations of 2 4-D, BAP and kinetin failed to induce calluses.

Table 5.1. Influence of IBA, NAA, Picloram and BAP on % callus induction from seed, nodal/ internodal and leaf explants of *Calophyllum inophyllum* L.

Medium Code	Auxins (μM)			Cytokinin (μM)	Explants		
	IBA	NAA	Picloram	BAP	Seed	Nodal/ Internodal	Leaf
A1	4.90	----	----	----	87.25	80.83	32.13
A2	9.80	----	----	----	90.49	80.23	43.20
A3	14.70	----	----	----	88.25	73.66	29.20
A4	19.60	----	----	----	79.38	87.50	33.00
A5	4.90	----	----	4.44	79.32	15.35	48.80
A6	9.80	----	----	4.44	80.99	20.87	39.10
A7	14.70	----	----	4.44	80.10	21.66	51.00
A8	19.60	----	----	4.44	96.01	34.00	55.20
B1	----	10.74	----	----	85.73	69.86	54.10
B2	----	21.48	----	----	87.73	73.20	54.80
B3	----	32.22	----	----	79.27	61.68	56.06
B4	----	42.96	----	----	78.47	67.86	62.59
B5	----	10.74	----	8.88	88.66	66.40	66.96
B6	----	21.48	----	8.88	87.08	73.20	54.83
B7	----	32.22	----	8.88	86.39	67.73	28.03
B8	----	42.96	----	8.88	82.00	69.74	26.43
C1	----	----	8.28	8.88	N R	75.63	78.72
C2	----	----	16.56	8.88	N R	78.50	62.47
C3	----	----	24.84	8.88	N R	86.38	86.66
C4	----	----	33.12	8.88	N R	84.33	67.05

Statistical analysis ANOVA:

	Med.	Exp.	Med. X Exp.
SE	a 2.45	a 11.85	a 3.22
	b 3.34	b 3.74	b 3.13
±CD	a N S	a 11.13	a 44.55
	b N S	b N S	b N S
	a N S	a **	a **
	b N S	a N S	b N S

Each value represents the mean of three replicates. Two way statistical analysis was performed separately for A1- B8 and C1- C4 medium. a- Values for A1- B8; b- Values for C1-C8.
(**) Significant at 1% level; (*) Significant at 5% level; N S- Non significant at 1% & 5% level. N R- No Result.

Maximum response for callus induction was 96.01% for seed explants, 87.50 % for nodal/ internodal explants, and 86.66 % for leaf explants on WPM medium supplemented with IBA 19.60 μM + BAP 4.44 μM , IBA 19.60 μM, and picloram 24.84 μM + BAP 8.88 μM respectively (Table 5.1). Supplementation of IBA in the range 4.90 – 19.60 μM alone and in combination with BAP 4.44 μM constant, induced calluses in the ranges 79.32% - 96.01 % from seed explants; 15.35 % - 87.50 % from nodal/ internodal explants and 29.20 % - 55.20 % from leaf explants. Addition of picloram in the range 8.28 – 33.12 μM along with BAP 8.88 μM constant did not induce calluses from seed explants (Table 5.1). Same range of picloram along with BAP induced callus in the range 75.63 – 86.38 % from nodal / internodal explants and 62.47 – 86.66 % from leaf explants (Table 5.1). Almost same highest callus induction responses of 86.38 % for nodal/ internodal explants and 86.66 % for leaf explants were achieved when media were supplemented with picloram 24.84 μM along with BAP 8.88 μM (Table 5.1). Maximum 88.66 % seed explants, and 66.96 % leaf explants formed calluses on supplementation of NAA 10.74 μM along with BAP 8.88 μM, while highest of 73.20 % nodal/ internodal explants formed calluses on supplementation of NAA 21.48 μM alone or along with BAP 8.88 μM (Table 5.1). Upon supplementation of NAA in the range 10.74 – 42.96 μM alone and in combination with BAP 8.88 μM, callus inductions from seed, nodal/ internodal and leaf explants were recorded in the ranges 78.47 % - 88.66 %, 61.68% - 73.20 %, and 26.43 % - 66.96 % respectively (Table 5.1). For production of many other plant secondary metabolites from number of other plant species, NAA and BAP were widely used and reported. Komaraiah et al., (2001) used the combinations of NAA, IAA and BAP for induction of callus cultures from leaf explants of *Plumbago rosea* for production of plumbagin.

Combinations of NAA were also used for establishment of callus cultures for the production of triterpenes in *Eriobotrya japonica* (Taniguchi et al., 2002). Combinations of NAA, IBA, picloram and BAP were successfully used in induction of callus cultures and production of secondary metabolites in number of plant species. Ramirez et al., (1992) used hormones for the induction of callus cultures of *Canavalis ensiformis* for the production of L-Canavanine. Callus cultures were induced in *Allium sativum* for the production of alliin by using hormones IAA, NAA and kinetin in MS medium (Malpathak and David, 1996). Using NAA and BAP in either of LS or B5 or White' medium, calli were induced in *Duboisia leichhardtii* for the production of tropane alkaloids (Yamada and Endo, 1984). In the present study, two way statistical analysis of variation revealed that variation in picloram concentrations in the range of 8.28 – 33.12 μM keeping BAP 8.88 μM constant could not give significantly different callus induction response. Significantly different callus induction responses were recorded for all three explants and their interactions with the different media combinations were significantly different (Table 5.1).

Table 5.2. Influence of different hormone combinations and concentrations on % induction of callus cultures from seed, nodal/ internodal and leaf explants of *Calophyllum inophyllum* L

Medium Code	Auxins (μM)	Cytokinin (μM)		Explants		
	2,4-D	BAP	Kinetin	Seed	Nodal/ Internodal	Leaf
D1	4.52	----	----	N R	N R	N R
D2	9.04	----	----	N R	N R	N R
D3	13.57	----	----	N R	N R	N R
D4	18.09	----	----	N R	N R	N R
D5	4.52	4.44	----	N R	Ng	Ng
D6	9.04	4.44	----	N R	Ng	Ng
D7	13.57	4.44	----	N R	Ng	Ng
D8	18.09	4.44	----	N R	Ng	Ng
D9	4.52	----	4.65	N R	N R	N R
D10	9.04	----	4.65	N R	N R	N R
D11	13.57	----	4.65	N R	N R	N R
D12	18.09	----	4.65	N R	N R	N R
E1	----	4.44	4.65	N R	N R	N R
E2	----	8.87	4.65	N R	N R	N R
E3	----	13.31	4.65	N R	N R	N R
E4	----	17.85	4.65	N R	N R	N R
E5	----	4.44	4.65	N R	N R	N R
E6	----	4.44	9.30	N R	N R	N R
E7	----	4.44	13..95	N R	N R	N R
E8	----	4.44	18.60	N R	N R	N R

Each value represents the mean of three replicates; N R- No Result; N g- negligible**.**

5.3.2. Pattern of dipyranocoumarins' expression in callus cultures:

Sequential extraction of callus samples with acetone and mixture of chloroform and water ensured the maximum extractions of dipyranocoumarins under study; while HPLC method developed with mixture of petroleum ether and ethyl acetate as a mobile phase and μPorasil HPLC column for quantitative analysis ensured the maximum separation of dipyranocoumarins. Upon loading 15μl of each of 1 mg /ml solution of standard samples of dipyranocoumarins, inophyllum B, R_T 9.17 min; inophyllum P**,** R_T 7.35 min; calophyllolide, R_T 5.00 min; inophyllum C, R_T 8.06 min; inophyllum A, R_T 13.30 min. and inophyllum D**,** R_T 8.93 min. were eluted in a isocratic mode

of 20 min run time. The details of HPLC method development, validation and chromatograms of standards of inophyllums are given in Chapter 3.

In the HPLC profiles of the extracts of all three types of calluses, peaks corresponding to the peaks of dipyranocoumarins were observed showing the expressions of dipyranocoumarins in callus cultures. When compared to chromatogram of extract of leaf callus, chromatograms of extracts of seed and nodal/ internodal calluses were much complex (Fig. 5.14, 5.15 and5.16). For the expression of dipyranocoumarins including anti- HIV- 1 inophyllum B and P in callus cultures, calluses induced from seed explants on medium range A1- A6 were best suited. Among A1 – A6, media, A2 was most suitable combination, since highest inophyllum B (40.59 mg %), calophyllolide (45.23 mg %), inophyllum C (142.32 mg %), and inophyllum D (44.72 mg %) were recorded from calluses induced from seed explants on A2 medium (Table 5.3). Highest inophyllum P (141.35 mg %) and inophyllum A (73.73 mg %) from seed calluses were recorded on A6 and A1 medium respectively (Table 5.3).

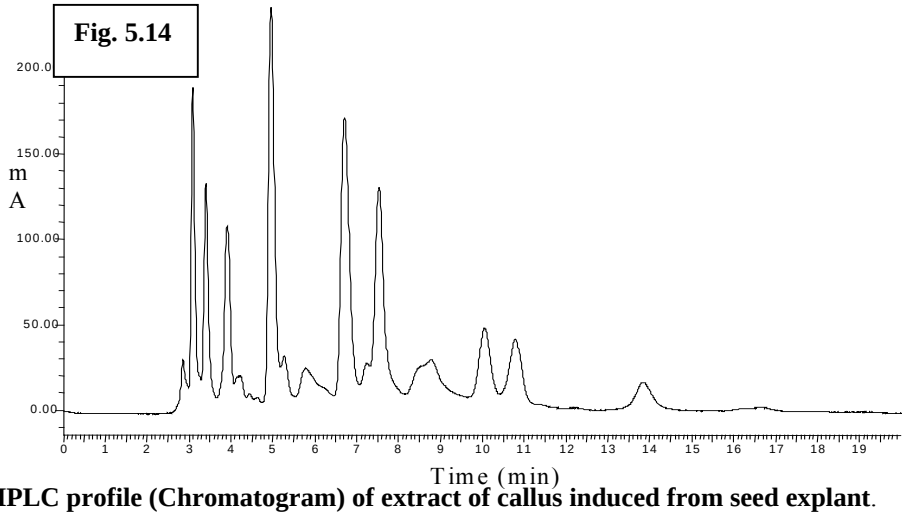


Fig. 5.14 HPLC profile (Chromatogram) of extract of callus induced from seed explant.

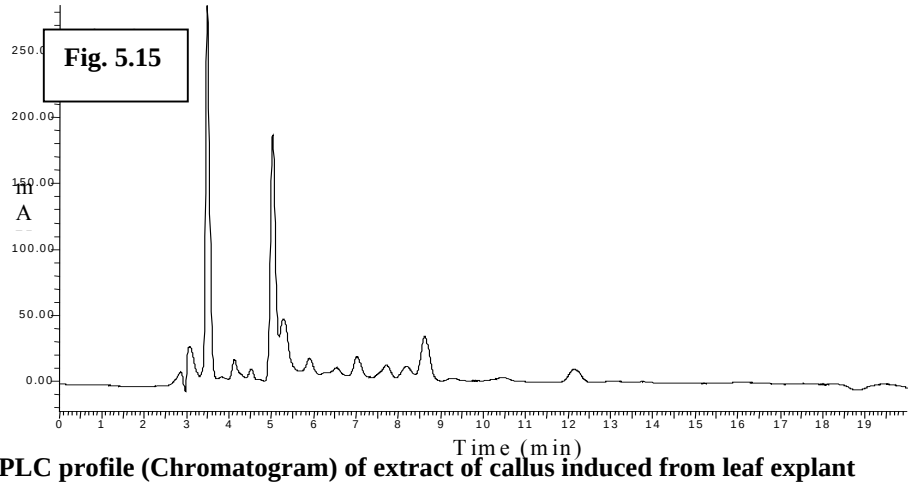


Fig. 5.15. HPLC profile (Chromatogram) of extract of callus induced from leaf explant

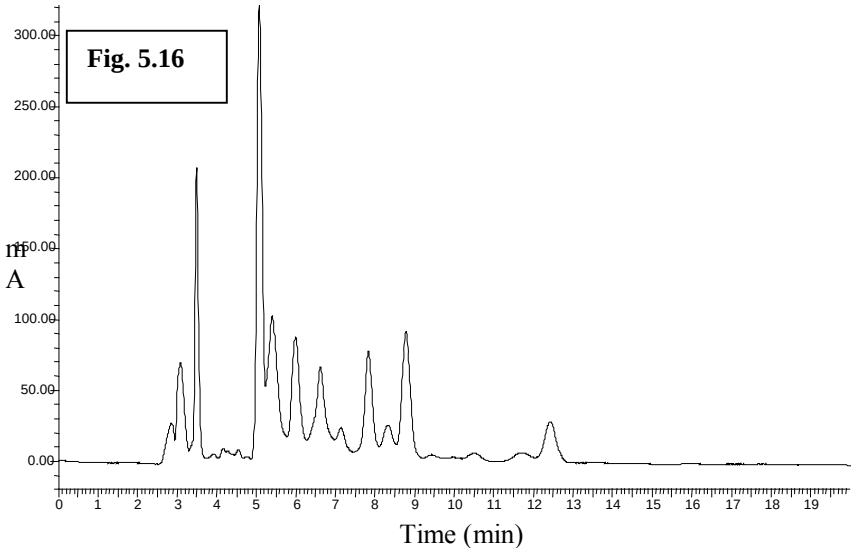


Fig. 5.16. HPLC profile (Chromatogram) of extract of callus induced from nodal/ internodal explant

Compared to calluses induced from seed explants, calluses induced from nodal/ internodal explants expressed much lower quantities of dipyranocoumarins (Table 5.5). Although lower in quantities, similar to that of seed calluses, pattern of higher expression from nodal / internodal calluses was also recorded on A1 – A6 medium (Table 5.5). In nodal/ internodal calluses, maximum inophyllum B (13.52 mg %) on A5, inophyllum P (20.43 mg %) on A4, calophyllolide (32.04 mg %) on A1, inophyllum C (8.73 mg %) on A5, inophyllum A (1.70 mg %) on A3 and inophyllum D (3.3314 mg %) on A1 were recorded (Table 5.5). In nodal/ internodal calluses, highest expressions of inophyllum B, and C were recorded on medium combination A5 whereas highest calophyllolide and inophyllum D were recorded on medium combination A1 (Table 5.5). Similarly, in leaf calluses, highests inophyllum B, inophyllum P, and calophyllolide (19.23, 148.93, and 16.12 mg % respectively) were estimated on A2, A6, and A3 respectively, whereas highest of inophyllum C (1.83 mg %), inophyllum A (6.63 mg %) were estimated on B1, and inophyllum D (0.59 mg %) on B4 (Table 5.4). In seed calluses induced on B1-B8 medium combination, inophyllum B in the range 0.02 – 21.67 mg %, inophyllum P in the range 0.009 – 29.14 %, inophyllum C in the range 0.035 – 23.66 %, Inophyllum A in the range 0.026 – 21.18 mg %, inophyllum D in the range 0.002 – 0.065 mg % and calophyllolide in the 9.59 – 45.19 mg %were estimated (Table 5.3). Similarly in nodal / internodal calluses induced on B1-B8 medium combination, inophyllum B in the range 0.01 – 0.35 mg %, inophyllum P in the range 0.016 – 0.44 mg %, inophyllum C in the range 0.011 – 0.605 %, Inophyllum A in the range 0.058 – 0.61 mg %, inophyllum D in the range 0.007 – 0.067 mg % and calophyllolide in the 0.030 – 1.71 mg % were estimated. Overall medium combinations B1- B8 is less significant or useful for the expression of dipyranocoumarins in callus cultures

induced from seed and nodal/internodal explants whereas only maximum inophyllums C and A on B1 and inophyllum D on B4 in leaf calluses were recorded (Tables 5.3, 5.5 and 5.4).

Table 5.3. Quantitative analysis of dipyranocoumarins in callus cultures induced from seed explants. (Dipyranocoumarin content in mg/100 g FW of callus mass).

Medium code	Inophyllum B	Inophyllum P	Inophyllum C	Inophyllum A	Inophyllum D	Calophyllolide
A1	17.855±3.09	0.297±0.04	41.063±6.09	73.732±12.76	3.450±0.41	24.809±1.71
A2	40.596±4.96	45.836±3.31	142.32±7.2	59.848±0.12	44.727±4.71	45.237±3.04
A3	14.953±1.61	78.149±13.2	52.751±4.63	22.621±3.85	0.102±0.011	35.363±1.94
A4	32.037±1.82	4.991±0.49	17.665±2.14	0.257±0.14	4.069±0.63	39.675±1.69
A5	16.484±2.57	81.858±13.6	46.098±3.81	4.762±0.71	0.175±0.01	24.906±2.35
A6	7.234±1.21	141.350±16.0	84.340±7.53	46.414±8.02	0.561±0.05	29.409±8.18
A7	0.388±0.02	31.618±5.35	20.450±2.62	29.510±5.08	5.833±0.99	16.028±6.18
A8	0.420±0.04	26.675±5.59	10.989±0.25	14.764±1.75	0.009±0.001	6.719±1.79
B1	7.314±1.99	0.363±0.05	12.512±1.27	9.648±0.22	0.032±0.002	22.450±2.80
B2	14.806±3.47	0.009±0.001	17.827±5.44	8.233±1.00	0.012±0.004	15.102±1.47
B3	5.884±0.06	0.037±0.005	11.529±8.82	13.740±3.47	0.003±0.0002	15.881±5.20
B4	21.676±2.13	29.143±5.03	23.667±2.64	21.187±5.14	0.065±0.001	9.596±1.75
B5	3.170±0.33	0.017±0.006	2.017±0.50	0.040±0.01	0.002±0.001	11.576±1.33
B6	0.020±0.01	0.104±0.01	17.117±0.78	8.277±1.14	0.004±0.0003	27.660±3.65
B7	5.158±0.49	0.036±0.002	0.035±0.004	0.026±0.005	0.011±0.001	45.196±9.99
B8	0.147±0.04	0.979±0.07	8.233±0.66	0.029±0.009	0.002±0.0007	35.069±11.8
C1	N R.	N R.	N R.	N R.	N R.	N R.
C2	N R	N R	N R	N R	N R	N R
C3	N R	N R	N R	N R	N R	N R
C4	N R	N R	N R	N R	N R	N R
All values are in gm % (mg / 100 g callus FW) Each value represents the ±S. D. mean of three replicates						

Table 5.4. Quantitative analysis of dipyranocoumarins in callus cultures induced from leaf explants. (Dipyranocoumarin content in mg/100 g FW of callus mass).

Medium code	Inophyllum B	Inophyllum P	Inophyllum C	Inophyllum A	Inophyllum D	Calophyllolide
A1	2.211±0.51	3.262±0.42	0.182±0.01	0.335±0.04	0.141±0.04	1.328±0.89
A2	19.237±1.04	1.459±0.36	0.050±0.03	0.052±0.007	0.026±0.006	0.247±0.02
A3	3.831±0.29	0.090±0.01	0.050±0.0009	0.186±0.01	0.008±0.004	16.125±1.01
A4	0.660±0.05	15.861±5.32	0.112±0.01	0.344±0.02	0.085±0.005	1.189±0.12
A5	0.881±0.06	3.432±0.50	0.011±0.001	0.596±0.04	0.051±0.03	2.890±0.29
A6	0.333±0.02	148.94±23.49	1.228±0.19	0.485±0.07	0.136±0.01	3.747±0.28
A7	0.044±0.004	0.638±0.05	0.214±0.017	0.030±0.008	0.052±0.005	1.843±0.23
A8	0.382±0.05	0.274±0.03	0.188±0.02	0.017±0.004	0.263±0.09	2.068±0.33
B1	0.219±0.009	0.042±0.004	1.833±0.20	6.638±2.02	0.029±0.007	4.874±0.33
B2	0.043±0.01	0.031±0.009	0.096±0.001	2.118±0.30	0.005±0.0009	1.927±0.66
B3	0.175±0.09	0.088±0.03	0.708±0.06	0.069±0.001	0.013±0.003	0.029±0.009
B4	0.268±0.02	0.320±0.15	0.654±0.05	0.743±0.04	0.592±0.26	0.201±0.05
B5	0.020±0.009	0.002±0.0007	0.083±0.004	0.172±0.07	0.019±0.0005	1.187±0.09
B6	0.0467±0.008	0.966±0.28	0.0047±0.001	0.029±0.002	0.016±0.005	1.256±0.50
B7	0.224±0.09	0.589±0.03	0.192±0.09	0.193±0.05	0.113±0.02	1.246±0.16
B8	0.063±0.02	0.525±0.05	0.017±0.001	0.043±0.02	0.009±0.003	0.583±0.16
C1	0.126±0.06	2.633±0.43	0.834±0.10	0.064±0.008	0.548±0.008	2.555±1.83
C2	0.662±0.37	13.956±1.69	1.006±0.43	0.476±0.098	0.061±0.005	2.870±1.00
C3	7.612±1.10	3.092±1.62	0.971±0.42	0.534±0.19	0.409±0.14	4.100±0.50
C4	0.523±0.04	1.078±0.63	0.701±0.07	0.974±0.08	0.903±0.12	2.932±1.42
All values are in gm % (mg / 100 g callus FW) Each value represents the ±S. D. mean of three replicates						

Medium combinations C1- C4 were not as good as A1- A8, since in any of these combinations, expression of inophyllum B was not more than in the ranges of 0.03 – 0.76 mg % from nodal/ internodal explants, 0.12 – 7.61 mg % from leaf explants, inophyllum P, 0.48- 11.33 mg % from nodal/ internodal explant, 1.07- 13.95 mg % from leaf explant, calophyllolide, 4.80- 8.42 mg % from nodal/ internodal explant, 2.55- 4.10 mg % from leaf explant, inophyllum C, 0.10- 0.34 mg % from nodal/ internodal explant, 0.70- 1.00 mg % from leaf explant, inophyllum A, 0.04- 0.66 mg % from nodal/ internodal explants, 0.06 – 0.97 mg % from leaf explants and

inophyllum D, 0.12 – 0.55 mg % from nodal/ internodal, 0.06 – 0.90 mg % from leaf explant (Table 5.4 and 5,5).

Table 5.5. Quantitative analysis of dipyranocoumarins in callus cultures induced from nodal/ internodal explants. (Dipyranocoumarin content in mg/100 g FW of callus mass).

Medium code	Inophyllum B	Inophyllum P	Inophyllum C	Inophyllum A	Inophyllum D	Calophyllolide
A1	4.180±0.056	5.513±0.92	0.190±0.05	0.058±0.01	3.330±0.43	32.049±2.99
A2	8.147±1.01	10.409±1.78	2.174±1.95	0.305±0.03	0.497±0.07	19.576±1.90
A3	1.420±0.22	9.099±0.78	1.483±1.29	1.703±0.27	0.495±0.08	8.308±0.78
A4	1.167±0.88	20.432±2.44	0.798±0.12	0.183±0.01	0.649±0.05	4.894±0.37
A5	13.527±1.28	2.655±0.22	8.730±0.96	0.077±0.02	0.061±0.01	24.037±10.67
A6	3.321±0.32	5.360±2.35	0.357±0.05	0.074±0.05	0.313±0.02	5.308±1.89
A7	0.365±0.04	1.012±0.03	0.529±0.07	0.103±0.01	0.064±0.005	3.720±0.26
A8	0.247±0.03	1.817±0.15	1.317±0.14	0.181±0.03	0.012±0.009	3.431±0.37
B1	0.022±0.009	0.065±0.012	0.011±0.002	0.391±0.05	0.014±0.003	1.526±0.82
B2	0.013±0.001	0.016±0.008	0.070±0.03	0.112±0.009	0.015±0.009	1.711±0.73
B3	0.047±0.002	0.056±0.02	0.062±0.05	0.058±0.02	0.014±0.005	0.818±0.05
B4	0.304±0.03	0.060±0.03	0.024±0.004	0.543±0.04	0.008±0.003	0.150±0.05
B5	0.079±0.009	0.126±0.04	0.079±0.01	0.618±0.05	0.019±0.009	0.030±0.001
B6	0.043±0.007	0.065±0.01	0.605±0.05	0.392±0.02	0.007±0.002	1.191±0.11
B7	0.322±0.02	0.320±0.02	0.096±0.07	0.267±0.12	0.067±0.02	0.039±0.01
B8	0.352±0.01	0.443±0.04	0.050±0.02	0.092±0.007	0.074±0.008	0.126±0.01
C1	0.037±0.002	11.334±1.72	0.117±0.01	0.048±0.02	0.256±0.02	4.800±1.25
C2	0.520±0.04	3.631±0.51	0.101±0.05	0.663±0.10	0.482±0.06	8.330±1.01
C3	0.764±0.11	0.482±0.13	0.342±0.04	0.062±0.03	0.553±0.08	8.235±4.43
C4	0.583±0.04	2.836±1.68	0.218±0.02	0.185±0.01	0.125±0.01	8.420±0.71
All values are in gm % (mg / 100 g callus FW) Each value represents the ±S. D. mean of three replicates						

Table 5.6. Statistical analysis (Two Way ANOVA) for inophyllums' contents in callus cultures induced from seed, leaf and nodal internodal explants on different medium combinations.

Two Way ANOVA	Inophyllum B			Inophyllum P			Calophyllolide		
	Medium	Explant	Med X Exp.	Medium	Explant	Med X Exp.	Medium	Explant	Med.XExp.
S.E	0.00148a	0.0032a	0.00128a	0.0061a	0.007a	0.047a	0.0018a	0.0083a	0.0026a
	0.00095b	0.00087b	0.00089b	0.0017b	0.00030b	0.0017b	0.00054b	0.0021b	0.00092b
±C.D	0.0135a	0.0058a	NS a	0.048a	NS a	NS a	0.014a	0.006a	0.025a
	NS b	NS b	NS b	NS b	NS b	NS b	NS b	NS b	NS b
	a,* *	a,* *	NS a	a*	NS a	NS a	a**	a**	a**
	NS b	NS b	NS b	NS b	NS b	NS b	NS b	NS b	NS b
	Inophyllum C			Inophyllum A			Inophyllum D		
	Medium	Explant	Med X Exp.	Medium	Explant	Med X Exp.	Medium	Explant	Med.X.Exp.
S.E.	0.0031a	0.010a	0.0036a	0.0022a	0.0078a	0.0027a	0.00093a	0.0011a	0.00093a
	0.00044b	0.00034b	0.00013b	0.00060b	0.00067b	0.00056b	0.00020b	0.00023b	0.00019b
±C.D	NS a	0.016a	NS a	NS a	0.017a	NS a	0.0086a	0.0028a	0.015a
	NS b	0.00063b	NS b	0.0010b	0.00071b	0.0014b	NS b	NS b	NS b
	NS a	a,* *	NS a	NS a	a,* *	NS a	a,* *	a,* *	a,* *
	NS b	b,* *	NS b	b,* *	b,* *	b,* *	NS b	NS b	NS b

Statistical analysis was performed separately for A1- B8 and C1- C4 medium. a- Values for A1- B8; b- Values for C1-C8. (**) Significant at 1% level; (*) Significant at 5% level; NS- Non significant at 1% & 5% level S. E. – Standard Error. N R- No Result.

In our chemo diversity study, data collected for contents of inophyllum B and P in leaf and seed tissues collected from different locations along the Western Ghats of India revealed that only 1.55 mg inophyllum B and 0.87 mg inophyllum P per 100 g seed tissue were estimated, whereas only 0.01 mg inophyllum B and 0.006 mg inophyllum P per 100 g leaf tissue were estimated.

Statistically for the production of calophyllolide and inophyllum D, medium combinations A1-B8, all explants and their interaction are significantly different, while only explants were significantly different for inophyllum C and A expression; whereas only explants and media for inophyllum B and only media for inophyllum P were significantly different (Table 5.6). For the production of different secondary metabolites in different plant species, callus cultures have been widely used and reported (Vanisree et, al, 2004). Although there are many repots on induction of callus cultures for production of different secondary metabolites from different plant species, reports on production of coumarins in callus cultures are very few. Kai et al, (2006) studied the accumulation of coumarins scopolectins and scopolin in roots and aerial plant parts as well as in callus cultures of *Arabidopsis thaliana*. Major work on *in vitro* production of coumarins has been done in plant *Ammi majus.* Ekiert and Gomolka (2000) studied expression of coumarin compounds in callus cultures of *A. majus.* The levels of coumarins in callus and cell suspension cultures of *A. majus* were successfully elicited (Hamerski et al., 1990; Staniszewska et al., 2003). There are no reports on *in vitro* callus induction in plants belonging to genus *Calophyllum* for the production of anti HIV dipyranocoumarins i.e. inophyllums. We, for the first time report that establishing callus cultures can be promising method to produce inophyllums. Moreover contents of inophyllums can be enhanced further by medium manipulation, cell suspension culture and elicitation with different biotic as well as abiotic elicitors.

5. 4 CONCLUSIONS:

A method for callus induction from different explants of *C. inophyllum* was described. WPM basal medium was most suitable basal medium for seed germination and callus induction. Three types of explants viz. seed, leaf and nodal/ internodal explants were found most effective explants in inducing calluses. Combinations of 2, 4-D, kinetin and BAP were not suitable for callus induction, whereas combinations of IBA, NAA, picloram and BAP in media were most effective in inducing callus. Calluses induced from seed explants were white or brown, friable, while calluses from leaf and nodal/ internodal explants were dark brown and nodular compact. Induction of calluses from leaf and nodal/ internodal explants took place ten days earlier as compared to induction from seed explants. Frequency of callus inductions from seed explants on medium incarporated with IBA alone and with BAP was almost same to that of frequency achieved by incorporating NAA alone and with BAP in the medium. IBA 19.60 µM along with BAP 4.44 µM and NAA 10.74 µM along with BAP 8.88 µM were optimum for callus induction from seed explants. All concentrations of picloram along with BAP consistently resulted in higher frequencies of callus induction from leaf and nodal/ internodal explants. Picloram 24.84 µM with BAP 8.88 µM

was the optimum for callus induction from these explants i.e. leaf and nodal/ internodal explants. Supplementation of only IBA in the medium was as good as supplementation of picloram and BAP for callus induction from nodal/ internodal explants. Repeated sub culturing or transferring was required to keep calluses in viable condition. Unlike leaf and nodal/ internodal calluses, seed callus became necrolytic after two sub cultures and ceased further growth.

Sequential extraction of callus masses with acetone and mixture of chloroform and water was optimum extraction method for dipyranocoumarins. HPLC method developed with μPorasil HPLC column, mixture of ethyl acetate and petroleum ether as mobile phase and recording of chromatograms at 245 nm were the optimum parameters for HPLC analyses and separated all dipyranocoumarins under study in a single isocratic mode of 20 min run time. Highest anti HIV inophyllum B (40.59 %) was expressed in callus induced from seed explant on medium containing 9.80μM IBA, while highest inophyllum P (141.35 mg %) was estimated in seed callus induced on medium containing IBA 9.80 μM along with BAP 4.44 μM.

This report described the protocol to study the *in vitro* expression of anti HIV phytochemicals like inophyllums. It also emphasized the use of callus cultures as an initial *in vitro* system for studying *in vitro* expression pattern of such phytochemicals. This study demonstrated the use of HPLC method for quantitative and qualitative analyses of inophyllums. HPLC method reported in this study can be further modified and extended for preparative isolation of inophyllums from plant material as well *in vitro* grown cultures. This study not only described the simple protocol for induction of callus cultures and their analysis but will also be of immense help in establishing and selecting hyper producing cell lines or callus cultures. Such selected cultures would be then used in establishing protocol for cell suspension culture and elicitation to enhance *in vitro* expression and production of inophyllums.

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CHAPTER 6. ESTABLISHMENT OF CELL SUSPENSION CULTURES FOR INCREASED BIOMASS AND DIPYRANOCOUMARINS' EXPRESSION.

6.1. INTRODUCTION:

Until the work of Zenk and co-workers, it had been considered for long time that unlike differentiated cells or specialized organs, undifferentiated cell such as callus and cell suspension cultures were not able to produce secondary metabolites (Krikorian et al., 1969). Zenk and co- workers experimentally demonstrated that this theory was wrong by observing dedifferentiated cell cultures of *Morinda citrifolia* yielding 2.5 g of anthraquinones per liter of the medium (Zenk, 1991). This finding opened the new era to a large community of plant cell culturist who extensively studied the possible use of plant cell culture for production of secondary compounds.

For production of secondary metabolites in cultured cells / tissues, after choosing the most promising plants, the real work begins with *in vitro* induction of callus cultures. This work mainly consists of determining the medium that is the best suited for callus induction. Once calli are obtained, it is well known that they can undergo somaclonal variations, usually during several subculture cycles. Somaclonal variations are variations arising *in vitro* in the plant system due to culture conditions. Due to such somaclonal variations, secondary metabolite production is often variable from one subculture cycle to another. It becomes necessary to screen the different callus lines according to their aptitude to provide an efficient metabolite production. Hence each callus line must be assessed separately for its growth speed as well as intracellular and extracellular metabolite concentration. This allows the evaluation of the productivity of each cell line so that only the best ones can be taken for cell suspension culture.

6.1.1. Cell suspension culture:

Cell suspension culture could be defined as "a process that allows rapidly dividing homogeneous suspensions of cells to grow in liquid nutrient media". For initiating cell suspension cultures, prerequisite is callus cultures. Callus cultures are initiated from explants which are isolated from plant material. Such explants are then plated on a solid growth medium which supports the growth of explant. Growth media that consist of carbon source, minerals, phytohormones and antioxidants, are tailored specific for different species. Under suitable conditions, the explants grow into undifferentiated mass of cells known as callus. The calli are subsequently transferred from solid to liquid media resulting in cell suspension culture, which are then incubated under agitation and controlled temperature. Such plant cell suspension cultures consist of undifferentiated cells derived from explant tissue. These cultures can be indefinitely maintained in undifferentiated state by frequent sub culturing or with the addition of phytohormones to the media, such as auxines and cytokinins. This scientific method can be exploited for mass propagation of quality planting materials by micropropagation (i.e. *in vitro*

clonal propagation), mass production of quality and useful secondary metabolites, development of new varieties (via mutagenesis, etc.) as well as the production and enhancement of natural pharmaceutical, nutraceutical and cosmaceutical compounds. Cell suspension culture systems could be used for the large-scale culture of plant cells from which secondary metabolites can be extracted. The principal advantage of this method is that it may ultimately provide a continuous, reliable source of natural products year-round. In addition, compounds from such tissue cultures may be more easily purified because of simple extraction procedures and the absence of significant amounts of pigments, thus possibly reducing production and processing costs.

Although production of plant secondary metabolites via cell suspension culture is renewable, environmental friendly and easy to process for product recovery, the primary challenges impeding regular commercial application of plant cell culture technology are low and variable yields of metabolite accumulation. Some metabolites do not accumulate in appreciable quantities in undifferentiated cells, which necessitate manipulation of genes within the biosynthetic pathways to utilize the cell suspension culture for bulk production. In most of the cases, manipulating the genes within the biosynthetic pathways remains unrealistic due to a lack of complete knowledge regarding secondary metabolic pathways. Moreover, plant cell suspension cultures are subject to the hydrodynamic force resulting from mechanical agitation. Plant cells are much larger than mammalian cells or microbes that make them extremely susceptible to shear forces in surrounding fluid. This susceptibility in shear forces reduces cell viability, release intracellular components and result in change in metabolism and morphology. Much improvement is needed to be made for increasing yields of secondary metabolites that accumulate at low level. However, little progress has been made in understanding and controlling the unstable secondary metabolite production pattern. The maintenance of consistently high production level has proven to be difficult, and gradual loss of secondary metabolite productivity over the time has long been known as an obstacle (Deusneumann et al, 1984; Qu et al, 2005). In some cases, metabolite accumulation in cell suspension cultures may be limited by feed back inhibition and product degradation. Also many secondary metabolites may be toxic to cultures at high level.

These difficulties apart, cell suspension culture still remains method of choice for secondary metabolite production. Cell suspension culture method has been successfully employed in the production of secondary metabolites from numerous plant species (Chapter 1, Table 1.2). Expression of secondary metabolites can be enhanced by number of ways, such as exogenous supply of hormones, nutrient manipulation, elicitation and precursor feeding. Manipulation of culture environment in many cases was found to be effective in increasing the product accumulation. The expression of many secondary metabolite pathways is easily altered by external factors such as nutrient level, stress factor and growth regulators (Rao et al, 2002). Many of the

constituents of plant cell culture media were shown to be the important determinants of growth and accumulation of secondary metabolites (Misawa, 1985; Stafford et al., 1986).

In the present study, experiments were conducted to establish cell suspension cultures in *C. inophyllum*. Induction of callus cultures from seed, leaf and nodal/ internodal explants and pattern of expression of dipyranocoumarins in those callus cultures have been discussed in previous (Chapter 5). Using those callus cultures, cell suspension cultures were established to study the effects of hormones and nutrient manipulations. Effect of hormones and medium components such as nitrate, sulphate, vitamins and sucrose on biomass growth and dipyranocoumarins' expression in cell suspension cultures of leaf and nodal / internodal callus would be discussed.

6.2. EXPERIMENTAL PROTOCOLS:

6.2.1. Induction of callus cultures:

A protocol for induction of callus cultures has been described in previous chapter (Chapter 5). For optimum induction of callus cultures, agar solidified WPM basal medium with previously optimized hormone combinations and concentrations for each explant type were used. Calluses were induced from seed explants on WPM basal medium supplemented with IBA 19.60 µM + BAP 4.44 µM, from nodal/ internodal explants on WPM basal medium supplemented with IBA 19.60 µM and from leaf explants on WPM basal medium supplemented with picloram 24.84 µM + BAP 8.88 µM. These callus cultures were maintained by sub culturing and transferring after every 45 days on their respective fresh medium containing same hormone combinations and concentrations until their further use for initiating suspension cultures.

6.2.2. Initiation of cell suspension cultures:

Cell suspension cultures were established by inoculating the weighed quantities of 2-3 month old callus masses in 250 ml Erlenmayer flasks containing 100 ml liquid WPM medium. Initially cell suspension cultures were established by inoculating all three types of calluses i. e. seed, nodal/ intenodal and leaf calluses. These cultures were incubated on gyratory shaker rotating at 120 rpm. The temperature and photoperiod was mainained at 25±1°C under cool white fluorescent continuous light (35 µmol m^{-2} s^{-1}; Philips, India). The sub-culturing of suspension cultures to fresh media was done after every 30 days of incubation.

6.2.3. Study of growth kinetics:

Effect of different incubation period (No. of Days) on growth of biomass and expression pettern of dipyranocoumarins in cell suspension cultures of nodal/ internodal and leaf

calluses was studied to optimize incubation period required for optimum biomass growth and maximum expression of dipyranocoumarins. Cell suspension cultures were initiated by transferring weighed quantities (about 2-3 g) of calluses in liquid WPM without any phytohormones and medium component manipulations. These cell suspension cultures were then harvested after 10, 20, 30, 40 50 and 60 days of incubations for estimation of biomass growth and extracted for HPLC analysis for the study of dipyranocoumarins' expression.

6.2.4. Medium manipulations:

For studying the effects of different phytohormones, hormone combinations and their concentrations which were used previously for callus induction and resulted in good callus inductions, were also studied in cell suspension cultures for their effects on growth of biomass and expression pattern of dipyranocoumarins. Initially, all three types of calluses were inoculated in liquid WPM basal media with 2 % sucrose and incorporated with IBA (4.90 – 19.60 μM), IBA + BAP (4.90 – 19.60 μM + 4.44 μM), Picloram + BAP (8.28 – 33.12 μM + 8.88 μM).

For studying the effects of different medium component manipulation, concentrations of initial sucrose, total nitrate, total sulphate and total vitamins were varied in liquid WPM medium. From the composition of WPM basal medium (Given in Chapter 2), separate stocks of total nitrate (NH_4NO_3- 0.4 g/L + Ca $(NO_3)_2$- 0.556 g/L), sulphate ($MgSO_4$ - 0.370 g/L + K_2SO_4- 0.990 g/L + $MnSO_4$ - 2943 μg/L + $ZnSO_4$ – 8600 μg/L + $CuSO_4$ – 2650 μg/L) and vitamins (Nicotinic acid- 0.5 mg/L + Thymine HCl –1.0 mg/L + Pyridoxin HCl - 0.5 mg/L + Glycin – 2.0 mg/L + Inositol – 100 mg/L) were prepared. Sucrose concentration was varied in the range of 0.5-6.0 % whereas total sulphate, total nitrates and total vitamins were varied in the range of nil - 4.0 fold (nil – 5X) increase. All these media combinations with varied concentrations of total sulphate; total nitrates and total vitamins were inoculated with leaf and nodal/ internodal calluses to initiate suspension cultures of leaf and nodal / internodal calluses.

6.2.5. Extraction of cell suspension cultures:

Except for the experiments to optimize incubation time, extraction of cell suspension cultures was done between 45 - 50 days of incubation. Suspension cultures were filtered through Whatmann's filter paper No-1 to collect biomass and culture filtrate. Culture filtrate was partitioned with chloroform in separating funnel. After thorough mixing, separating funnel was kept aside for 10 min to separate two immiscible solvents. Thereafter, chloroform layer was separated and concentrated. This chloroform extract of culture filtrate was then dissolved in HPLC mobile phase and used for HPLC analysis. Remaining biomass collected after filtering the cell

suspension cultures were weighed and biomass growth was determined by using following formula.

$$\% \text{ Biomass growth} = \frac{\text{Final Weight} - \text{Initial Weight}}{\text{Initial Weight}} \times 100$$

After determining the % biomass growth, biomass was extracted with extraction method described in chapter 5.

6.2.6. HPLC analysis of cell suspension cultures:

Chloroform extracts of culture filtrate and biomass were dissolved in 1.0 ml HPLC mobile phase (25% ethyl acetate in petroleum ether, v/v). These samples were then analyzed with previously standardized HPLC method to study the expression pattern of dipyranocoumarins. The details of the HPLC method, its develoment and validation have been discussed in Chapter 3 and 5.

6.3. RESULTS AND DISCUSSION:

For induction of callus cultures, the conditions like basal medium, hormone combinations and their concentrations and explant types were studied and discussed in previous chapter (Chapter 5). During callus induction, it was observed that callus masses induced from seed explants were initially white, friable and irregular in appearance and turned dark brown colored on subsequent sub-culturing and transferring. After two sub-cultures or transfers, these seed calluses became more friable, brown, necrolytic and ceased further growth. When cell suspension cultures were established by transferring such friable, brown seed calluses to liquid WPM medium, there was no biomass growth. Either callus masses did not remain viable or completely turned necrolytic and ceased further growth. Integrity of seeds callus masses in cell suspension culture was also a critical factor. In cell suspension cultures of seed callus, integrity of cell aggregates was disturbed which was also a responsible factor for seed callus biomass not growing in cell suspensions. On the contrary, similar problem was not observed with cell suspension cultures of leaf and nodal / internodal calluses. For selecting the callus masses to initiate suspension culture, % induction from each explant and quality of callus masse were considered as selection criteria. Because cell suspension culture of seed callus did not result any growth of biomass, cell suspension cultures with seed calluses were not initiated for further studies. Since callus induction responses from nodal/ internodal explants on WPM incorporated with IBA 19.60 µM and from leaf explants on WPM incorporated with picloram 24.84 µM + BAP 8.88 µM were maximum, these media combinations were used to induce callus cultures from these explants. These callus cultures were then used for initiating cell suspension cultures.

Integrity of callus masses in cell suspension culture was prerequisite for effective growth. At the time of inoculation, the integrities of leaf and nodal / internodal calluses played important role. Chopping or making leaf and nodal / internodal calluses into very small pieces before transferring to liquid media determined the biomass growth. Very small pieces of these callus masses poorly grew in cell suspension cultures. Callus masses of approximate size more than 3X3 mm and their aggregates were best suited for growth in suspension cultures. Age of the callus masses used for initiating suspension cultures also played a significant role in growth of biomass. Suspension cultures initiated with calluses of less than two month old age resulted in very poor and minimum biomass growth, whereas suspension cultures initiated with 2 – 3 month old calluses showed significantly enhanced biomass growth.

Simultaneous extractions of culture filtrates were also carried out and analyzed with HPLC to estimate the release of dipyranocoumarins in medium. HPLC analyses of the culture filtrate showed that dipyranocoumarins were not released in media. Dipyranocoumarins being insoluble in highly polar solvent like water might have contributed to their not being released in medium. HPLC profiles of all the suspension cultures revealed that there were no significant differences in HPLC profiles of the suspension cultures. HPLC profiles of the extracts of the biomasses from all suspension cultures were more or less similar showing no expression of new metabolites. Chromatograms were different in terms of only the levels of dipyranocoumarins under study. This indicated that secondary metabolites spectrum in suspension cultures of *C. inophyllum* was not changed by cell suspension conditions and medium component manipulation.

6.3.1. Time course of biomass Growth:

In order to study the effect of incubation period on biomass growth and to optimize the number of incubation days required for maximum biomass growth and expression of dipyranocoumarins in suspension cultures of both callus types, experiments were carried out by initiating cell suspension cultures with both, leaf and nodal / internodal calluses. In this regard, growth of biomass and accumulation of dipyranocoumarins were estimated after incubation period of every 10 days up to 60 days. Phytohormones are known to influence the biomass growth as well as production of secondary metabolites in different plant species (reviewed in Chapter 1, Table 1.1 and 1.2). Accumulation of secondary product like phenylpropanoids were shown to be affected by phytohormones independently from their effect on growth (Funk and Brodelius, 1990; Meyer and Van Staden, 1995). To rule out any such possibilities of hormonal interference and influence during the study of growth kinetic, cell suspension cultures were initiated by inoculating calluses in liquid WPM basal medium without any growth hormones. The results for effect of incubation

period on biomass growth in cell suspension cultures of leaf and nodal/ internodal callus have been given in Fig. 6.1.

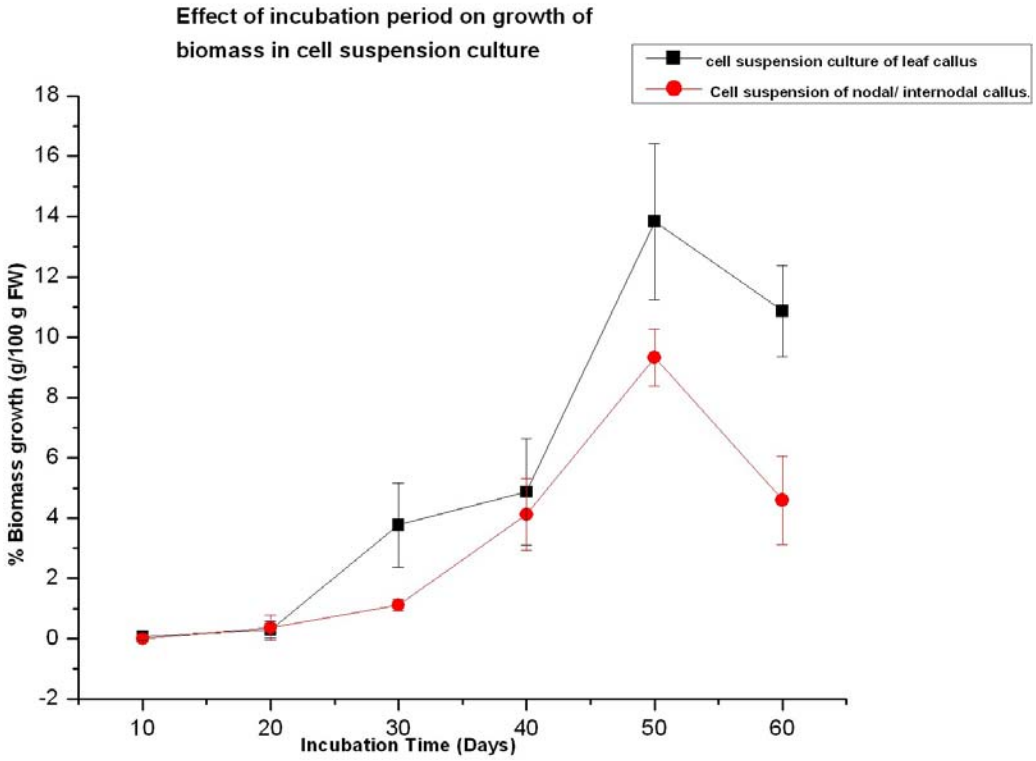

Fig. 6.1. Effect of incubation period on biomass growth in suspension cultures of leaf and nodal / internodal callus.

The time course of biomass growth in cell suspension cultures of leaf callus and nodal / internodal callus followed typical growth curve like pattern. In cell suspension cultures of both calluses, biomass did not increase till 20 days. This lag phase was subsequently followed by exponential phase in which biomass started growing in cell suspension after 20 days of incubation. During 20 – 40 days, biomass growth was comparatively higher in suspension culture of leaf callus than nodal / internodal callus. Exponential growth of biomass was followed up to 50 days. Maximum biomass growth in suspension cultures of leaf callus (13.83 %) and nodal / internodal callus (9.32 %) was observed at 50 days incubation. At the incubation of 50 days, biomass growth in suspension cultures of leaf callus was higher than suspension culture of nodal / internodal callus. Further incubations for more than 50 days did not result biomass growth in suspension cultures of both callus types. Compared to the herbs or shrub plant species, higher woody plant species are known to be slow in their growth. Sometime their slow growth rate is also reflected in *in vitro* growing cultures. *C. inophyllum*, being slow growing woody tree species, its *in vitro* cultures grew slowly. This is why for maximum biomass growth in suspension cultures required incubation period up to 50 days. This results suggested that incubation period of 50 days was optimum for

biomass growth in cell suspension cultures of leaf and nodal / internodal calluses. Therefore, in the next experiments for study of effects of hormones and medium component manipulations on biomass growth and expression patteren of dipyranocoumarins, incubation for 50 days was considered as optimum and extraction of cell suspension cultures were carried out between 45- 50 days of incubation.

6.3.2. Expression pattern of dipyranocoumarins during growth curve:

During the study of biomass growth curve, biomass harvested at different incubation period were also extracted and analyzed with the HPLC method for studying the expression pattern of dipyranocoumarins in cell suspension cultures of both, leaf and nodal/ internodal calluses. The results for effect of incubations period on expression of inophyllums A, B, C, D, P and calophyllolide have been given graphically in Fig 6.2, 6.3, 6.4, 6.5, 6.6 and 6.7 respectively.

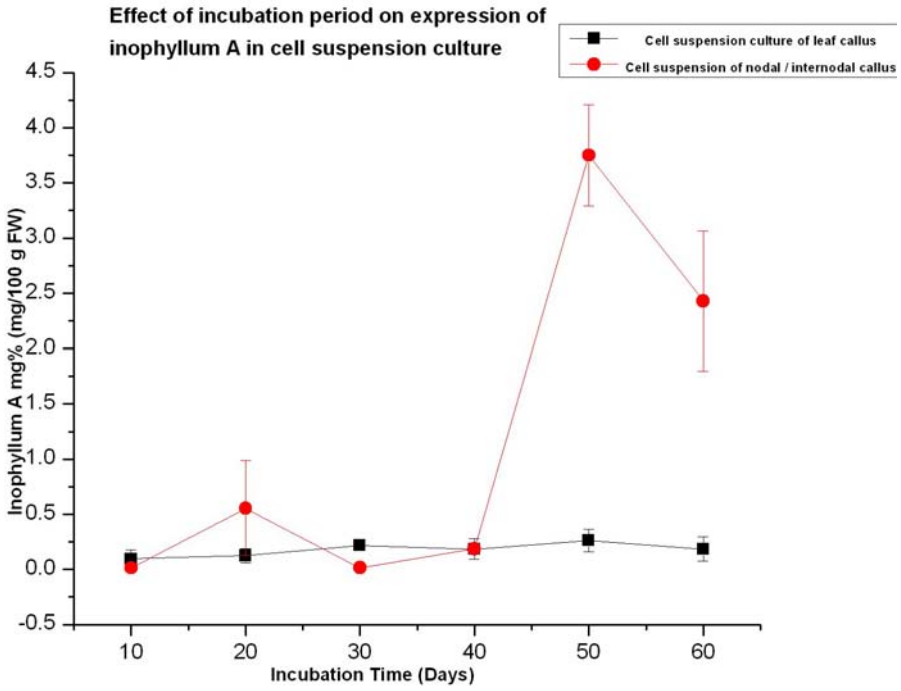

Fig. 6.2. Effect of incubation period on expression of inophyllum A in suspension cultures of leaf and nodal / internodal callus.

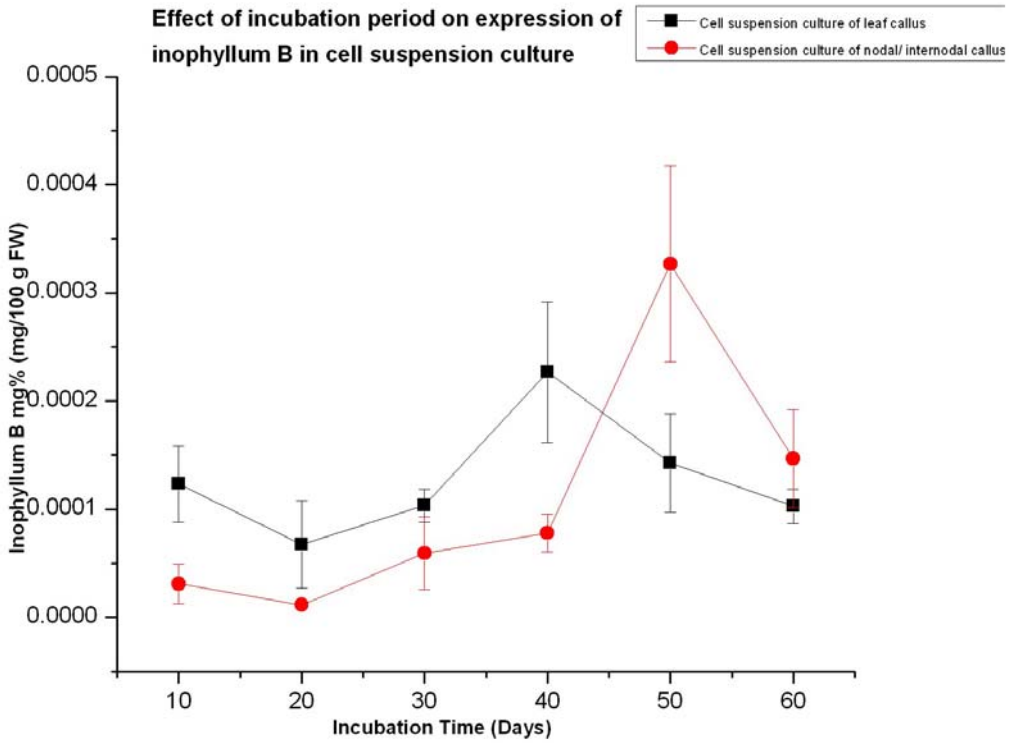

Fig. 6.3. Effect of incubation period on expression of inophyllum B in suspension cultures of leaf and nodal / internodal callus.

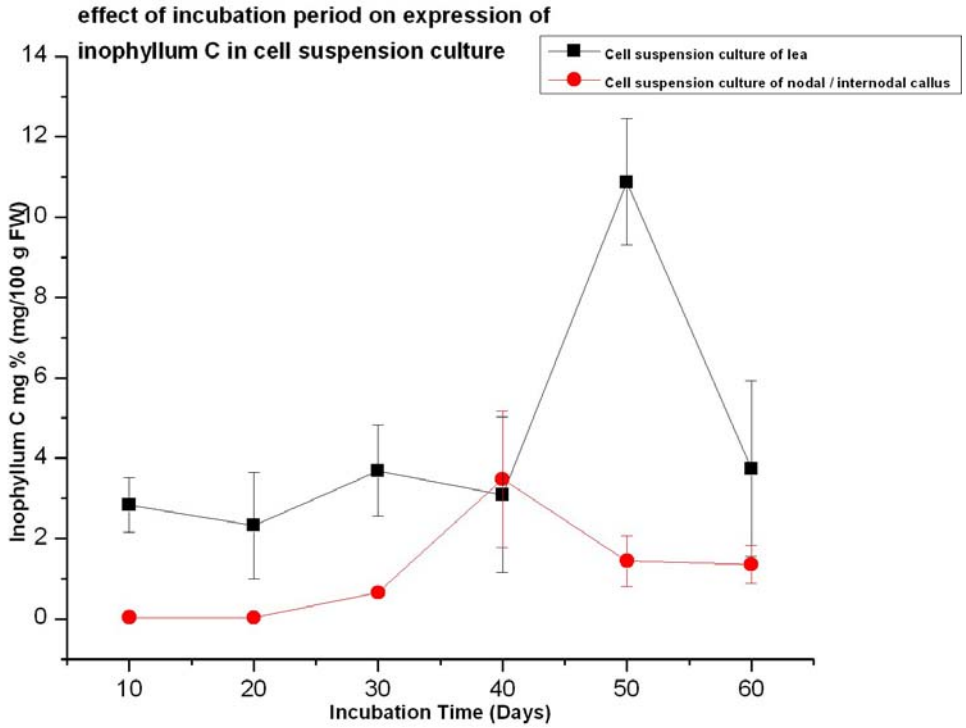

Fig. 6.4. Effect of incubation period on expression of inophyllum C in suspension cultures of leaf and nodal / internodal callus

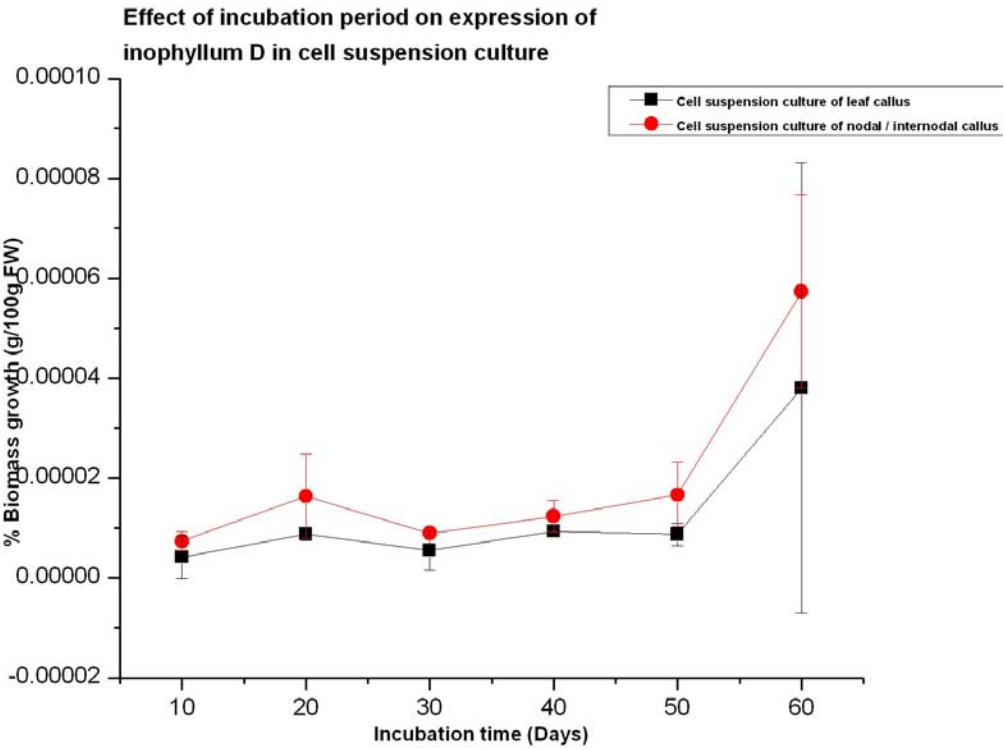

Fig. 6.5. Effect of incubation period on expression of inophyllum D in suspension cultures of leaf and nodal / internodal callus.

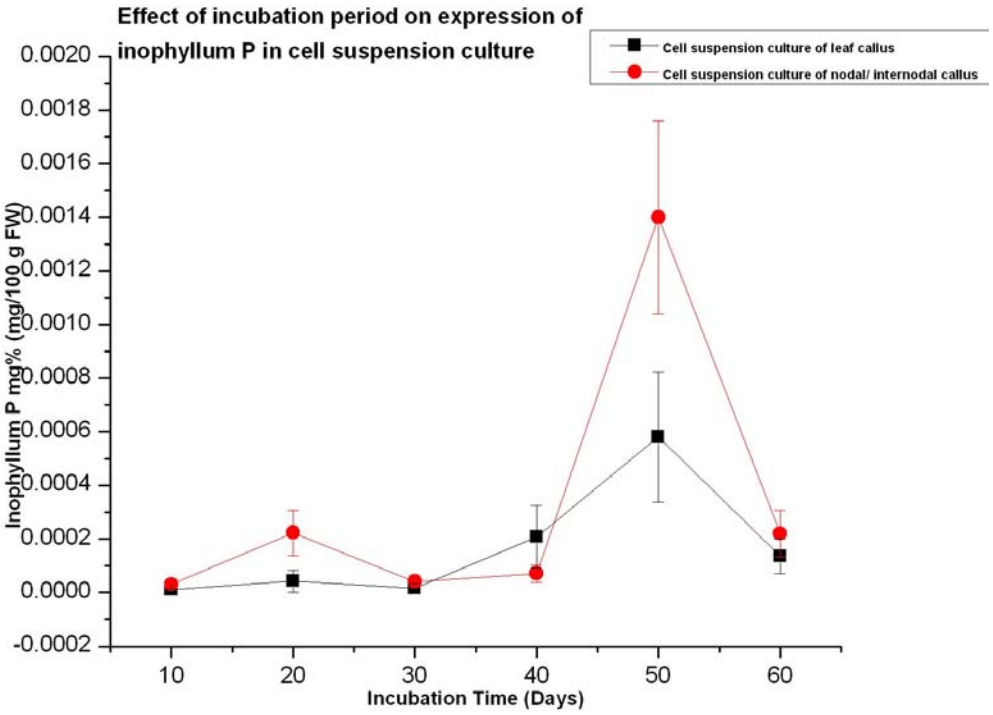

Fig. 6.6. Effect of incubation period on expression of inophyllum P in suspension cultures of leaf and nodal / internodal callus.

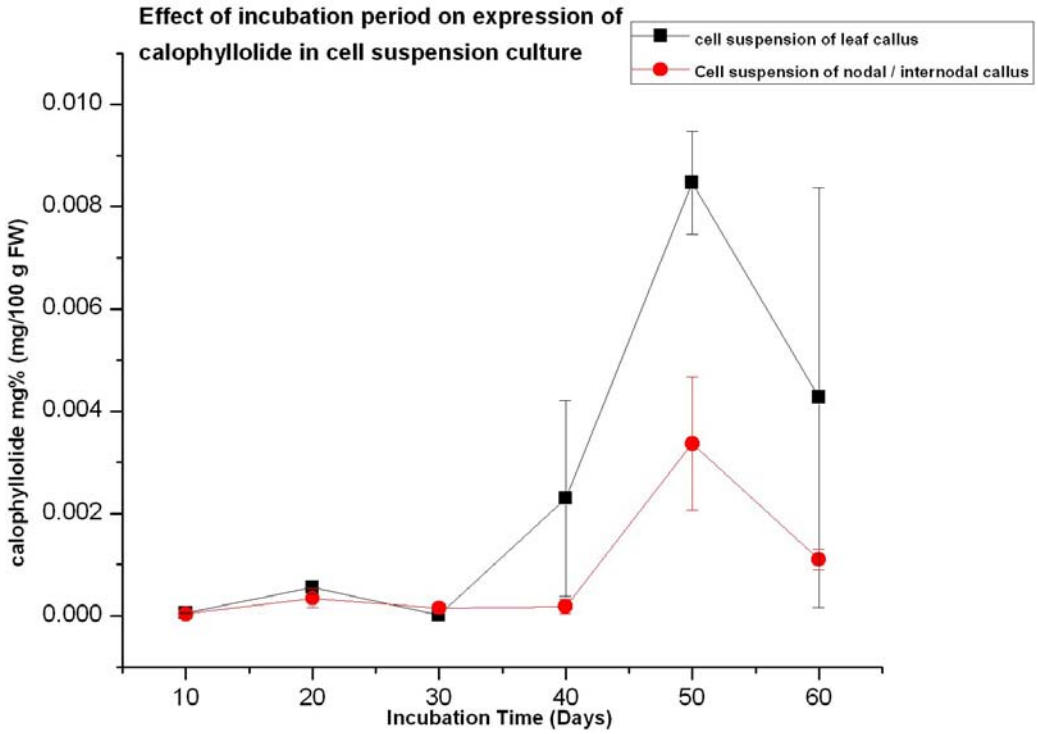

Fig. 6.7. Effect of incubation period on expression of Calophyllolide in suspension cultures of leaf and nodal / internodal callus.

6.3.2.1. Inophyllum A:

In suspension cultures of leaf and nodal / internodal callus, maximum accumulation of inophyllum A was observed on 50 days incubation (Fig. 6.2). In cell suspension of nodal / internodal callus, during 10- 20 days, expression of inophyllum A was slightly increased, then it was decreased during 20- 30 days. Incubation for more than 30 days resulted further increase in expression of inophyllum A (Fig. 6.2). Increase in expression of inophyllum A in suspension cultures of nodal / inetrnodal during 30 – 40 days was less when compared to 40 – 50 days incubation (Fig. 6.2) i.e. maximum accumulation (3.75 mg %) was between 40 – 50 days. In suspension cultures of leaf callus, inophyllum A expression was increased during 10 – 30 days, decreased during 30 – 40 days and then increase to maximum (0.26 mg %) during 40 – 50 days (Fig. 6.2).

6.3.2.2. Inophyllum B:

Expression pattern of inophyllum B in cell suspensions of both callus types between 10 – 30 days followed the similar pattern of accumulation (Fig. 6.3). Maximum accumulation of inophyllum B (0.00026 mg %) was estimated during 30 – 40 days in cell suspension of leaf callus, whereas it was 0.000326 mg % during 40 – 50 days in cell suspension of nodal / internodal callus.

Incubation for more than 40 days and 50 days of suspension cultures of leaf and nodal / internodal calluses respectively did not result further increase in expression of inophyllum B (Fig. 6.3).

6.3.2.3. Inophyllum C:

In suspension cultures of leaf callus, expression of inophyllum C was slightly decreased during 10 – 20 days. This initial decline was followed by increase during 20 – 30 days and again slight decrease during 30 – 40 days. Maximum increase in inophyllum C expression (10.87 mg %) was observed during 40 – 50 days. Maximum inophyllum C was accumulated on 50 days of incubation (Fig. 6.4). Pattern of expression of inophyllum C in suspension culture of both callus types during 10 – 30 days was almost similar. It was during 30 – 40 days; maximum inophyllum C (3.47 mg %) was accumulated in suspension cultures of nodal/ internodal callus and its accumulation started decreasing thereafter (Fig. 6.4).

6.3.2.4. Inophyllum D:

The expression pattern of inophyllum D in cell suspension cultures of both callus types was similar (Fig. 6.5). In both of these suspension cultures, accumulation of inophyllum D increase during 10 – 20 days followed by slight decrease during 20 – 30 days and increased during 30 – 60 days incubation. Maximum accumulation of inophyllum D in suspension cultures of leaf callus (3.8×10^{-5} mg %) and nodal / internodal callus (5.7×10^{-5} mg %) was estimated during 50 – 60 days (Fig. 6.5).

6.3.2.5. Inophyllum P:

During 10 – 30 days of incubation, inophyllum P followed the similar pattern of expression in cell suspension of both callus types. It was slightly increased during 10 – 20 days followed by slight decrease during 20 – 30 days. During 30 – 40 days, there was increase in accumulation of inophyllum P and it further increased to maximum 0.00058 mg % and 0.0014 mg % in suspension cultures of leaf and nodal / internodal callus respectively during 40 – 50 days (Fig. 6.6).

6.3.2.6. Calophyllolide:

In suspension cultures of both callus types, accumulation pattern of calophyllolide was similar to accumulation pattern of inophyllum P. In suspension cultures of these callus types, accumulation of calophyllolide was slightly increased during 10 – 20 days, then slightly decreased during 20 – 30 days and significantly increased to maximum 0.0084 mg % during 30 – 50 days. Like inophyllum P, maximum (0.0033 mg %) accumulation of calophyllolide was estimated during

40 – 50 days (Fig. 6.7). At 50 days, level of accumulation in suspension culture of leaf callus was higher than suspension culture of nodal / internodal callus.

Overall expression pattern of dipyranocoumarins over the incubation period of 60 days revealed that except the expression of inophyllum B in suspension cultures of leaf callus, expression of inophyllum C in suspension cultures of nodal/ internodal callus (which was maximum during 30 – 40 days) and expression of inophyllum D in suspension cultures of both callus types (which was maximum during 50 – 60 days), expressions of most of the dipyranocoumarins in suspension cultures of both callus types were maximum during 40 – 50 days of incubations. It was concluded that incubation period for about 40 – 50 days was best suited for the maximum expression of dipyranocoumarins. Hence, in the next all experiments, incubation period for about 45 – 50 days was considered optimum.

6.3.3. Effect of hormones on biomass growth and expression pattern of dipyranocoumarins in cell suspension culture of leaf callus:

WPM basal medium supplemented with picloram 24.88 µM + BAP 8.88 µM was found to be best suited for induction of callus cultures from leaf explants (discussed in Chapter 5). For initiating cell suspension cultures of leaf callus, callus masses were induced and grown on this medium. As and when required, callus masses were maintained by sub culturing on the same medium. Callus masses of leaf explants induced on this medium were then transferred to liquid WPM medium to initiate cell suspension cultures of leaf callus. Since maximum biomass growth and higher expression of most of the dipyranocoumarins in suspension cultures of both callus types was observed during 40 – 50 days of incubation, harvesting and extraction of all suspension cultures were done between 45 – 50 days of incubation. The results for effects of hormones on biomass growth and expression of dipyranocoumarins are given in Table 6.1.

Maximum 54.60% biomass growth was resulted in WPM supplemented with higher concentration of IBA i. e. 19.60 µM alone. This biomass growth was almost more than three times (3.6 times) the biomass growth resulted in control medium (without hormones). On addition of BAP 4.44 µM to the same medium reduced biomass growth to 33.50 % (2.21 times). In comparison of IBA, picloram (8.28 – 33.12 µM) along with BAP (8.88µM) constant was not much effective for biomass growth. Maximum 16.24 % (1.07 times) biomass growth was resulted in a medium containing high picloram (33.12 µM) with BAP (8.88µM). Overall, addition of IBA alone in the range 4.90 – 19.60 µM was found to be best suited and optimum for biomass growth in suspension cultures of leaf callus. This concentration range of IBA increased biomass in the range of 19.76 – 54.60 % (1.30- 3.6 times). Addition of BAP 4.44 µM constant to the medium incorporated with same range of IBA reduced biomass growth to the range 19.44 – 33.50% (1.28 -

2.21 times). Picloram in the range of 8.28 – 33.12 µM along with BAP 8.88 µM constant was not as effective for biomass growth as was IBA alone in range of 4.90 – 19.60 µM. Only picloram 16.56 and 33.12 µM along with BAP 8.88 µM resulted only 15.24 – 16.24 % biomass growth.

Although significantly higher biomass growth was resulted in medium containing IBA (4.90 – 19.60 µM), expression of inophyllum A with this growth in biomass was reduced. Supplementation of IBA alone did not show positive influence on expression of inophyllum A. With the exception of IBA 14.70 µM + BAP 4.44 µM, presence of BAP 4.44 µM with the same range of IBA also did not enhance the expression of inophyllum A. Accumulation of inophyllum A was slightly (1.5 times) higher (0.3 mg%) in medium incorporated with IBA 14.70 µM + BAP 4.44 µM than control (0.2 mg%). Picloram in the range 8.28 – 33.12 µM + BAP 4.44 µM, which did not result in good biomass growth as compared to IBA alone or in combination of BAP, however resulted in maximum expression of inophyllum A. Maximum 59.01 mg % (295.05 times) and 8.33 mg % (41.65 times) inophyllum A was expressed in a medium containing picloram 8.28µM and 16.56 µM respectively along with BAP 8.88 µM constant. This expression of inophyllum A was much higher than the expression in control medium. This type of increased biomass growth and reduced expression of inophyllum A clearly suggested the inverse relationship between biomasss growth and expression of inophyllum A.

Expression of inophyllum B, similar to the expression of inophyllum A was also reduced with most of the hormone combinations tried. Highest 0.033 mg % (25.38 times) inophyllum B was expressed in medium containing IBA 14.70 µM only. Addition of BAP 4.44 µM along with IBA 14.70 µM reduced the inophyllum B expression to 0.007 mg % (5.38 times). When compared to the expression of inophyllum B in control (0.0013 mg %), rest of the IBA combinations reduced the expression of inophyllum B. However, reduction in inophyllum B expression in medium supplemented with picloram in the range 8.28 – 33.12 µM + BAP 8.88 µM (which resulted minimum biomass growth) was comparatively less when compared to medium containing either IBA alone or along with BAP (which resulted maximum biomass growth). This trend of expression of inophyllum B also suggested the inverse relationship between biomass growth and expression of inophyllum B.

Table 6.1. Effects of hormones on expression (accumulation) pattern of dipyranocoumarins in suspension cultures of leaf callus.

Hormones (μM)			% Biomass increase	Dipyranocoumarins (mg / 100 g biomass FW)					
IBA	Picloram	BAP		InophyllumA	Inophyllum B	Inophyllum C	Inophyllum D	Inophyllum P	Calophyllolide
0.0	0.0	0.0	15.14±3.33	0.20±0.15	0.0013± 9X10^{-4}	1.19±0.284	1.4 X10^{-5}±2 X10^{-6}	0.0031±7.3X10^{-4}	0.00046±3.5X10^{-4}
4.90	0.0	0.0	24.45±6.20	0.13±0.097	1.1X10^{-4}±6.9X10^{-5}	0.051±0.037	6 X10^{-5}±0.0011	0.015±0.002	0.0391±0.0028
9.80	0.0	0.0	19.76±13.57	0.012±0.07	2 X10^{-4}±0.00013	0.115±0.092	5.15X10^{-5}±3.5X10^{-5}	0.072±0.013	0.020±0.013
14.70	0.0	0.0	34.86±30.87	0.044±0.014	0.033±0.0175	3.89±0.775	4.1 X10^{-5}±5.7 X10^{-6}	8 X10^{-5}±5 X10^{-5}	6 X10^{-5}±4 X10^{-4}
19.60	0.0	0.0	54.60±19.80	0.049±0.026	2.3X10^{-5}±1.22	5.01±1.224	9.3 X10^{-5}±4.2 X10^{-5}	2 X10^{-5}±1 X10^{-5}	4 X10^{-5}±1 X10^{-4}
4.90	0.0	4.44	21.64±14.75	0.049±0.027	3.2X10^{-5}±1.5X10^{-5}	0.030±0.0034	2.4 X10^{-5}±1.9 X10^{-5}	0.0030±0.0020	3.1X10^{-4}±1.8X10^{-4}
9.80	0.0	4.44	19.44±18.98	0.101±0.100	7.5 X10^{-5}±1.4 X10^{-5}	0.043±0.0049	4 X10^{-5}±3X10^{-5}	0.010±0.0014	0.0084±0.0024
14.70	0.0	4.44	31.75±8.88	0.300±0.131	0.0070±0.00134	0.014±0.009	5.6 X10^{-5}±4.8 X10^{-5}	9 X10^{-5}±7 X10^{-5}	5 X10^{-5}±4 X10^{-5}
19.60	0.0	4.44	33.50±27.10	0.0925±0.072	10.2X10^{-5}±1.2X10^{-5}	0.014±0.009	2.9 X10^{-5}±0.00072	4 X10^{-5}±2 X10^{-5}	9 X10^{-5}±1.7X10^{-5}
0.0	8.28	8.88	13.44±9.56	59.01±45.11	0.00018±1.5X10^{-4}	0.1181±1.1X10^{-4}	0.0052±7.6 X10^{-4}	1.6X10^{4}±1.3X10^{-5}	0.0034±0.0015
0.0	16.56	8.88	15.24±8.41	8.33±1.44	0.00042±1.4 X10^{-4}	1.44±0.244	0.0025±0.0010	1.1 X10^{-4}±7 X10^{-5}	0.0026±2.9 X10^{-4}
0.0	24.84	8.88	9.18±6.88	0.25±0.21	0.0028±0.0016	1.87±0.4440	0.033±0.0005	0.0029±9 X10^{-4}	0.0024±2.1 X10^{-4}
0.0	33.12	8.88	16.24±11.4	0.26±0.14	0.0069±0.0015	2.33±0.495	5 X10^{-5}±3 X10^{-5}	1.5 X10^{-4}±7 X10^{-5}	8 X10^{-4}±1.7 X10^{-4}

Values in mg % (mg/100 g biomass)., All values are mean ± S.D of four replicates (cultured flaks).

Expression of inophyllum C in suspension cultures of leaf callus did not show inverse relationship between biomass growth and expression of inophyllum C. Medium containing high IBA i.e. 14.70 and 19.60 µM accumulated 3.89 mg % (3.26 times) and 5.01 mg % (4.21 times) inophyllum C respectively. For expression of inophyllum C, picloram along with BAP (when compared to IBA alone) was comparatively less suited since 1.44 mg % (1.21 times), 1.87 mg % (1.57 times) and 2.33 mg % (1.99 times) inophyllum C was expressed in medium containing picloram 16.56 µM + BAP 8.88 µM, picloram 24.84 µM + BAP 8.88 µM and picloram 33.12 µM + BAP 8.88 µM respectively. Still these expressions were higher than the expressions in the control medium

In a media containing either IBA alone or IBA along with BAP constant, expression of inophyllum D was slightly increased. In a media containing IBA in the range 4.90 – 19.60 µM alone expressed inophyllum D in the range $4.1x10^{-5}$– $9.3x10^{-5}$ mg % (2.92 – 6.64 times). Same media when incorporated with BAP 4.44 µM constant expressed inophyllum D in the range $2.4x10^{-5}$– $5.6x10^{-5}$ mg % (1.71 – 4.0 times). However higher expressions of inophyllum D was estimated in media containing picloram and BAP. Picloram in the range 8.28 – 33.12 µM along with BAP 8.88 µM enhanced inophyllum D expression in the range 5 $x10^{-5}$ – 0.033 mg % (3.57 – 2357 times). Maximum 0.033 mg % (2357 times) inophyllum D was expressed in suspension cultures of leaf callus containing picloram 14.84 µM along with BAP 8.88µM. Compared to expression in media containing IBA in the range 4.90 – 19.60 µM alone and along with BAP 4.44 µM constant (which resulted maximum biomass growth), expression in media containing picloram in the range 8.28 – 33.12 µM along with BAP 8.88 µM constant (which resulted less biomass growth) was higher. This pattern of accumulation of inophyllum D also suggested the inverse relationship between biomass growth and inophyllum D expression.

Expression pattern of inophyllum P and calophyllolide was found to be erratic. Highest 0.072 mg % (23.22 times) inophyllum P and 0.0391 mg % (85 times) calophyllolide were estimated in media containing IBA 9.80 µM and 4.90µM respectively. Higher concentrations of IBA (14.70 and 19.60µM) alone or with BAP (4.44 µM) constant reduced expression of both, inophyllum P and calophyllolide. All of the media combinations containing picloram with BAP reduced inophyllum P expression. This expression pattern of inophyllum P did not indicated the inverse relationship between biomass growth and expression of inophyllum P. Media containing picloram in the range 8.28 – 33.12 µM along with BAP 8.88 µM constant expressed calophyllolide in the range 8 $X10^{-4}$ – 0.0034 mg % (1.79 – 7.39 times). Since the expression of calophyllolide in these media was consistently higher than in control, there was little inverse relationship between biomass growth and expression of calophyllolide. Similar type of inverse relationship was not found for the expression of inophyllum P.

Evident from overall expression pattern of dipyranocoumarins in the suspension cultures of leaf callus, it was concluded that that there was inverse relationship between biomass growth and expression of inophyllums A, B, D and calophyllolide while this trend of expression was not observed for inophyllums C and P.

6.3.4. Effect of hormones on biomass growth and expression pattern of dipyranocoumarins in cell suspension culture of nodal / internodal callus:

With all the hormone combinations tried in suspension cultures of nodal / internodal callus, biomass growth was more than control medium. Compared to suspension culture of leaf callus, growth of biomass in suspension culture of nodal / internodal callus was consistently more. Maximum 59.27 % (4.98 times) biomass increase was noted in medium containing IBA 14.70 µM + BAP 4.44 µM. In the media containing IBA (4.90 – 19.60 µM), biomass was increased in the range 22.65 – 45.47 % (1.90 – 3.82). Addition of BAP 4.44 µM to the same media resulted further increase in the range of biomass growth. Presence of BAP 4.44 µM constant along with IBA in the range 4.90 – 19.60 µM enhanced biomass to the range 43.26 – 59.27 % (3.63- 4.98 times). Similar effect of presence of BAP on biomass growth was not observed in suspension cultures of leaf callus. IBA (4.90 – 19.60 µM) along with BAP (4.44 µM) seemed to be best suited for biomass growth in suspension culture of nodal/internodal callus. Effect of picloram along with BAP on biomass growth in suspension cultures of both callus types was almost similar. Picloram in the range 8.28 – 33.12 µM along with BAP 8.88 µM constant enhanced the biomass in the range 12.29 – 21.46 % (1.03 – 1.80 times). Similar to the suspension culture of the leaf callus, increase in the biomass in suspension culture of nodal / internodal callus was not directly proportional to the concentrations of hormones tried.

Unlike the expression of inophyllum A in suspension cultures of leaf callus (where inophyllum A expression was reduced), incorporation of hormones IBA alone or with BAP in suspension culture of nodal / internodal callus increased the expression of inophyllum A. Incorporation of IBA in the range 4.90 – 19.60 µM alone accumulated inophyllum A in the range 0.072 – 0.356 mg% (1.09 – 5.39 times). Maximum 0.356 mg % (5.39 times) inophyllum A was expressed in media supplemented with IBA 9.80 µM alone. Addition of BAP 4.44 µM constant along with IBA in the range 4.90 – 19.60 µM increased the expression range to 0.148 – 0.945 mg % (2.24 – 14.31 times). Maximum 0.945 mg % (14.31 times) inophyllum A was expressed in media supplemented with IBA 9.80 µM and BAP 4.44 µM. This effect of presence of BAP on expression of inophyllum A was similar to the effect on biomass growth. Like cell suspension of leaf callus, maximum expression of inophyllum A was estimated in medium containing picloram and BAP (Table 6.1 and 6.2). Maximum 6.67 mg % (101.06 times) inophyllum A was estimated in

suspension culture of nodal / internodal callus supplemented with picloram 16.56 µM + BAP 8.88 µM. Picloram in the range 8.28 – 33.12 µM along with BAP 8.88 µM constant resulted expressions of inophyllum A in the range 0.075 – 6.67 mg % (1.13-101.06 times).

Maximum 0.245 mg% (1065 times) inophyllum B was estimated in suspension culture supplemented with IBA 14.70 µM + BAP 4.44 µM. Lower concentrations of IBA alone (4.90 and 9.80 µM) reduced the inophyllum B expression while higher concentration of IBA alone (14.70 and 19.60 µM) increased the inophyllum B expression. Consistently higher range of inophyllum B i.e. 0.147 – 0.245 mg % (639.13 – 1065 times) was expressed in suspension culture supplemented with IBA in the range 4.90 – 19.60 µM along with BAP 4.44 µM constant. Supplementation of picloram along with BAP resulted negligible expression of inophyllum B.

Among all the dipyranocoumarins estimated, the level of expression of inophyllum C was higher than other dipyranocoumarins in suspension culture of nodal /internodal callus. Maximum 22.18 mg % (616 times) inophyllum C was estimated when suspension cultures were supplemented with IBA 4.90 µM alone. Supplementation of IBA alone in the range 4.90 – 19.60 µM expressed inophyllum C in the range 0.045–22.18 mg% (1.25 - 616 times).

Incorporation of BAP 4.44 µM with same combinations of IBA reduced the expression, but still it was consistently higher than in the control medium. IBA in the range 4.90 – 19.60 µM along with BAP 4.44 µM constant expressed inophyllum C in the range 1.94 – 6.21 mg % (53.88 – 172.5). Although less than the expressions in media containing IBA alone and IBA + BAP, expressions of inophyllum C in media containing picloram + BAP still was higher than control medium. Picloram in the range 8.28 – 33.12 µM + BAP 8.88 µM expressed inophyllum C in the range 0.045 – 0.44 mg% (1.25 – 12.22 times)

Table 6.2. Effects of hormones on expression (accumulation) pattern of dipyranocoumarins in suspension cultures of nodal / internodal callus.

Hormones (μM)			% Biomass Increase	Dipyranocoumarins (mg / 100 g biomass FW)					
IBA	Picloram	BAP		InophyllumA	Inophyllum B	Inophyllum C	Inophyllum D	Inophyllum P	Calophyllolide
0.0	0.0	0.0	11.89±5.32	0.066±0.021	2.3 $X10^{-4}$±1.3 $X10^{-5}$	0.036±0.016	2.85±1.067	2.6 $X10^{-4}$±1.2$X10^{-4}$	0.0078±1.6 $X10^{-4}$
4.90	0.0	0.0	35.11±12.49	0.072±0.033	8.1 $X10^{-5}$±1 $X10^{-5}$	22.18±8.19	0.00726±0.00125	1 $X10^{-4}$±6.59 $X10^{-5}$	1.2 $X10^{-4}$±1.6$X10^{-5}$
9.80	0.0	0.0	24.56±13.21	0.356±0.211	2.2 $X10^{-4}$±1.2 $X10^{-4}$	0.150±0.080	7.4 $X10^{-5}$±2.6$X10^{-5}$	1.1$X10^{-4}$±6.5 $X10^{-5}$	15.4 $X10^{-5}$±2 $X10^{-5}$
14.70	0.0	0.0	22.65±11.54	0.089±0.073	0.119±0.044	0.045±0.009	4.3 $X10^{-5}$±2.7$X10^{-5}$	7.8 $X10^{-5}$±2.4$X10^{-5}$	0.00147±0.00104
19.60	0.0	0.0	45.47±13.26	0.099±0.067	0.104±0.074	11.65±09.55	2.8 $X10^{-5}$±1 $X10^{-5}$	9.6 $X10^{-5}$±4.3$X10^{-5}$	0.00224±0.00124
4.90	0.0	4.44	43.26±39.21	0.5467±0.15	0.185±0.012	3.451±0.45	15.6$X10^{-5}$±1.5$X10^{-5}$	4.5 $X10^{-5}$±1.5$X10^{-5}$	0.00158±9 $X10^{-4}$
9.80	0.0	4.44	53.19±42.15	0.945±0.048	0.147±0.101	6.21±2.19	0.00156±9.1 $X10^{-4}$	1.6 $X10^{-4}$±1.2$X10^{-4}$	0.00651±0.00267
14.70	0.0	4.44	59.27±35.18	0.148±0.541	0.245±0.186	2.657±0.947	94.7$X10^{-5}$±2.5$X10^{-4}$	1.9 $X10^{-5}$±1.2$X10^{-5}$	9.5$X10^{-4}$±1.6 $X10^{-4}$
19.60	0.0	4.44	48.21±29.61	0.165±0.0974	0.195±0.094	1.948±0.45	8.75$X10^{-4}$±1.2$X10^{-4}$	9.4 $X10^{-5}$±1.1$X10^{-5}$	8.1$X10^{-4}$±3.1$X10^{-5}$
0.0	8.28	8.88	18.26±15.26	0.102±0.085	1.1 $X10^{-5}$±1.6 $X10^{-6}$	0.44±0.11	3.4 $X10^{-5}$±2.5 $X10^{-5}$	2.2 $X10^{-5}$±0.0011	2.8$X10^{-5}$±1.4 $X10^{-5}$
0.0	16.56	8.88	14.55±9.78	6.67±0.786	8.6 $X10^{-5}$±5$X10^{-5}$	0.10±0.01	4.3 $X10^{-5}$±2.8 $X10^{-5}$	9.2 $X10^{-5}$±3 $X10^{-5}$	5.2 $X10^{-5}$±4.1$X10^{-5}$
0.0	24.84	8.88	21.46±12.41	0.32± 0.25	3.3 $X10^{-4}$±1.1 $X10^{-4}$	0.146±0.020	5 $X10^{-5}$±3.7 $X10^{-5}$	1.7 $X10^{-4}$±4 $X10^{-4}$	9.8$X10^{-5}$±1.4 $X10^{-5}$
0.0	33.12	8.88	12.29±6.61	0.0757±0.065	1.3 $X10^{-5}$±3.5 $X10^{-5}$	0.045±0.026	2 $X10^{-5}$±1.2 $X10^{-5}$	1.3 $X10^{-5}$±0.0012	0.0024±0.0019

Values in mg % (mg/100 g biomass)., All values are mean ± S.D of four replicates (cultured flaks)

It was noted that in suspension cultures of nodal / internodal callus, the expression pattern of inophyllums D, P and calophyllolide was erratic. When compared to expression of these dipyranocoumarins in control medium, expressions in medium supplemented with IBA, IBA+ BAP and picloram + BAP were reduced. This clearly suggested that in suspension cultures of nodal / internodal callus, there was the inverse relationship between biomass growth resulting from hormonal influence and accumulation of inophyllums D, P and calophyllolide. From overall expression pattern of dipyranocoumarins in the suspension cultures of leaf callus, similar type of inverse relationship between biomass growth and expression of dipyranocoumarins like inophyllums A, B, D and calophyllolide was also noted. This trend of expression was not observed for inophyllum C in suspension culture of leaf callus. Secondary metabolites are known to accumulate at later stage of growth cycle when growth slows down or reaches plateau (Biondi et al., 2004.) In general phytohormones exert negative effect on secondary metabolite formation (Rhodes et al., 1994; Arroo et al., 1995). Negative influence of the phytohormones is some time due to stimulation of growth. Similar type of inverse relationship between biomass growth and accumulation of secondary products has been reported in several species of Solanaceae (Lindsey and Yeomann, 1983), *Morinda citrifolia* (Hagendroon et al., 1997) and *Rheum ribes* (Farzami Sepehr and Ghorbanli, 2002). In root cultures of *Solanum khasianum,* inverse relationship between biomass growth and solasodine production was also observed (Jacob and Malpathak, 2005). Solasodine production was noted to be decreased due to biomass growth resulting from supplementation of phosphate. Thengane et al, (2003) reported that incubation of callus cultures of *Nothapodytes foetida* beyond 45 days resulted gradual increase in callus mass, but this increase in callus mass resulted declined alkaloid (camptothecin) production. Based on overall expression pattern of dipyranocoumarins in suspension cultures of both callus types, it can be concluded that irrespective of the callus type used for initiating suspension cultures, biomass growth resulting from hormonal influence had inverse relationship with expression of some of the dipyranocoumarins like inophyllums A, B, D, P, calophyllolide and direct relationship with only inophyllum C. Similar type of observation where some, but not all metabolites were reduced due to biomass growth resulting from hormonal influence was made by Biondi et al., 2004**.** In *Zanthoxylum stenophyllum,* some of the metabolites but not all were shown to have reduced accumulation because of highest stimulation of growth (Biondi et al., 2004). The inverse relationship between biomass growth and expression of dipyranocoumarins could be explained with the help of finding reported by Abenavoli and co workers. Abenavoli et al., (2003) studied the effects of coumarins on the growth of Carrot (*Daucus carota* L.cv. Saint Valery) cells in suspension cultures and found that coumarin inhibited the growth of Carrot cells in suspension cultures. These workers hypothesized that coumarins in nature may act as inhibitors of the cell

cycle and/or as a senescence-promoting substance. In the present study, whatever levels of coumarins were present in the callus cultures used for initiating suspension cultures, that might have been sufficient for the suppression of biomass growth in suspension cultures. However hormonal influence might have overcome this inhibitory influence of initial coumarins level that might have led to increased biomass growth. Thus enhanced biomass growth in suspension cultures of *C. inophyllum* can be attributed to overcoming effects of hormones in the influence of some initial levels of dipyranocoumarins. Possibly, because of these reasons, direct relationship between biomass growth and level of dipyranocoumarins expression was not noted in this study. However to prove these possibilities, further work at molecular level would be helpful.

6.3.5. Statistics (Two Way ANOVA):

Two way ANOVA for dipyranocoumarins variation resulting from hormonal influence in suspension cultures of both callus types revealed that for the expression of inophyllums A and C, medium, suspension types and interaction between medium and suspension type were significant at 1 % level. For expression of inophyllums P and B, media were significantly different at 1 % level; suspension types were significant at 5% level, whereas interaction between them was at 1% level.

Table 6.3. Two way ANOVA for dipyranocoumarins variation resulting from hormonal influence in suspension cultures of both callus types

Two way ANOVA	Inophyllum A			Inophyllum B			Inophyllum C		
	Med.	Sus type	MedXSus type	Med.	Sus type	MedXSus type	Med.	Sus type	MedXSus type
S.E.	1.521	3.214	1.95	0.0009	0.0011	0.0012	0.0013	0.0019	0.0011
C.D.	6.541	2.514	12.47	0.0082	0.0013	0.0108	0.0088	0.0026	0.0117
Significance	**	**	**	**	*	**	**	**	**
	Inophyllum D			Inophyllum P			Calophyllolide		
	Med.	Sus type	MedXSus type	Med.	Sus type	MedXSus type	Med.	Sus type	MedXSus type
S.E.	NS	NS	NS	0.0021	0.0024	0.00265	NS	NS	NS
C.D.	NS	NS	NS	0.0169	0.0035	0.0221	NS	NS	NS
Significance	NS	NS	NS	**	*	**	NS	NS	NS

Med- Medium containing hormone, Sus type- Suspension type, Med X Sus type - Interaction between medium and Suspension type. (**) Significant at 1% level; (*) Significant at 5% level; NS- Non significant at 1% & 5% level S. E. – Standard Error.

For expression of inophyllum D and calophyllolide, neither of media, suspension type and interaction between them was significant at 1 % and 5% level i. e. non significant at both 1% and 5% levels (Table 6.3).

6.3.6. Effect of sucrose on dipyranocoumarins' expression in suspension cultures:

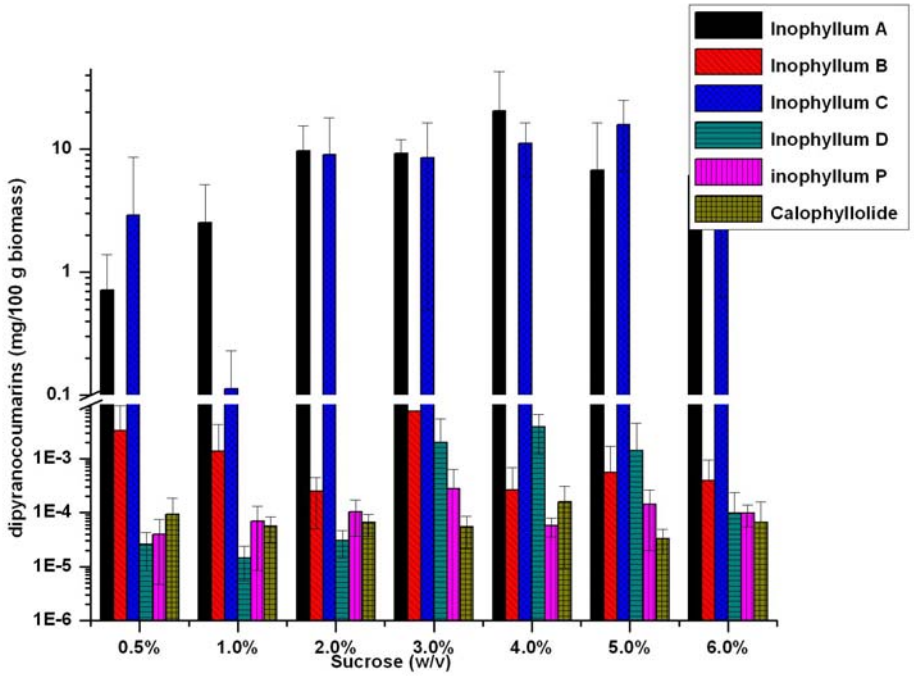

Fig. 6.8. Effect of initial sucrose concentrations on expression of dipyranocoumarins in suspension cultures of leaf callus.

Carbohydrates in general and especially sucrose in particular, is an important carbon and energy sources for most plant cell. To study the effect of sucrose on biomass growth and expression pattern of dipyranocoumarins, initial sucrose concentration was varied in the range 0.5 – 6.0 % in suspension cultures of both callus types. The results for effect of initial sucrose concentrations in suspension cultures of leaf and nodal / internodal callus have been given graphically in figures 6.8 and 6.9 respectively.

Variation in initial sucrose concentration did not result in biomass growth in suspension cultures of both callus types. In suspension culture of leaf callus, reducing the concentration of sucrose than the control i.e. 2. 0 % resulted in the decreased expression of inophyllums A and C (Fig. 6.8); however, more than 2.0 % increase in sucrose concentration was found to have major influence on expression of inophyllums A and C. In comparison to the expression of inophyllum A (9.69 mg%) and inophyllum C (9.05 mg %) in control medium (2.0 % sucrose), highest 20.56 mg % (2.12 times) inophyllum A and 15.85 mg % (1.75 times) inophyllum C expressions were estimated in suspension cultures of leaf callus incorporated with 4.0 and 5.0 % sucrose respectively (Fig. 6.8). Sucrose was noted to have negligible influence on expression on expression patterns of other dipyranocoumarins in suspension cultures of leaf callus (Fig. 6.8).

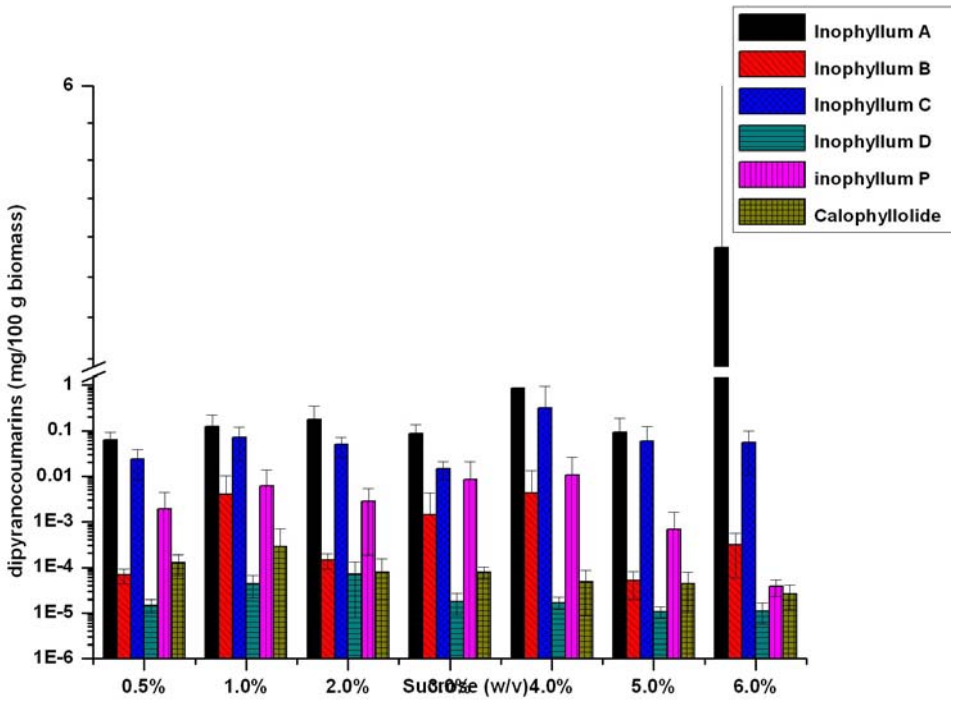

Fig. 6.9. Effect of initial sucrose concentration on expression of dipyranocoumarins in suspension cultures of nodal / internodal callus.

The levels of inophyllums A and C in suspension cultures of nodal / internodal callus were less than the suspension cultures of the leaf callus. Compared to the influence of sucrose in suspension cultures of leaf callus, sucrose was observed to have comparatively more enhancing effect on expression of inophyllum A and inophyllum C in suspension cultures of nodal / internodal callus. Highest sucrose concentration (6.0%) resulted in highest 5.52 mg % (89.03 times) inophyllum A expressions, whereas highest of 0.311 mg % (13.23 times) inophyllum C was expressed in suspension culture incorporated with 4.0 % sucrose (Fig. 6.9). Similar to the expression in suspension cultures of leaf callus, expression patterns of other dipyranocoumarins in suspension cultures of nodal / internodal callus were not significantly influenced by variation in sucrose concentrations (Fig. 6.9).

Previously production of number of secondary metabolites by plant cells were shown to be affected by initial sugar concentration in a number of plant cell cultures, for example, in accumulation of alkaloid by *Catharanthus roseus* (Merillon et al., 1984) and *Holarrhena antidysenterica* cells (Panda et al., 1992), ajmalicine by *C. roeseus* (Zenk et al., 1977a; Schlatmann et al., 1995b), rosmarinic acid by *Anchusa officinalis (*Su and Humphrey, 1990) and *Coleus blumei* (Zenk et al., 1977b; Gertlowski and Petersen, 1993), carotenoid by carrot (Yun et al., 1990), anthocyanins by *Vitis vinifera* (Cormier et al., 1990) and *Perilla frutescens* (Zhong et al., 1994; Zhong and Yoshida, 1995), as well as nicotine by *Nicotiana tobaccum* (Mantel1 et al., 1983). In

addition, sugar feeding was also reported to enhance shikonin formation (Srinivasan and Ryu, 1993) and anthocyanin accumulation (Zhong and Yoshida, 1995) by plant cell cultures. Although, these reports suggested that initial sucrose concentration was important to the growth of plant cells, but its effect was dependant on specific cell lines. In the present study, suspension cultures of both callus types showed negative influence and did not result in biomass growth. Overall expression pattern of dipyranocoumarins revealed that in suspension cultures of *C. inophyllum* initiated from leaf and nodal / internodal callus, sucrose positively influenced the expression of only inophyllum A and inophyllum C; whereas expression pattern of other dipyranocoumarins under study was not significantly influenced and remained almost same as control.

6.3.7. Effect of nitrate on biomass growth and dipyranocoumarins' expression:

In an attempt to enhance the biomass growth and expression of dipyranocoumarins in suspension cultures of both callus types, concentration of total nitrate in WPM basal medium was varied. Concentration of total nitrate was increased up to four fold (5X) the normal concentration of nitrates in WPM. The results for effect of total nitrate on biomass growth and dipyranocoumarins' expression in suspension cultures of leaf callus and nodal internodal callus have been given graphically in Fig.6.10 and 6.11 respectively.

These experiment were conducted with the objectives of increasing the biomass in suspension cultures. However in the present study, like the effect of sucrose, variation in total nitrate concentrations did not result in biomass growth in suspension cultures of either of the callus types. In suspension culture of leaf callus, similar to the effect of sucrose, nitrate was also observed to have major influence on expression of inophyllums A and C and comparatively little influence on expression of inophyllums B, D, P and calophyllolide. Maximum 5.81 mg% (2.80 times) inophyllum A was expressed in medium containing total nitrate four fold (4X) higher than the normal nitrate. Except with 3 fold increase in total nitrate, expression of inophyllum A was directly proportional to the increase in total nitrate. There was little effect on expression of inophyllum B. Compared to the expression of inophyllum B (0.000273 mg %) with normal nitrate (control), one fold increase in nitrate increased the expression of inophyllum B to 0.000481 mg % (1.76 times). More than one fold increase in nitrate concentration reduced the expression of inophyllum B.

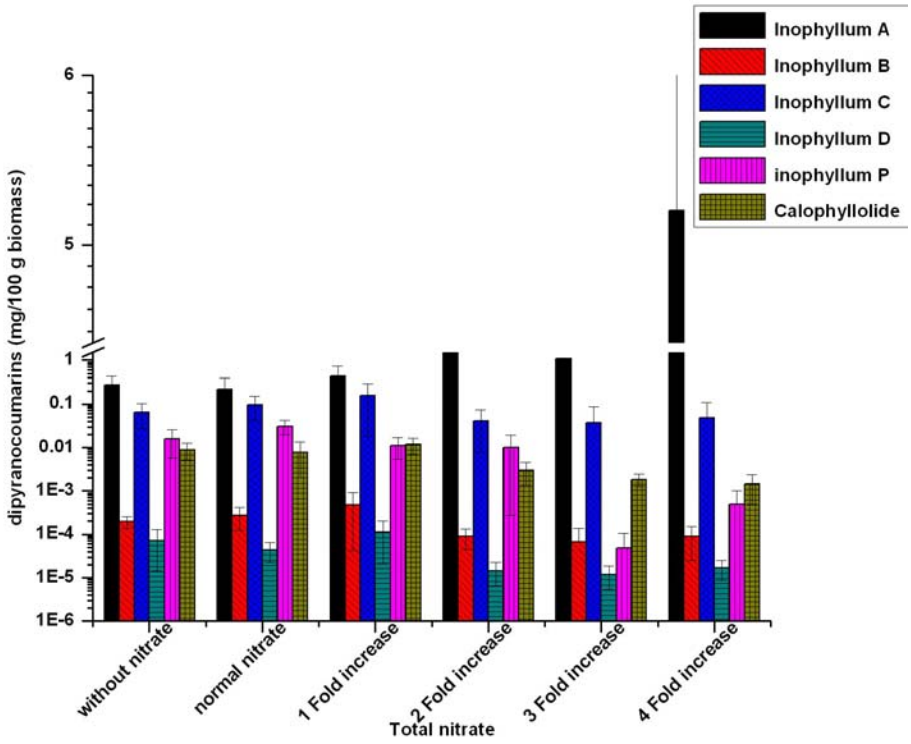

Fig. 6.10. Effect total nitrate concentration on expression of dipyranocoumarins in suspension cultures of leaf callus.

Like expression of inophyllum B, highest expression of inophyllum C, inophyllum D and calophyllolide were estimated to be 0.154 mg % (2.36 times), 0.000112 mg % (1.57 times) & 0.0116 mg % (1.31 times) respectively in medium containing one fold total nitrate, whereas further increase in total nitrate reduced their expression. For maximum expression of inophyllum P, control medium (without nitrate manipulation) was found to be optimum since highest 0.0308 mg% inophyllum P was estimated in this control medium.

In suspension cultures of nodal / internodal callus, one fold increase in nitrate concentration resulted maximum 2.93 mg % (32.55 times) inophyllum A, 0.00477 mg % (1.323 time) inophyllum P and 0.0010 mg % (4 times) calophyllolide expression. Further increase in nitrate was not as effective as was one fold increase for expression of inophyllums A, P and calophyllolide. Like the expression of inophyllum P in suspension cultures of leaf callus, maximum 1.60 mg % inophyllum C expression was estimated with normal nitrate concentration (control). Although the level of expression of inophyllum A was higher in suspension cultures of leaf callus than nodal / internodal callus; the number of time increase in expression was more in suspension cultures of nodal / internodal callus. (Fig 6.10 and 6.11). Four fold increase in total nitrate concentration resulted maximum 0.000305 mg % (3.21 times) inophyllum B and 0.000077 mg % (1.2 times) inophyllum D expression.

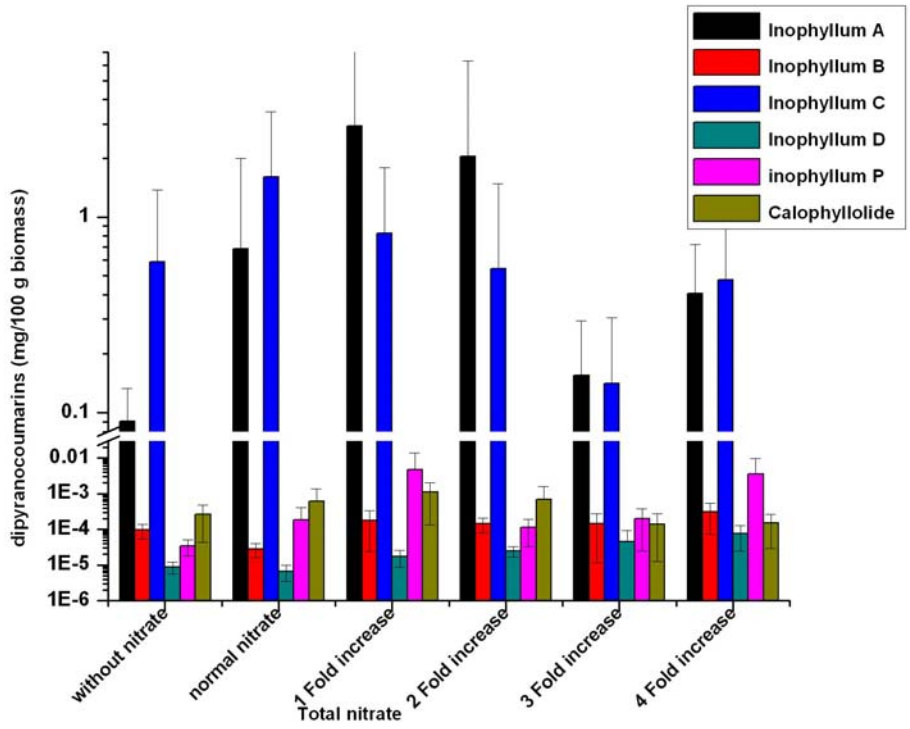

Fig. 6.11. Effect total nitrate concentration on expression of dipyranocoumarins in suspension cultures of nodal / internodal callus.

For increased expression of inophyllum A, suspension cultures of leaf callus required higher concentrations of nitrate (3 – 6 fold) whereas suspension cultures of nodal / internodal callus required only 1-2 fold increase in nitrate concentrations. Overall, in suspension cultures of both callus types, variation in total nitrate concentrations had significant influence on the expression pattern of inophyllums C and A and had comparatively little influence on inophyllums B, D, P and calophyllolide in suspension culture of both callus types.

Nutritive factors like nitrogen supply were shown to have influence on alkaloid production. The nitrogen status of the cell determines the composition of its major components and directly or indirectly affects the spectrum and quantity of the secondary products. These effects could be guided by changes in metabolic pathways. Therefore it is likely that overall metabolic adaptation of plant cells could be affected by nitrogen status (Okazaki et al., 2008). The contribution of nitrate and nitrogen metabolism to the regulation of phenylpropanoid synthesis was studied (Fritz et al., 2006). It was concluded that stimulation of phenylpropanoid metabolism was triggered by changes of nitrate, rather than downstream nitrogen metabolites, and was mediated by induction of a set of enzymes in the early steps of the phenylpropanoid biosynthetic pathway. Nitrate was known to act as signal regulating the activity of many enzymes and transporter, including nitrate reductase, phosphoenolpyruvate carboxylase, malate dehydrogenase, sucrose

phosphate synthase and the nitrate transporter involved in regulating carbon and nitrogen balance in plants (Scheible et al, 1997). These different roles might have contributed for influence of nitrate on dipyranocoumarins expression pattern. Bensaddek and co-workers reported that the contents of secondary metabolites like scopolamine and hyoscyamine in hairy root cultures of *Atropa belladonna* were strongly influenced by increase in nitrate concentration (Bensaddek et al., 2001). Nitrate was also investigated for its effect on production of azadirachtin. As a part of media optimization, effect of nitrate as a source of nitrogen on cell growth and azadirachtin production in *Azadirachta indica* was studied (Prakash and Srivastava, 2005). These authors concluded that nitrate alone as a nitrogen source was favourable for biomass growth and azadirachtin production. In cell cultures of *Pinax quinquefolium*, when nitrate was used as the sole source of nitrogen, high production of ginseng saponin and polysaccharides was achieved. Further it was concluded that nitrate and ammonia had different effect on cell physiology and primary and secondary metabolism (Zhong and Wu, 1998). Though these reports have suggested that nitrate as nitrogen source positively influences the biomass growth and production of secondary metabolites, in the present study, nitrate was noted to have no effect on biomass growth in suspension cultures of *C. inophyllum*.

6.3.8. Effect of variation in total sulphate on biomass growth:

WPM basal medium contains macronutrients like $MgSO_4$, K_2SO_4 and micronutrients like, $MnSO_4$, $ZnSO_4$ and $CuSO_4$. To study the effects of these salts, combined stocks of these sulphates was added to suspension cultures of both callus types. The results for the effect of total sulphate on biomass growth in suspension cultures of both callus types have been given in Fig.6.12.

Unlike variation in total nitrate and initial sucrose, which did not result in biomass growth, variation in total sulphate concentration resulted in biomass growth in suspension cultures of both callus types. In suspension culture of leaf callus, one fold increase in sulphate concentration resulted in 61.22 % (2.42 times) biomass growth, which was more than double the biomass growth in control medium (Fig. 6.12). Rest of the concentrations of sulphate in suspension cultures of leaf callus did not result in biomass growth. However in suspension culture of nodal /internodal callus, it was three fold increase in sulphate concentrations that resulted the maximum biomass 38.23% (6.2 times) growth. This growth of biomass was more than six times the growth of biomass in control medium.

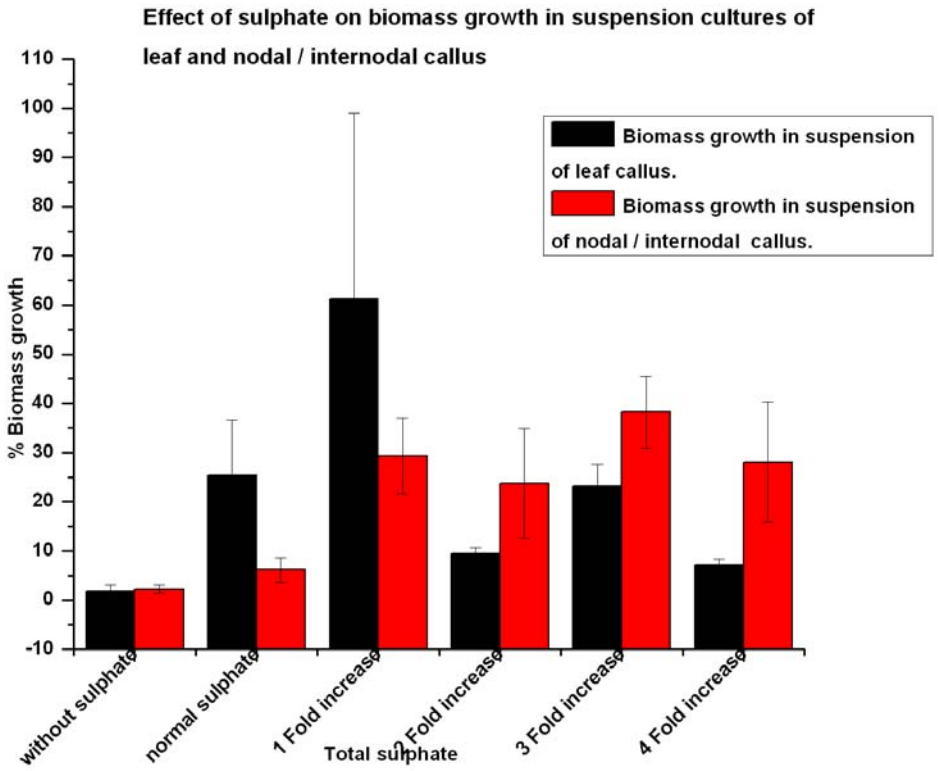

Fig. 6.12. Effect total sulphate concentration on biomass growth in suspension cultures of leaf and nodal / internodal callus.

Variation in total sulphate in the range 1.0 – 4.0 fold enhanced biomass growth in the range 23.72 – 38.23 % (3.85 – 6.20 times) in suspension cultures of nodal / internodal calluses (Fig. 6.12). Jacob and Malpathak (2005) studied the effect of different components of MS and B5 on growth and solasodine production in hairy root cultures of *Solanum khasianum*. Among the different components, sulphate was also the medium component, which was studied for its effect. These authors reported that $MgSO_4$ concentration greatly influenced the growth and was directly related to the growth of hairy roots. In the present study, similar effect of sulphate on biomass growth in the suspension cultures of *C. inophyllum* was observed. This effect of sulphate could have been mediated by certain transporters involved in uptake and translocation of sulphate. Takahashi and co workers (2000) studied such transporters in *Arabidopsis thaliana* and reported that sulphate transporters *Sultr1:1, Sultr2:1, Sultr2:2,* are involved in uptake and translocation of sulphate. Possibly, similar types of transporters could have been involved in sulphates' action in enhancing biomass growth in the present study.

6.3.9. Effect of sulphate on dipyranocoumarins' expression:

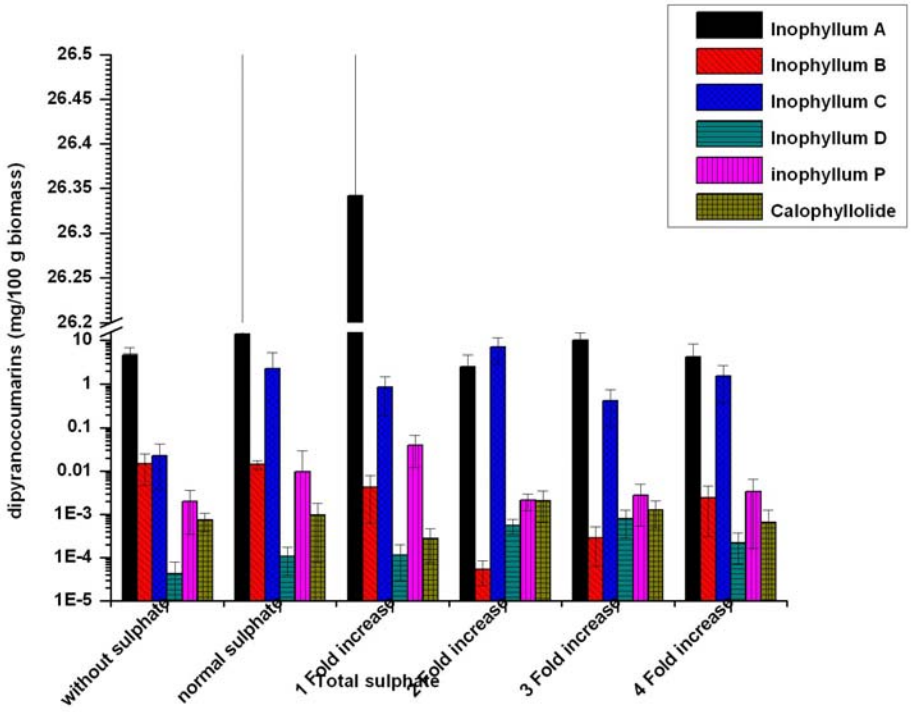

Fig. 6.13. Effect total sulphate concentration on dipyranocoumarins' expression in suspension cultures of leaf callus.

Variation in total sulphate in suspension culture of leaf callus was found to have influence on expression pattern of dipyranocoumarins. Like nitrate and sucrose, variation in sulphate also had major influence on expression pattern of inophyllum A and inophyllum C. Increase of sulphate to one fold almost doubled the expression of inophyllum A (Fig. 6.13). In control medium, 13.76 mg % inophyllum A was expressed whereas increasing the sulphate concentration to one fold resulted 26.34 mg % (1.94 times) inophyllum A expression. There was no noticeable increase and had negative influence on expression pattern of inophyllum B; rather increase in sulphate concentration decreased the inophyllum B expression. Sulphate was observed to have enhancing effect on expression of inophyllum C. In control medium, 2.30 mg % inophyllum C was estimated which was enhanced to 7.16 mg % (3.11 times) on addition of two-fold sulphate. This increase in the inophyllum C expression was more than three times than the expression in control medium. Sulphate was noticed to have little influence on expression of inophyllums D, P and calophyllolide (Fig. 6.13).

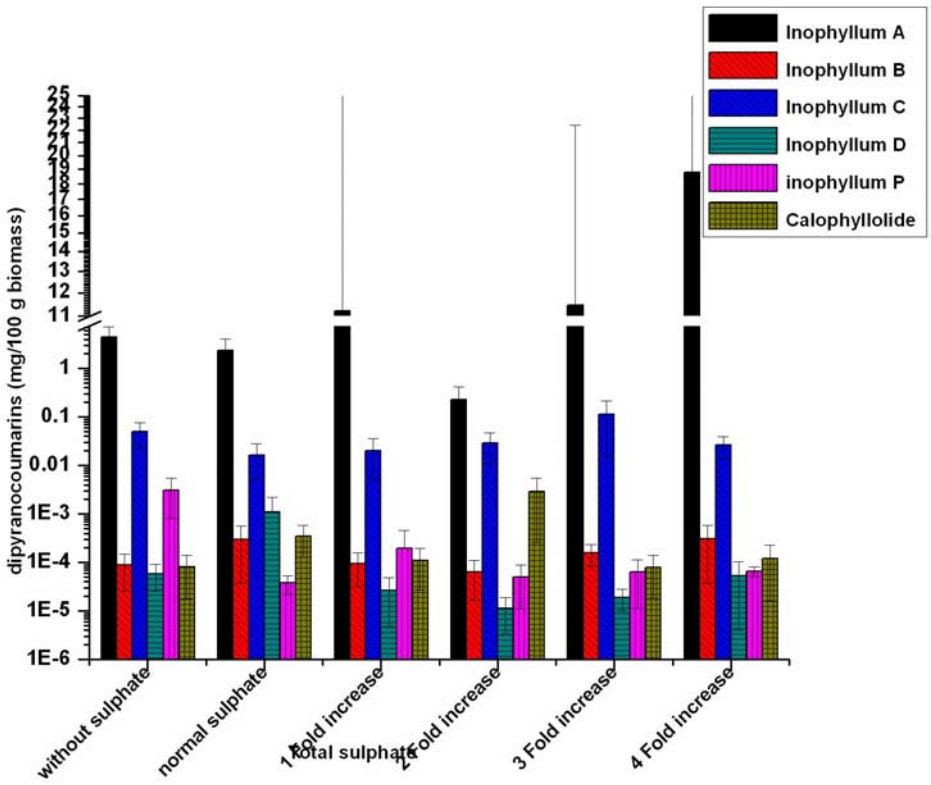

Fig. 6.14. Effect total sulphate concentration on dipyranocoumarins' expression in suspension cultures of nodal/ internodal callus.

Like in suspension cultures of leaf callus, sulphate was also observed to have major influence on expression of inophyllum A in suspension cultures of nodal /internodal callus. Highest 18.77 mg % (9.32 times) inophyllum A was estimated on supplementation of four fold sulphate (Fig. 6.14). This increase in inophyllum A was more than nine times than the expression in control medium. In the control medium, 0.016 mg % inophyllum C was expressed which on supplementation of three fold sulphate rose to 0.11mg % (6.87 times). Similar to the suspension cultures of leaf callus, expression pattern of other dipyranocoumarins (inophyllums B, D, P and calophyllolde) in suspension cultures of nodal /internodal callus did not change by variation in sulphate (Fig. 6.13).

The overall expression pattern in suspension cultures of both callus resulting from variation in total sulphate revealed that, among the dipyranocoumarins estimated, sulphate had positive influence on expression pattern of only inophyllums A and C, whereas expression pattern of rest of the dipyranocoumarins was not influenced by variation in sulphate concentrations.

6.3.10. Effect of vitamins on dipyranocoumarins' expression:

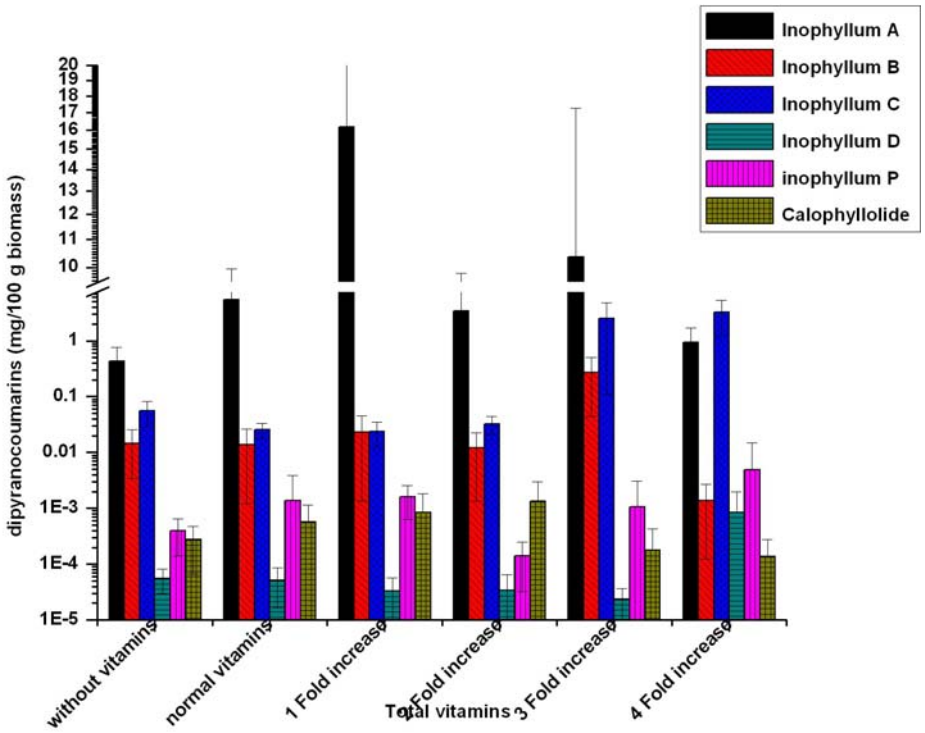

Fig. 6.15. Effect total vitamin concentration on dipyranocoumarins' expression in suspension cultures of leaf callus.

Like sucrose and nitrate and unlike sulphate, variation in total vitamins concentration in WPM basal medium did not show increase in biomass growth in suspension culture of either callus types. In suspension culture of leaf callus, vitamins were observed to have major influence on the expression of inophyllums A, B and C and little influence on expression of inophyllums D, P and calophyllolide. Maximum 16.19 mg % (2.95 times) inophyllum A was estimated in suspension of leaf callus with one fold increase in vitamins concentration (Fig. 6.15). This increase in inophyllum A was almost three times the expression in control medium (5.48 mg%). Vitamins also positively influenced the expression of inophyllum B. Maximum 0.11 mg % (7.97 times) inophyllum B was expressed in cultures supplemented with 3 fold vitamins. This expression of inophyllum B was almost eight times the expression in control medium. Maximum 3.26 mg % (163 times) accumulation of inophyllum C was estimated in suspension cultures incorporated with four fold vitamins which was much higher than in the control medium (0.02 mg %) (Fig.6.15). Vitamins were noted to have better influence on expression of inophyllum D whereas on the expression pattern of other dipyranocoumarins (inophyllums P and calophyllolide), vitamins were observed to have little influence. Compared to the expression of inophyllums D, P and calophyllolide in control medium (0.0000511, 0.0013 and 0.000569 mg % respectively), four fold increase in the vitamins

enhanced the expression of inophyllums D and P to 0.00085 mg % (16.63 time) and 0.0049 mg % (3.76 times) respectively, whereas two fold increase in vitamins enhanced calophyllolide expression to 0.00134 mg % (2.35 times).

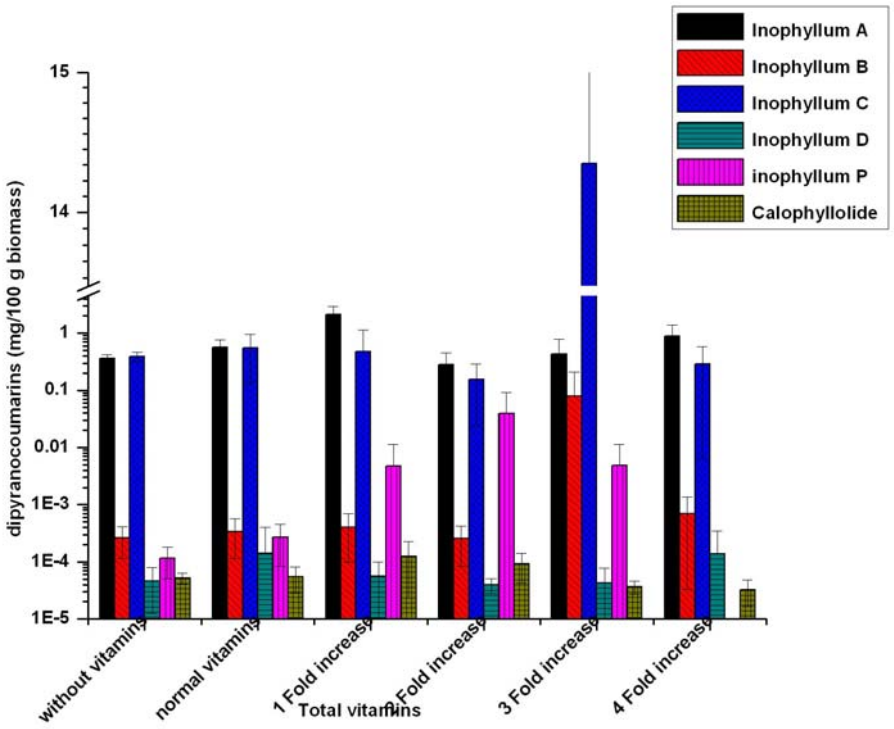

Fig. 6.16. Effect total vitamins concentration on dipyranocoumarins' expression in suspension cultures of nodal / internodal callus.

In suspension cultures of both callus types, expression pattern of inophylum A was almost similar. Although pattern was similar, expression level was less in suspension cultures of nodal /internodal callus than suspension cultures of leaf callus. Maximum 2.11 mg % (4.12 times) inophyllum A was estimated on incorporation of one fold vitamin. This expression was four times more than the expression in control medium. Maximum expression of inophyllums B and C was resulted on three fold increase in vitamins. Compared to the expression of inophyllum B in control (0.000341 mg %), three fold increase in vitamins enhanced inophyllum B expression to 0.07998 mg% (234 times). Highest inophyllum C (14.34 mg %) was also expressed on three fold increase in vitamins (Fig. 6.15). This was the highest (265 times) expression of inophyllum C when compared to enhanced expression of inophyllum C with sulphate and nitrate variation. Expression of remaining dipyranocoumarins was not significantly influenced by addition of vitamins. Overall expression pattern resulting from variation in vitamins showed that like the effect of variation in sucrose, nitrate and sulphate, vitamins also had positive and major influence on

expression of inophyllums A and C. However vitamins were also observed to have positive influence on expression of inophyllum B.

If the effects of all the hormones and manipulations of medium components are taken into consideration, supplementation of IBA 19.60 µM in suspension cultures of leaf callus resulted maximum 3.6 times increase in biomass whereas in suspension cultures of nodal / internodal callus, supplementation of three fold sulphate enhanced biomass growth to maximum 6.2 times. In comparison, for increased biomass growth, supplementation of three fold sulphates in the suspension cultures of nodal/ internodal callus was found to be most optimum condition. For the increased expression of inophyllum A, hormones picloram with BAP were most suited in suspension cultures of both callus types. In suspension cultures of leaf callus, picloram 8.28 µM with BAP 8.88 µM resulted highest 295.05 times increased expression of inophyllum A and in suspension cultures of nodal/ internodal callus, picloram 16.56 µM with BAP 8.88 µM resulted highest 101.06 times increased inophyllum A expression. As compared to the medium component manipulation, hormones IBA with BAP were noted to induce highest expression of inophyllum B in suspension cultures of both callus types. IBA 14.70 µM alone was the best suited since it enhanced 25.38 times inophyllum B expression in suspension cultures of leaf callus. When IBA in the same concentration i.e. 14.70 µM along with BAP 4.44 µM was incorporated in suspension cultures of nodal / internodal callus, maximum 1065 times inophyllum B expression was increased. Variation in vitamins were noted to have maximum influence on expression of inophyllum C. Highest 136 times and 265 times inophyllum C expression was increased in suspension cultures of leaf and nodal / internodal callus on supplementation of four and three fold vitamins respectively. Similar to the expression of inophyllum A in suspension cultures of leaf callus, picloram with BAP were also found best suited for increasing inophyllum D expression in suspension cultures of leaf callus. Maximum 2357 times inophyllum D expression was increased when cultures were supplemented with picloram 24.84 µM with BAP 8.88µM. On the contrary, in suspension cultures of nodal / internodal callus, all of the hormone combinations and media component manipulations had either negligible or no inducing effect of inophyllum D expression. Only one fold increase in nitrate slightly (1.2 times) increased inophyllum D expression. Like the expression of inophyllum D, for the expression of inophyllum P also, most of the hormone combinations and medium component manipulations did not induce inophyllum P expression. Only IBA 9.80 µM in suspension cultures of leaf callus enhanced 23.22 times inophyllum P expression whereas only one fold increase in nitrate enhanced 1.32 times inophyllum P expression in suspension cultures of nodal / internodal callus. Almost similar to the expression of inophyllum P, expression of calophyllolide was also maximum on supplementation of IBA and one fold nitrate in suspension cultures of leaf and nodal / internodal callus respectively. IBA 4.90 µM in suspension cultures of

leaf callus increased maximum 85.05 times expression of calophyllolide while one fold nitrate in suspension cultures of nodal / internodal callus increased maximum 4.0 times expression of calophyllolide.

Effect of media components on growth rate and formation of secondary metabolites in cultured cells were studied by number of workers. Farzami and workers studied the influence of IBA and BAP on callus growth and reported that IBA and BAP were best suited for growth of callus cultures of *Rheum ribes.* They also studied the effects of sucrose, vitamins and nitrates on anthraquinone formation and found that growth rate was inversely proportional to anthraquinone formation (Farzami and Ghorbanli, 2002). Similarly Suzuki and co workers studied the influence of nutritional factors such as sucrose, vitamins and inorganic nitrogen source on anthraquinone formation in suspension cultures of *Rubia cordifolia* (Suzuki et al., 1984).

6.3.11. Statistics (Two Way ANOVA):

To find out most suitable medium component manipulation and suspension type for maximum expression of dipyranocoumarins, two way ANOVA was performed (Table 6.4). Two way ANOVA for dipyranocoumarins variation resulting from medium component manipulation in suspension cultures of both callus types revealed that for expression of inophyllums A and C, all three variable i.e. medium manipulation, suspension type and interaction between them were significantly different at 1 % level.

Table 6.4. Two way ANOVA for dipyranocoumarins variation resulting from medium component manipulation in suspension cultures of both callus types

Two way ANOVA	Inophyllum A			Inophyllum B			Inophyllum C		
	Med. Man.	Sus type	Med man. X Sus type	Med. Man.	Sus type	Med man. X Sus type	Med. Man.	Sus type	Med man. X Sus type
S.E.	1.62	3.514	1.54	0.0012	0.0013	0.0015	0.0019	0.0022	0.0018
C.D.	5.954	2.315	14.25	0.0091	0.0018	0.0138	0.00101	0.0027	0.0126
Significance	**	**	**	**	*	**	**	**	**
	Inophyllum D			Inophyllum P			Calophyllolide		
	Med. Man.	Sus type	Med an. X Sus type	Med. Man.	Sus type	Med man. X Sus type	Med. Man.	Sus type	Med man. X Sus type
S.E.	NS	NS	NS	0.0031	NS	0.00268	NS	NS	NS
C.D.	NS	NS	NS	0.0159	NS	0.0252	NS	NS	NS
Significance	NS	NS	NS	**	NS	**	NS	NS	NS

Med. Man- Medium component manipulation, Sus type- Suspension type, Med.Man X Sus type - Interaction between medium component manipulation and Suspension type. (**) Significant at 1% level; (*) Significant at 5% level; NS- Non significant at 1% and 5% level S. E. – Standard Error. N R- No Result.

For expression of inophyllum B, medium manipulation and its interaction with suspension type was significant at 1 % level and suspension type was significant at 5% level. For expression of

inophyllum P, suspension type was non significant but medium manipulation and its interaction with suspension types were significant at 1% level. None of the medium component, suspension type and their interactions was significant for the expression of both, inophyllum D and calophyllolide (Table 6.4).

6.4 CONCLUSIONS:

The effects of hormone IBA, picloram**,** BAP and medium components such as sucrose, nitrate, sulphate and vitamins on biomass growth and expression pattern of dipyranocoumarins in suspension cultures of leaf and nodal / internodal callus was studied and described. Seed callus, because of their necrolytic nature and inability to grow further did not grow in suspension culture; while leaf and nodal / internodal calluses were best suited for initiation of cell suspension cultures. In suspension cultures of both callus types, incubation period for 50 days was optimum for maximum biomass growth and expression of dipyranocoumarins since maximum biomass growth and expressions of most of the dipyranocoumarins were estimated on 50 days incubation. Among the hormones IBA, picloram and BAP studied in suspension cultures of both callus types, IBA 19.60 µM resulted maximum 54.60% (3.6 times) biomass growth in suspension cultures of leaf callus whereas IBA 14.70 µM + BAP 4.44 µM resulted maximum 59.27 % (4.95 times) biomass growth in suspension cultures of nodal / internodal callus. In comparison to IBA + BAP, picloram + BAP was not that much suitable for biomass growth. Although picloram + BAP resulted biomass growth only in the range 9.18 – 16.24 %, highest 59.01 mg % (295 times) and 6.67 mg % (101.06 times) inophyllum A was expressed on media containing picloram (8.28 µM) + BAP (8.88 µM) and picloram (15.56 µM) + BAP (8.88 µM) in suspension cultures of nodal / internodal callus respectively. Among all the combinations and concentrations of hormones and medium component manipulations, these concentrations of picloram and BAP were the best suited for highest expression of inophyllum A in suspension cultures of both callus types. Similar inverse relationship between biomass growth and expression of inophyllums B, D, and calophyllolide was also observed in suspension cultures of leaf callus. In suspension cultures of nodal/internodal callus also, inverse relationship between biomass growth and expression pattern of inophyllums D, P, and calophyllolide was noted. For maximum expression of inophyllum B, IBA 14.70 µM alone in suspension cultures of leaf callus and IBA 14.70 µM + BAP 4.44 µM in suspension cultures of nodal / internodal callus were best suited.

Variation in sucrose did not result in biomass growth in suspension cultures of both callus types. In suspension cultures of leaf callus, sucrose was found to have little influence on expression pattern on inophyllums A and C. In suspension culture of nodal / internodal callus, sucrose was observed to have major influence on expression of inophyllum A and inophyllum C.

Expression patterns of other dipyranocoumarins in suspension cultures of both callus types were not influenced by variation in initial sucrose concentrations.

Like sucrose, variation in total nitrate also did not enhance biomass growth, but did increase the expression of inophyllums A and C and had little influence on expression pattern of other dipyranocoumarins (inophyllums B, D, P and calophyllolide). On the contrary, variation in total sulphate was found to increase biomass growth in suspension cultures of both callus types. One fold increase in sulphate concentration resulted maximum 61.22% (2.42 times) biomass growth in suspension cultures in leaf callus whereas, maximum 38.23 % (6.2 times) biomass was increased on supplementation of three fold sulphate in suspension cultures of nodal / internodal callus. Like nitrate and sucrose, variation in vitamins did not increase biomass in suspension cultures of both callus types, had maximum influence on expression of inophyllums A, B and C and had little influence on expression of other dipyranocoumarins. Maximum 234 times inophyllum B expression was increased on supplementation of 3 fold vitamins in suspension cultures of nodal / internodal callus.

Part of this work has been communicated to journal Process Biochemistry

K.D. Pawar. and S. R. Thengane.
Influence of hormones and medium components on expression of dipyranocoumarins in cell suspension cultures of *C. inophyllum* L.
(Communicated to Process Biochem).

CHAPTER 7. STUDY OF EFFECT OF DIFFERENT ELICITORS ON DIPYRANOCOUMARINS' EXPRESSION IN CELL SUSPENSION CULTURES OF *CALOPHYLLUM INOPHYLLUM*.

7.1. INTRODUCTION:

In spite of great advances made in the area of plant biotechnology for production of useful secondary metabolites, the levels obtained do not always attain commercial application. Among the numerous plants, valuable metabolites obtained using cultured plant cells, only the production of shikonin in *Lithospermum erythrorhizon* cell cultures (Fujita et al., 1984), Purpurina in *Rubia akane* (Alfermann et al., 1995) and taxol in *Taxus cuspidata* (Di Cosmo and Misawa, 1995) gained commercial application. Different strategies were used by different workers to enhance the production of plant secondary metabolites. Elicitation of plant cells in culture represents one of the useful biotechnological tools to improve the production of valuable secondary metabolites. The close relationship between plant secondary metabolism and defense response is widely recognized. Plants respond to attack of pathogens, insects and herbivores or to other biotic and abiotic stresses by activating an array of defense mechanisms including induction of biosynthesis of secondary metabolites as phytoalexins, hypersensitive responses and structural defensive barriers, such as lignin deposition on cell wall, are some of the responses (Hutcheson et al., 1998; He et al., 2002; Mert-Turk, et al., 2002; Durango et al., 2002). Keen (1975), studied the induction by *Phytophthora megasperma* of phytoalexin accumulation in Soyabean and showed that small molecules of pathogenic origin triggered the same response as pathogen itself. These compounds were termed “elicitors”. Elicitor is the term that refers to chemicals from various sources, biotic or abiotic, as well as physical factors, that can trigger a response in living organisms resulting in accumulation of secondary metabolites. Such elicitors are useful tools for improving the production of plant valuable compounds (Zhao et al., 2001; Zhang et al., 2004).

7.1.1. Classification of Elicitors:

Many compounds or stimuli of different origin and structures that enhance the production of useable secondary metabolites in plant cell cultures have been identified. Each type of elicitor according to its characteristics can induce specific response that depends on the interaction between elicitor and plant cell culture. Generally, elicitors are classified on the basis of their origin and molecular structure. Elicitors can be classified as biotic or abiotic.

7.1.1.1. Biotic elicitors:

The biotic elicitors have biological origin, derived from the pathogen or from the plant itself (sometimes called endogenous elicitor). Biotic compounds can be of defined composition when their molecular structures are known, or have a complex composition when they comprise several different molecular classes making impossible to define an unique chemical identity. e.g. Chitosan, Alginate, Pectin, Chitin, Elicitin, Fungal homogenate, Yeast extract, Fungal spores etc.

7.1.1.2. Abiotic elicitors:

Abiotic elicitors do not have a biological origin and are grouped in physical factors and chemical compounds. Sodium orthovanadate, Vanadyl sulphate and Heavy metal salts are the examples of chemical abiotic elicitor whereas thermal stress, osmotic stress, UV irradiation and wounding are the examples of physical abiotic elicitors.

The classification described above only takes into consideration the nature of the elicitor. But these elicitors can also be classified according to the interaction between plant and elicitor into two groups: 'general elicitors' which are able to trigger defense responses both in host and non-host plants and 'race specific elicitors' which induce responses leading to disease resistance only in specific host cultivars, depending on the simultaneous presence of avirulence and resistance genes in the pathogen and plant, respectively.

7.1.2. Factors influencing elicitation:

The effectiveness of elicitation technique to stimulate the production of secondary metabolites depends on a complex interaction between the elicitor and the plant cell. Factors like elicitor specificity, elicitor concentration, treatment interval and culture conditions determine the success of elicitation techniques.

7.1.2.1. Elicitor specificity:

There are reports that the same elicitor can stimulate secondary metabolism in different cell cultures and, on the other hand, that certain plant cultures are responsive to diverse elicitors. Treatment of a particular culture with different elicitors will result in the accumulation of the same compounds, since these are specific of each plant culture. Although, the class of metabolite depends on the plant species, the kinetics of induction or accumulation levels varies with different elicitors. If the elicitor signal is detected by the plant cell via a specific receptor, the selectivity of the response of a given plant species for a determined elicitor would depend to a great extent on the presence of such molecular entity and on the transduction pathways that each elicitor activates (Vasconsuelo and Boland, 2007).

7.1.2.2. Elicitor concentration and treatment interval:

The concentration of elicitors is a factor that strongly affects the intensity of the response and the effective dose, which varies according to the plant species, can only be found empirically. It has been demonstrated that elicitor levels, which exert stimulatory effects in certain plant systems when applied to others are devoid of activity, reflecting different sensibilities of the

molecular components involved in elicitation. In general the elicitor is in contact with the system until harvest, but the time required for maximum secondary metabolite accumulation is a characteristic of each plant species and normally is preceded by an increase in activity of the metabolic enzymes involved. These facts emphasize the importance of determining empirically the optimum conditions of elicitation time and elicitor concentration for each system in particular.

7.1.2.3. Culture conditions:

Elicitation responses due to different factors are variable that necessitates, the optimization of medium composition and culture conditions. Optimization of medium composition and culture coditions represents an important aspect in elicitation technique. The most appropriate moment to add the elicitor is during the exponential phase of growth when the enzymatic machinery is in the maximum operative status, the response to the elicitor being, in consequence, more efficiently achieved. Another factor is the presence of growth regulators in the medium, which can markedly affect the elicitation of secondary metabolism.

In the present work, both biotic and abiotic elicitors have been used to enhance the production of dipyranocoumarins This chapter envisages the results on elicitation and describes the effects of abiotic elicitors like calcium, copper, cadmium and chromium in suspension cultures of leaf and nodal / internodal callus. Results on isolation and identification of fungi endophytic to *C. inophyllum*, use of dried cell powder and culture filtrate of isolated fungi as biotic elicitors and effects of the biotic elicitation on expression of dipyranocoumarins are also discussed.

7.2 EXPERIMENTAL PROTOCOL:

7.2.1. Isolation of endophytic fungi:

For using fungi as biotic elicitors, fungi endophytic to *C. inophyllum* were isolated from mature leaves. The mature leaves of *C. inophyllum* were collected from plant growing in botanical garden, National Chemical Laboratory, Pune, India. Leaves were first washed thoroughly with running tap water and then with liquid detergent Labolene. Thereafter, leaves were treated with 10 % Savlon for 10 min. and washed thoroughly with sterile double distilled water. After Savlon treatment, next treatments were given in Laminar Air Flow Chamber. In LAF chamber, leaves were first surface sterilized with 80% ethanol (v/v) for 20 seconds followed by thorough washing with sterile double distilled water. Finally, leaves were surface sterilized with 15% hydrogen peroxide (H_2O_2, v/v) for 10 min. Treatment with H_2O_2 was followed by thorough washing with sterile double distilled water. Such surface sterilized leaves were then cut into small pieces of

2 x 2 cm size and were injured with sterile surgical blade prior to its inoculation. Leaf pieces were aseptically transferred to Petri dishes containing agar-agar (2%) solidified Potato Dextrose Medium (PDA). Plates were incubated at 37° C for 7 – 10 days. After 7 – 10 days, fungal mycelia that grew on the surface of the PDA medium were picked using nicrome wire loop and transferred onto PDA slants. Mixed fungal cultures were made into pure culture by repeatedly transferring onto slants or plates.

7.2.2. Identification of fungi:

Pure fungal cultures isolated from *C. inophyllum* were microscopically identified at Division of Mycology, Agharkar Research Institute, Pune, India.

7.2.3. Preparation of biotic elicitors and elicitation medium:

For eliciting the suspension cultures of leaf and nodal/ internodal callus with biotic elicitor, dried cell powder (DCP) and culture filtrates (CF) of isolated fungi were used. Fungi were cultured in potato dextrose broth in larger volumes. These cultures were incubated as static cultures at room temperature for two weeks. For harvesting the mycelia mat that floated on the surface of medium, culture vessels were autoclaved at 121° C for 10 min. Then cultures were filtered to collect biomass and washed with sterile double distilled water. Fungal biomass was then air dried for two days and crushed to a fine powder in liquid nitrogen. Such dried cell powder (DCP) was then incorporated as biotic elicitors in liquid WPM medium. Concentration of DCP in liquid WPM medium was varied in the range 20 – 800 mg/100 ml medium (i. e. 20, 40, 60, 80, 100, 200, 400, 600 and 800 mg/100 ml medium).

After collecting the biomass, remaining culture filtrate was collected and centrifuged at 10000 rpm for 10 min. Such a biomass free culture filtrates of fungi were also used as biotic elicitors. Concentration of culture filtrates in liquid WPM medium was varied in the range 10 – 50 % (v/v)

7.2.4. Preparation of abiotic elicitation medium:

For elicitation with abiotic elicitor in suspension cultures of leaf and nodal/ internodal callus, heavy metals such as copper, cadmium and chromium were incorporated in liquid WPM medium. Salts of these heavy metals such as $CuSO_4$, $CdCl_2$ and $K_2Cr_2O_7$ were used as source of these heavy metals. 0.5 M stock solutions of all these salts of heavy metals were prepared and incorporated in liquid WPM medium to final desired concentrations. Concentrations of these heavy metals were varied in the range 0.1 – 20 mM (i e. 0.1, 0.5, 1.0, 5.0, 10, 20 mM). Calcium was also used as abiotic elicitor. $CaCl_2$ was used as a source of calcium. Concentration of calcium was

varied in the range 1.0 – 4.0 mM. All these abiotic elicitors were incorporated before autoclaving the media.

7.2.5. Harvesting of elicited biomass and HPLC analysis:

For the HPLC analysis of elicited biomass, cell suspension cultures were harvested after 45 – 50 days of incubation. To collect biomass, cell suspension cultures were filtered through Whatmann's filter paper No.1. Collected biomass was then washed with sterile distilled water and weighed. Culture filtrate was also collected and used for the HPLC analysis. Biomass and culture filtrates were extracted with the extraction procedure described in chapter 5 and 6. HPLC analyses of the extracted biomass and culture filtrates were performed using HPLC method described in previous chapters (Chapter 3, 5 and 6).

7.2.6. Data analysis:

Two way analysis of variance (Two way ANOVA) for the dipoyranocoumarins' contents (expression) resulting from elicitation with biotic and abiotic elicitor was performed using OriginPro 8 program and Microsoft Excel.

7.3. RESULTS AND DISCUSSION:

7.3.1. Biotic elicitation:

7.3.1.1. Isolation of endophytic fungi:

Secondary metabolites are involved in plant defense reactions and fungal – host interactions. The concentrations of the secondary metabolites differ as a result of endophytic infections. Considering these facts, we decided to isolate fungi endophytic to *C. inophyllum* and use them as the biotic elicitors to study the expression pattern of dipyranocoumarins'. For the isolation of endophytic fungi from leaf tissue, different surface sterilizing agents in different concentrations were tried for effective surface sterilizaton. Among the sodium hypo-chloride (NaOCl), mercury chloride ($HgCl_2$) and hydrogen peroxide (H_2O_2), 15 % H_2O_2 resulted in best surface sterilization. This was confirmed by inoculating the leaf pieces without injuring prior to transferring to the PDA medium. Fungal growth was not observed when leaf tissue was not injured with sterile surgical blade or needle prior to inoculation. However, fungal growth was observed when leaf tissue was injured and inoculated on PDA medium. Using 15% H_2O_2 (v/v) as a sterilizing agents, four fungi were isolated from leaf (Fig7.1b and.7.1a) and made into pure cultures. The experiments for isolation were repeated twice to check the reproducibility.

Fig.7.1 a. Leaf of *C. inophyllum.*

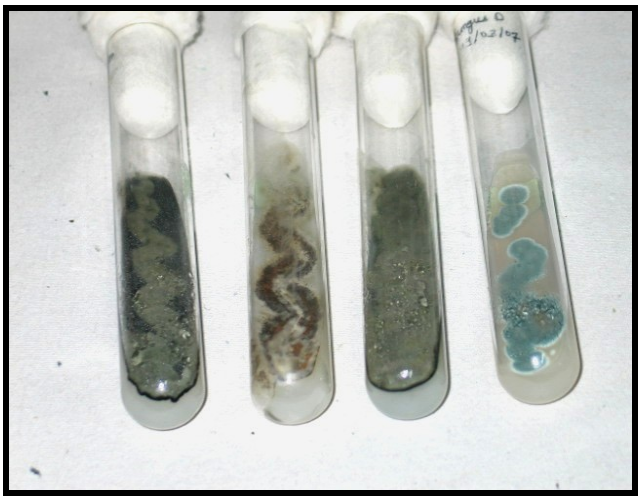

Fig.7.1 b Isolated endophytic fungi on slants

These four fungal isolates were microscopically identified at Mycology Division, Agharkar Research Institute Pune, India.

1. *Nigrospora sphaerica* (Sacc.) Mason**.** (Fig. 7.2).
2. *Phoma Species* (Fig. 7.3).
3. *Cladosporium cladosporioides* (Fresen.) de Vries (Fig. 7.4).
4. *Aspergillus sydowi* (Bain. and Sart.) Thom. & Church (Fig. 7.5).

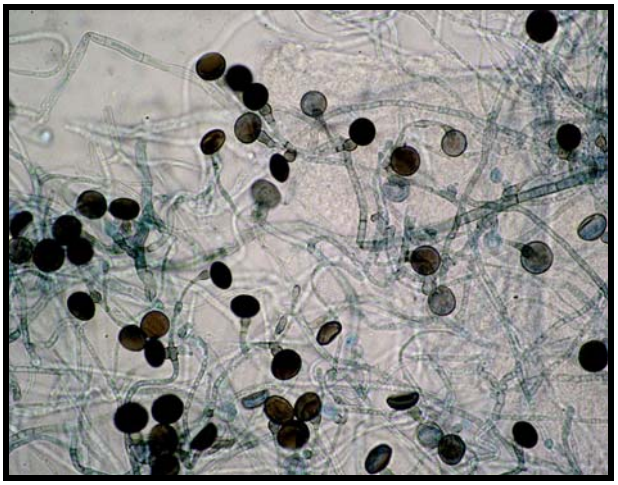

Fig. 7.2 Microscopic image of *Nigrospora sphaerica* (Sacc.) Mason.

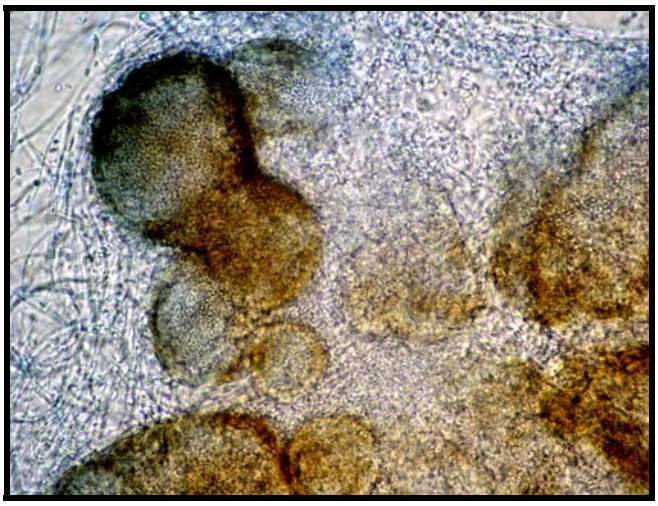

Fig. 7.3 Microscopic image of *Phoma Species*

Fig. 7.4 Microscopic image of *Cladosporium cladosporioides* (Fresen.) de Vries

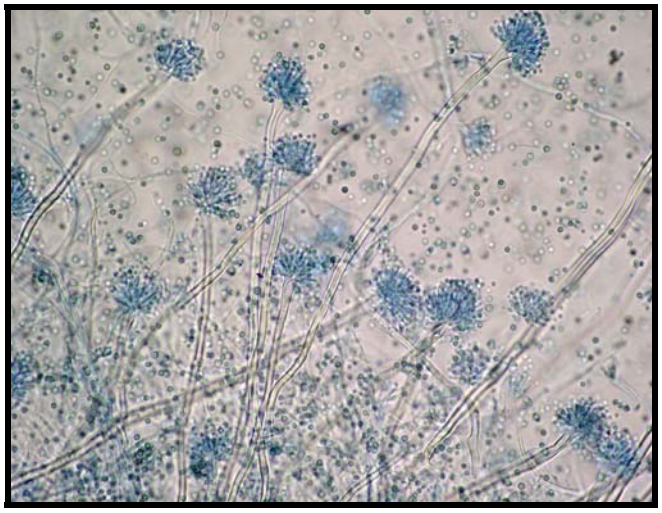

Fig. 7.5 Microscopic image of *Aspergillus sydowi* (Bain. & Sart.) Thom. & Church

Out of these four fungi, *Nigrospora sphaerica* (Fig 7.2), *Phoma Species* (Fig 7.3) and *Cladosporium cladosporioides* (Fig 7.4) were also reported as endophytic to number of plant species. *Cladosporium* and *Phoma* species with some other fungal species were the most abundant endophytes in twigs of *Fagus Sylvatica* (Danti et al., 2002). These two fungal species were also isolated as endophytes from woody tissues of *Quercus robur* and *Quercus cerris* (Gennaro et al., 2003; Ragazzi et al., 2001). *Nigrospora* was also reported as endophyte to many plant species. *Nigrospora* and *Phoma* species were isolated from *Eucalyptus globules* (Lupo et al., 2001) and *Tectona grandis* (Chareprasert et al., 2006). Because *Nigrospora sphaerica*, *Phoma Species* and *C. cladosporioides* were confirmed endophytes, out of these three endophytes, *N. sphaerica and Phoma Species* were further used as source of biotic elicitor. Dried cell powder (DCP) and culture filtrates of both of these fungi were incorporated in suspension cultures of both callus types.

7.3.1.2. Preparation of biotic elicitors and elicitation medium:

For large scale cultivation of isolated fungi, potato dextrose broth was found to be most suitable medium. Incubation of fungal cultures as static cultures at room temperature for more 7 – 10 days was sufficient and optimum conditions to ensure maximum biomass growth (Fig.7.6). For the preparation of DCP, after 7 – 10 days, cultures were autoclaved to prevent their further growth and sporulation. Autoclaving the cultures for short time i.e. only for 10 min. was done only to prevent the further growth and to ensure minimal or no damage to cell components that act as elicitor. For complete removal of the medium residues, biomass was washed with distilled water and then dried. Initially attempts were made to dry the biomass by keeping for air drying as well as keeping in the hot air oven at 40° C. While air drying, a problem of secondary growth i. e. fungal and bacterial contaminations were encountered. Drying in hot air oven was also not suitable since biomass dried in hot air oven was difficult to make into powder using morter and pestle. As an alternative, crushing the biomass to a powder in liquid nitrogen was best suited and used. Such biomasses of both fungi were then incorporated in suspension cultures of both callus types. Centrifugation of culture filtrates at 1000 rpm for 10 min. ensured the complete removal of the cell debris and suspended particles.

Fig. 7.6 Endophytic fungi cultured in larger culture volumes.

7.3.1.3. Effects of fungal dired cell powder as biotic elicitors on dipyranocoumarins' expression in suspension culture of leaf callus:

Since *Nigrospora sphaerica* and *Phoma* species were also isolated from numerous plant species**,** out of the four fungi isolated from *C. inophyllum,* these two fungal species were used as biotic elicitors to study their effects on expression pattern of dipyranocoumarins. Table 7.1 shows the results for effects of DCP of both fungi as biotic elicitors in suspension cultures of leaf callus.

Elicitation of the suspension cultures of leaf callus with DCP did not induce the biomass growth. Incorporation of DCP of *Phoma species* was found to have influence on expression of inophyllum A. Compared to expression of inophyllum A (0.018 mg %) in unelicited cultures; 600 mg DCP of *Phoma species* enhanced the expression of inophyllum A to 0.403 mg %. This expression of inophyllum A was 21.78 times more than the expression in unelicited cultures (control). DCP of *Phoma species* at all tested concentrations increased inophyllum A expression. Compared to DCP at lower concentrations (20 – 100 mg), higher concentrations (200-800 mg) induced maximum expression of inophyllum A in the range 0.052 – 0.403 mg % (2.81 – 21.78 times). DCP of fungus *N. sphaerica* was not effective for eliciting the expression of inophyllum A. Inophyllum A expression was reduced in cultures elicited with DCP of *N. sphaerica.* Compared to unelicited cultures, inophyllum A expression was decreased with all tested concentrations of DCP of *N. sphaerica* (Table 7.1).

DCP of both fungal species were observed to elicit the expression of inophyllum B. At all tested concentrations of DCP of *Phoma species,* expression of inophyllum B was

enhanced. At lower concentrations (20 – 80 mg), 0.0021 – 0.016 mg % (75 – 571.42 times) inophyllum B expression was resulted. This expression was more than the expression 4.2 $x10^{-5}$ – 0.00176 (1.5 – 62.85 times) resulted with higher concentrations (100 –800 mg) of DCP of *Phoma species*. DCP of *N. sphaerica* only at lower concentrations (20 –60 mg) enhanced inophyllum B expression in the range 0.0018 – 0.0029 mg % (5.55 –8.05 times). However, direct relationship between concentration of DCP and expression level of inophyllum B was not observe. 40 mg DCP of *Phoma species* resulted in 0.016 mg % (571.42 times) inophyllum B and 60 mg DCP of *N. sphaerica* resulted in 0.0029 mg % (8.05 times) inophyllum B expression. This indicated that 40 mg DCP of *Phoma species* as biotic elicitor was optimum concentration for eliciting the higher expression of inophyllum B in suspension cultures of leaf callus (Table 7.1).

Like expression of inophyllum B, expression of inophyllum C was also influenced by DCP of both fungal species. But compared to *Phoma species*, *N. sphaerica* had little influence on expression of inophyllum C. DCP of *Phoma species* at lower concentrations than 400 mg was noted to enhance inophyllum C expression in the range 0.089 – 0.834 mg % (2.40 – 22.54 times). In comparison to expression of inophyllum C (0.037 mg%) in unelicited cultures, DCP of *Phoma species* at 100 mg increased inophyllum C expression to maximum 0.83 mg % (22.54 times). DCP of *N. sphaerica* only at lower concentration of DCP (20 mg) resulted slight increase in inophyllum C expression from 0.265 mg % (in control) to 0.295 mg % (1.11 times). At higher concentration of *N. sphaerica*, inophyllum C expression was reduced (Table 7.1).

Expression pattern of inophyllum D in cultures elicited with both fungal species was quite similar to expression pattern of inophyllum C. For expression of inophyllum D also, *N. sphaerica* had little influence when compared to *Phoma species*. Elicitation of cultures with DCP of *Phoma species* in the range 20 – 100 mg, enhanced inophyllum D expression in the range 0.0015 – 0.0188 mg % (4.28 – 53.71 times). 60 mg DCP of *Phoma species* resulted in 0.018 mg % (53.71 times) inophyllum D whereas only 40 mg DCP of *N. sphaerica* could enhance inophyllum D expression to 0.0022 mg % (32.83 times) (Table 7.1).

Expression of inophyllum P was slightly influenced *by Phoma species* and was negatively influenced by *N. sphaerica.* All the tested concentrations of *Phoma species* enhanced inophyllum P expression in the range 2.9 x 10^{-5} – 0.00018 mg % (1.11 – 6.92 times). Maximum 0.00018 mg % (6.92 times) inophyllum P was estimated in cultures elicited with 200 mg DCP of *Phoma species*; whereas DCP of *N. sphaerica* at all tested concentrations reduced the inophyllum P expression (Table 7.1).

Table 7.1 Effect of fungal DCP as biotic elicitors on dipyranocoumarins' expression in suspension cultures of leaf callus.

Fungal Spp.	DCP (mg/100ml medium)	Dipyranocoumarins (mg/100 g elicited biomass FW)					
		Inophyllum A	Inophyllum B	Inophyllum C	Inophyllum D	Inophyllum P	Calophyllolide
Phoma spp.	0.0	0.0185±0.002	2.8 $x10^{-5}$±1.5 $x10^{-5}$	0.037±0.028	0.00035±0.00012	2.6 $x10^{-5}$±2.2 $x10^{-5}$	2.7 $x10^{-5}$±1.7 $x10^{-5}$
	20	0.0373±0.003	0.0021±0.0016	0.264±0.487	0.00502±0.0009	5.4 $x10^{-5}$±2.6 $x10^{-5}$	0.025±0.007
	40	0.042±0.021	0.016±0.0018	0.159±0.018	0.0034±0.0004	5.7 $x10^{-5}$±5.5 $x10^{-5}$	0.0177±0.002
	60	0.046±0.013	0.0054±0.0031	0.119±0.005	0.0188±0.021	3.7 $x10^{-5}$±2.1 $x10^{-5}$	0.0219±0.006
	80	0.065±0.027	0.0044±0.0007	0.18±0.011	0.0015±9 $x10^{-5}$	0.00012±6.7 $x10^{-5}$	0.268±0.520
	100	0.032±0.009	4.2 $x10^{-5}$±2.08 $x10^{-5}$	0.834±0.14	0.0058±0.00065	0.00010±7.7 $x10^{-5}$	0.0122±0.008
	200	0.390±0.051	5.9 $x10^{-5}$±4.1 $x10^{-5}$	0.089±0.013	5.4 $x10^{-5}$±7.3 $x10^{-5}$	0.00018±0.00015	0.000254±0.0001
	400	0.052±0.030	4.6 $x10^{-5}$±2.09 $x10^{-5}$	0.0189±0.008	0.00024±0.00016	2.9 $x10^{-5}$±1.2 $x10^{-5}$	2.9 $x10^{-5}$±1.6 $x10^{-5}$
	600	0.403±0.12	5.4 $x10^{-5}$±2.8 $x10^{-5}$	0.020±0.006	0.00021±0.0002	4.6 $x10^{-5}$±1.3 $x10^{-5}$	7.20 $x10^{-5}$±2 $x10^{-5}$
	800	0.172±0.106	0.00176±0.00032	0.0160±0.078	0.00019±0.00011	0.00011±8.3 $x10^{-5}$	0.0022±0.0004
Nigrospora sphaerica	0.0	0.434±0.18	0.00036±0.00012	0.265±0.068	6.7 $x10^{-5}$±1.9 $x10^{-5}$	0.088±0.017	8.2 $x10^{-5}$±5.09 $x10^{-5}$
	20	0.256±0.07	0.0018±0.0017	0.295±0.434	2.5 $x10^{-5}$±8.3 $x10^{-6}$	0.032±0.01	5.8 $x10^{-5}$±4.5 $x10^{-5}$
	40	0.161±0.03	0.0020±0.00012	0.120±0.11	0.0022±0.0004	0.029±0.023	0.00022±3.6 $x10^{-5}$
	60	0.061±0.021	0.0029±0.005	0.0540±0.043	6.3 $x10^{-5}$±4.5 $x10^{-5}$	0.017±0.003	0.00017±0.00012
	80	0.080±0.07	6.2 $x10^{-5}$±2.7 $x10^{-5}$	0.023±0.012	2.8 $x10^{-5}$±1.2 $x10^{-5}$	5.1 $x10^{-5}$±2.3 $x10^{-6}$	3.8 $x10^{-5}$±2.5 $x10^{-5}$
	100	0.087±0.036	6.2 $x10^{-5}$±3.02 $x10^{-5}$	0.025±0.017	2.09 $x10^{-5}$±5.4 $x10^{-6}$	4 $x10^{-5}$±1.1 $x10^{-5}$	9.03 $x10^{-5}$±8.3 $x10^{-5}$
	200	0.0875±0.053	5.1 $x10^{-5}$±2.1 $x10^{-5}$	0.031±0.022	2.3 $x10^{-5}$±1.5 $x10^{-5}$	0.00010±72. $x10^{-5}$	3.2 $x10^{-5}$±1.9 $x10^{-5}$
	400	0.0858±0.036	8.8 $x10^{-5}$±6.6 $x10^{-5}$	0.018±0.010	3.6 $x10^{-5}$±2.2 $x10^{-5}$	4.04 $x10^{-5}$±2.2 $x10^{-5}$	3 $x10^{-5}$±2.02 $x10^{-5}$
	600	0.060±0.037	4.2 $x10^{-5}$±1.9 $x10^{-5}$	0.032±0.017	2.5 $x10^{-5}$±2.2 $x10^{-5}$	4.4 $x10^{-5}$±2.09 $x10^{-5}$	0.00122±0.00023
	800	0.064±0.024	3.3 $x10^{-5}$±9.5 $x10^{-6}$	0.013±0.006	9.1 $x10^{-5}$±5.9 $x10^{-5}$	4.1 $x10^{-5}$±1.7 $x10^{-5}$	4.5 $x10^{-5}$±1.2 $x10^{-5}$

All values are mean ± S.D of four replicates

DCP of both fungal species had positive influence on the expression of calophyllolide and increased its expression. At all tested concentrations of DCP of *Phoma species***,** expression of calophyllolide was enhance in the range 2.9 $x10^{-5}$ – 0.268 mg % (1.07 – 9925 times). As compared to expression of calophyllolide in control ($2.7x10^{-5}$), 80 mg DCP of *Phoma species* enhanced calophyllolide expression to 0.268 mg % (9925 times). Expression pattern of calophyllolide resulting from DCP of *N. sphaerica* was erratic. 600 mg DCP of *N. sphaerica* enhanced calophyllolide expression from 8.2 $x10^{-5}$ mg % to 0.0012 mg % (14.63 times) (Table 7.1).

Overall expression pattern of dipyranocoumarins in suspension cultures of leaf callus indicated that in comparison, DCP of *Phoma species* as biotic elicitor was more effective than DCP of *Nigrospora sphaerica* for eliciting the expression of dipyranocoumarins. In suspension cultures of leaf callus, maximum 21.78 times inophyllum A, 571.42 times inophyllum B, 22.43 times inophyllum C, 53.71 times inophyllum D, 6.92 times inophyllum P and 9925 times calophyllolide were expressed when cultures were elicited with 600, 40, 100, 60, 200, 80 mg DCP of *Phoma species* respectively. Among all the dipyranocoumarins estimated, expression level of calophyllolide was increased up to 9925 times which was highest than the increased levels of any other dipyranocoumarins under study.

7.3.1.4. Effects of fungal dired cell powder as biotic elicitors on dipyranocoumarins' expression in suspension culture of nodal/ internodal callus:

The results for effects of fungal DCP (dried cell powder) on dipyranocoumarins' expression in suspension culture of nodal/ internodal callus are given in Table 7.2. In suspension cultures of nodal / internodal callus, DCP of both fungal species had positive influence on inophyllum A expression. However, compared to *N. sphaerica, Phoma species* was comparatively more effective for increasing expression of inophyllum A, since DCP of *Phoma species* resulted in many fold increase in expression of inophyllum A. Maximum 0.58 mg % (362.5 times) inophyllum A was expressed on eliciting the cultures with 60 mg DCP of *Phoma species*, whereas maximum 76.88 mg % (13.48 times) inophyllum A was expressed in cultures elicited with 80 mg DCP of *N. sphaerica.* This indicated that for increased expression of inophyllum A, DCP of *Phoma species* at lower concentrations was much more suitable. At all the concentrations of DCP tried, *Phoma species* increased inophyllum A expression in the range 0.03 – 0.58 mg% (18.75- 362.5 times), while only lower concentrations of DCP of *N. sphaerica* (20 – 80 mg) increased inophyllum A expression in the range 12.25 – 76.88 mg% (2.14-13.48 times). Higher concentrations of DCP of *N. sphaerica* (100 – 800 mg) had negative influence and decreased the expression of inophyllum A (Table 7.2).

For inophyllum B expression, DCP of both fungal species at higher concentration were found to have positive influence and increased the inophyllum B expression. Maximum 0.047 mg % (4968 times) and 0.0036 mg % (49.31 times) inophyllum B was expressed on eliciting the cultures with 400 mg DCP's of *Phoma species* and *N. sphaerica* respectively. However, between the two fungal species, *Phoma species* was found to be best suited because maximum inophyllum B expression (0.047 mg %) was achieved with it. At higher concentrations (200 – 800 mg), *Phoma species* and *N. sphaerica* increased the inophyllum A expression in the range 0.0014 – 0.047 mg % (145.83 - 4968 times) and 9.6 x 10^{-5} – 0.0036 mg % (1.31- 49.31 times) respectively (Table 7.2).

DCP of *Phoma species* at all tested concentrations was found to enhance the expression of inophyllum C while DCP of *N. sphaerica* at concentrations more than 60 mg was found to induce inophyllum C expression. Maximum 1.51 (321.27 times) and 0.441 mg % (18.92 times) inophyllum C was expressed in cultures elicited with 400 mg DCP of *Phoma species* and 800 mg DCP of *N. sphaerica.* Comparatively, *Phoma species* was more effective than *N. sphaerica* for inophyllum C expression (Table 7.2).

DCP of both of the fungal species had little positive influence on expression pattern of inophyllum D. Higher concentrations of DCP of both fungal species slightly increased inophyllum D expression. Maximum 0.0037 (308.33 times) and 9.5 x 10^{-5} mg % (6.55 times) inophyllum D was expressed on elicitation with 100 mg DCP of *Phoma species* and 400 mg DCP of *N. sphaerica* respectively. Rest of the concentration levels of both fungal species showed only slight influence on inophyllum D expression (Table 7.2).

In elicited cultures, expression pattern of inophyllum P and calophyllolide was almost similar to the expression of inophyllum D. Maximum 0.0063 mg % (157.10 times) inophyllum P was expressed on elicitation with 200 mg DCP of *Phoma species* whereas maximum 0.071 mg % (1690.47 times) inophyllum P was expressed in cultures elicited with 800 mg DCP of *N. sphaerica.* In comparison, DCP of *N. sphaerica* was best suited for inophyllum P expression (Table 7.2).

For the expression of calophyllolide, *Phoma species* at higher concentration (100 – 800 mg) resulted in higher expression of calophyllolide i.e. in the range 0.00010 – 0.030 mg % (2.56 – 769.23 times). Maximum 0.030 mg % (769.23 times) calophyllolide was expressed in cultures elicited with 800 mg DCP of *Phoma species*. Comparatively, *N. sphaerica* was less effective for calophyllolide expression. Highest 0.0069 mg % (246 times) calophyllolide was expressed on elicitation with 600 mg DCP of *N. sphaerica* (Table 7.2).

Table 7.2 Effect of fungal DCP as biotic elicitors on dipyranocoumarins' expression in suspension cultures of nodal/internodal callus.

Fungal Spp.	DCP (mg/100ml medium)	Dipyranocoumarins (mg/100 g elicited biomass FW)					
		Inophyllum A	Inophyllum B	Inophyllum C	Inophyllum D	Inophyllum P	Calophyllolide
Phoma spp.	0.0	0.0016±0.001	$9.6 \times 10^{-6} \pm 4.2 \times 10^{-6}$	0.0047±0.003	$1.2 \times 10^{-5} \pm 6.9 \times 10^{-6}$	$4.01 \times 10^{-5} \pm 1.02 \times 10^{-5}$	$3.9 \times 10^{-5} \pm 1.03 \times 10^{-5}$
	20	0.040±0.012	$4.6 \times 10^{-5} \pm 1.8 \times 10^{-5}$	0.0209±0.019	$1.2 \times 10^{-5} \pm 6.9 \times 10^{-6}$	$6.1 \times 10^{-5} \pm 5.7 \times 10^{-5}$	$1.5 \times 10^{-5} \pm 9.8 \times 10^{-6}$
	40	0.137±0.073	0.00083±0.00012	0.181±0.023	$4.4 \times 10^{-5} \pm 3.8 \times 10^{-5}$	$0.00011 \pm 7.6 \times 10^{-5}$	$6.04 \times 10^{-5} \pm 4.4 \times 10^{-5}$
	60	0.581±0.37	$5.61 \times 10^{-5} \pm 1.2 \times 10^{-5}$	0.146±0.253	$1.3 \times 10^{-5} \pm 3.3 \times 10^{-5}$	$5.2 \times 10^{-5} \pm 4.08 \times 10^{-5}$	$1.01 \times 10^{-5} \pm 5.9 \times 10^{-5}$
	80	0.133±0.10	$8.01 \times 10^{-5} \pm 6.9 \times 10^{-5}$	0.027±0.01	$3.5 \times 10^{-5} \pm 3.2 \times 10^{-5}$	$6.6 \times 10^{-5} \pm 4.9 \times 10^{-5}$	0.00046±0.00030
	100	0.421±0.26	$4.3 \times 10^{-5} \pm 2.7 \times 10^{-5}$	0.141±0.07	0.0037±0.0014	0.0023±0.00046	0.00091±0.00011
	200	0.034±0.010	0.032±0.021	0.426±0.06	$0.00073 \pm 6 \times 10^{-5}$	0.0063±0.0012	0.000103±0.00008
	400	0.075±0.059	0.0477±0.021	1.517±0.298	$8.6 \times 10^{-6} \pm 4.5 \times 10^{-6}$	$1.6 \times 10^{-5} \pm 4.3 \times 10^{-6}$	0.0159±0.0018
	600	0.068±0.020	0.00218±0.0024	0.910±0.51	0.00010±0.0001	$4.7 \times 10^{-5} \pm 2.7 \times 10^{-5}$	0.0166±0.0019
	800	0.077±0.020	0.0014±0.001	1.129±0.15	$0.00012 \pm 1.1 \times 10^{-5}$	0.00023±0.00018	0.0306±0.016
Nigrospora sphaerica	0.0	5.70±2.50	$7.3 \times 10^{-5} \pm 4.9 \times 10^{-5}$	0.0233±0.007	$1.45 \times 10^{-5} \pm 4.2 \times 10^{-6}$	$4.2 \times 10^{-5} \pm 1.7 \times 10^{-5}$	$2.8 \times 10^{-5} \pm 5.2 \times 10^{-6}$
	20	12.25±24.32	$5.02 \times 10^{-5} \pm 1.9 \times 10^{-5}$	0.022±0.009	$1.5 \times 10^{-5} \pm 6.5 \times 10^{-6}$	$2.2 \times 10^{-5} \pm 1.4 \times 10^{-5}$	$1.6 \times 10^{-5} \pm 3.05 \times 10^{-6}$
	40	23.51±10.19	$5.4 \times 10^{-5} \pm 2.7 \times 10^{-5}$	0.0224±0.018	$1.6 \times 10^{-5} \pm 1.2 \times 10^{-5}$	$3.9 \times 10^{-5} \pm 1.1 \times 10^{-5}$	$2.4 \times 10^{-5} \pm 5.5 \times 10^{-6}$
	60	74.95±10.54	$0.00012 \pm 2.7 \times 10^{-5}$	0.036±0.004	$1.8 \times 10^{-5} \pm 3.9 \times 10^{-6}$	$7.2 \times 10^{-5} \pm 5.3 \times 10^{-5}$	$2.7 \times 10^{-5} \pm 1.3 \times 10^{-5}$
	80	76.88±31.27	$8.8 \times 10^{-5} \pm 3.3 \times 10^{-5}$	0.065±0.030	$2.2 \times 10^{-5} \pm 8.2 \times 10^{-5}$	0.00082±0.00012	$7.06 \times 10^{-5} \pm 7.3 \times 10^{-5}$
	100	0.03±0.018	$8.41 \times 10^{-5} \pm 2.7 \times 10^{-5}$	0.054±0.056	$3.4 \times 10^{-5} \pm 2.8 \times 10^{-5}$	$4.4 \times 10^{-5} \pm 2.4 \times 10^{-5}$	$2.1 \times 10^{-5} \pm 8.3 \times 10^{-6}$
	200	0.433±0.33	$9.6 \times 10^{-5} \pm 2.7 \times 10^{-5}$	0.0289±0.007	$2.6 \times 10^{-5} \pm 1.1 \times 10^{-5}$	0.00043±0.00016	$2.9 \times 10^{-5} \pm 1.05 \times 10^{-5}$
	400	4.95±3.88	0.0036±0.0007	0.059±0.0027	$9.5 \times 10^{-5} \pm 3.4 \times 10^{-5}$	$2.15 \times 10^{-5} \pm 8.6 \times 10^{-5}$	0.00020±0.00003
	600	1.68±0.307	0.00023±0.0001	0.095±0.058	$4.5 \times 10^{-5} \pm 2.7 \times 10^{-5}$	$0.000132 \pm 4.1 \times 10^{-5}$	0.0069±0.0013
	800	0.27±0.04	0.00191±0.00025	0.441±0.816	$2.3 \times 10^{-5} \pm 1.2 \times 10^{-5}$	0.071±0.05	$2.2 \times 10^{-5} \pm 1.08 \times 10^{-5}$

All values are mean ± S.D of four replicates

Like the effect of DCP of *Phoma species* in suspension cultures of leaf callus, DCP of *Phoma species* was also found most effective and suitable for eliciting the expression of dipyranocoumarins in suspension cultures of nodal / internodal callus. In suspension cultures of nodal/ internodal callus, the increased levels of expression of inophyllums A (362.5 times) and B (4968 times) resulting from elicitation with DCP (60 and 400 mg) of *Phoma species* was higher than the expression in suspension cultures of leaf callus (600 and 40 mg respectively). For expression of inophyllum C, D and calophyllolide, elicitation of the suspension cultures of nodal / internodal callus with DCP of *Phoma species* was also most suitable condition. 400, 100 and 800 mg DCP of *Phoma species* induced 321.27 times inophyllum C, 308.33 times inophyllum D and 769.23 times calophyllolide expression respectively. Only for the expression of inophyllum P, DCP of *N. sphaerica* was found suitable in suspension cultures of nodal / internodal callus. 800 mg DCP of *N. sphaerica* resulted in 1690.47 times increased expression of inophyllum P. Thus in comparison to DCP of *N. sphaerica* as biotic elicitor, DCP of *Phoma species* in suspension cultures of nodal / internodal callus was the best suited condition for increased expression of dipyranocoumarins.

Fungi as biotic elicitors especially autoclaved mycelia have been successfully used for the elicitation of many different secondary metabolites in different plant species. To find out the effective elicitation factors for the production of antraquinone in cell suspension cultures of *Morinda elliptica*, fungal mycelial homogenates of *Aspergillus niger* and *Aspergillus flavus* were used (Chong et al., 2005). Roken et al., (1984) studied the effect of autoclaved fungal mycelia in cell suspension cultures of *Dioscorea deltoida* and reported that autoclaved fungal mycelia increased the production of steroid diosgenin. In the present study, autoclaved mycelia of *Phoma species* and *N. sphaerica* also increased the expression of dipyranocoumarins. The increased expression could have been mediated by the induction of enzymes involved in the biosynthesis of dipyranocoumarins. Such induction of enzymes of phytoalexin synthesis was studied in cultured Soyabean cells (Hille et al., 1982). These authors used fungal elicitors from *Phytophthora megasperma* and induced the enzymes of phytoalexin synthesis in cultured soyabean cells. Similar study aimed at inducing the enzymes of phytoalexin synthesis was carried out in suspension cultures of *Medicago sativa.* Walton et al., (1993) reported that fungal elicitors induced phytoalexin synthesis by generation of inositol 1, 4, 5- trisphosphate and hydrolysis of phosphatidylinositol 4, 5- bisphosphate (Walton et al., 1993). Namdeo et al., (2002) successfully studied the increased production of ajmalicine in suspension cultures of *Catharanthus roseus*. They used the fungal cell wall fragments of *Aspergillus niger*, *Fusarium moniliforme* and *Trichoderma viride* as elicitors and reported that cell wall fragments of *Trichoderma viride* resulted increase in ajamalicine production from 79 µg/ g DW to 166µg/ g DW. In *Catharanthus roseus* cell cultures,

crude extract of *Pythium aphanidermatum* was used as elicitors that increased tropane alkaloid production from 50 µmol/L to 75 µmol/L (Moreno et al., 1993). Using cryptogein, an elicitor from *Phytophthora cryptogea*, maximum 25 µg / ml capsidiol was induced in cell cultures of *Nicotiana tobacum* (Milat et al., 1991).

7.3.1.5. Statistical analysis (Two Way ANOVA):

Table 7.3 shows the results for statistical analysis of the expression of dipyranocoumarins in suspension cultures of both callus types elicited with DCP of both fungi. Two way ANOVA for the expression for dipyranocoumarins' in cultures elicited with fungal DCP revealed that DCP treatment (Elicitation medium), suspension type and the interaction between them was significantly different at 1% level (P=0.01) for inophyllum A.

For inophyllums B, D and P, only medium and its interaction with suspension type was significantly different at 1% (P=0.01) level and suspension types were not significant at 1% and were significant at 5% level. For inophyllum C and calophyllolide, elicitation medium, suspension type and interaction between them were non significant at both 1 % and 5 % levels (Table 7.3).

Table 7.3 Two way ANOVA for dipyranocoumarins' expression resulting from elicitation with DCP in suspension cultures of both callus types.

Two way ANOVA	Inophyllum A			Inophyllum B			Inophyllum C		
	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty
S.E.	2.078	4.101	2.864	0.0011	0.0016	0.0014	NS	NS	NS
C.D.	8.819	2.789	12.47	0.0091	0.0021	0.0129	NS	NS	NS
Significance	**	**	**	**	*	**	NS	NS	NS
	Inophyllum D			Inophyllum P			Calophyllolide		
	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty
S.E.	0.00041	0.00070	0.00050	0.00228	0.0029	0.00295	NS	NS	NS
C.D.	0.0052	0.00126	0.00747	0.0170	0.0040	0.0241	NS	NS	NS
Significance	**	*	**	**	*	**	NS	NS	NS

Med- Medium containing hormone, Sus type- Suspension type, Med X Sus type - Interaction between medium and Suspension type. (**) Significant at 1% level; (*) Significant at 5% level; NS- Non significant at 1% & 5% level S. E. – Standard Error.

7.3.1.6. Effects of fungal culture filtrates as biotic elicitors on dipyranocoumarins' expression in suspension culture of leaf callus:

In attempts to improve the expression of dipyranocoumarins, culture filtrates (CF) of both fungi were also added as biotic elicitors to suspension cultures of both callus types. The results for effects of fungal culture filtrates as biotic elicitors on dipyranocoumarins' expression in suspension cultures of leaf callus are given in Table 7.4. Like DCP of both fungi, CF also did not

enhance the biomass growth in elicited suspension cultures. HPLC analyses of elicited cultures revealed that culture filtrate of both fungi had maximum influence on expression of inophyllum A. Elicitation of cultures with CF of both fungi in the range 10 – 50 % (v/v) increased inophyllum A expression. Maximum 18.59 mg % (34.29 times) inophyllum A was expressed in cultures elicited with 20 % culture filtrate of *Phoma species*, whereas 61.25 mg % (258.43 times) inophyllum A was expressed in cultures elicited with 40 % CF of *N. sphaerica*. CF of *Phoma species* and *N. sphaerica* in the range 10 – 50 % enhanced the expression of inophyllum A in the ranges 1.5 – 18.59 mg % (2.76 - 34.29 times) and 0.32 – 61.25 mg % (135 - 258.43 times) respectively. When compared to DCP, CF of both fungi was more effective for eliciting the expression of inophyllum A (Table 7.2 and 7.4).

CF of both fungi were also effective and had positive influence on the expression of inophyllum B. Elicitation of cultures with CF of *Phoma species* and *N. sphaerica* in the range 10 – 50 % (v/v) expressed inophyllum B in the range 6.4×10^{-5} – 0.00022 mg % (1.23 – 4.23 times) and 0.00020 – 0.024 mg % (1.25 – 150 times) respectively. 10 % CF of *Phoma species* and 40 % CF of *N. sphaerica* increased inophyllum B expression to 0.00022 mg % (4.23 times) and 0.024 mg % (150 times) respectively. This effect of *N. sphaerica* indicated that CF of *N. sphaerica* was better than *Phoma species* for increasing inophyllum B expression (Table 7.4).

CF of both fungal species also elicited the expression of inophyllum C. These CF at lower concentrations were found best suited for eliciting inophyllum C expression since highest 5.11 mg % (176.2 times) and 4.29 mg % (39.35 times) inophyllum C was expressed in cultures elicited with 20 and 10 % CF of *Phoma species* and *N. sphaerica* respectively. CF of these fungi in the range 10 – 50 % expressed inophyllum C in the range 0.46- 5.11 mg % (16.10 - 176.2 times) and 0.35 – 4.29 mg % (3.88- 39.35 times) respectively. Comparatively, suspension cultures of leaf callus and CF of both fungi were more effective than DCP for eliciting the expression of inophyllum C. Similar type of effect of CF was also noted for expression of inophyllum A (Table 7.2 and 7.4).

For increased expression of inophyllum D, CF of *N. sphaerica* at higher concentrations was found better suited than CF of *Phoma species*. Pattern of inophyllum D expression resulting from elicitation with CF of *Phoma species* was erratic. Although, cultures elicited with 20 and 30 % CF of *Phoma species* increased inophyllum D expression, the level of increase was lower. All the cultures elicited with CF of *N. sphaerica* in the range 10 – 50 % enhanced the inophyllum D expression in the range 5.4×10^{-5} – 0.00021 mg % (1.16 – 4.54 times). With CF of *N. sphaerica*, maximum 0.00021 mg % (4.54 times) inophyllum D was expressed on elicitation with 30 % CF, whereas 20 % CF of *Phoma species* resulted maximum 0.0001 mg % (1.25 times) inophyllum D expression (Table 7.4).

Table 7.4 Effect of fungal culture filtrate as biotic elicitors on dipyranocoumarins' expression in suspension cultures of leaf callus.

Fungal Spp.	Culture filtrate % volume (v/v).	Dipyranocoumarins (mg/100 g elicited biomass FW)					
		Inophyllum A	Inophyllum B	Inophyllum C	Inophyllum D	Inophyllum P	Calophyllolide
Phoma spp.	0.0	0.542±0.30	$5.2 \times 10^{-5} \pm 2.5 \times 10^{-5}$	0.029±0.015	$8 \times 10^{-5} \pm 7.4 \times 10^{-5}$	$5.3 \times 10^{-5} \pm 2.6 \times 10^{-5}$	0.00060±0.00010
	10	16.73±2.76	$0.00022 \pm 7.4 \times 10^{-5}$	0.467±0.42	$5.12 \times 10^{-5} \pm 1.9 \times 10^{-5}$	0.0043±0.0083	0.00028±0.00017
	20	18.59±1.64	$0.00010 \pm 6.09 \times 10^{-5}$	5.11±4.61	$0.00010 \pm 5.5 \times 10^{-5}$	0.000173±0.00013	0.000102±0.0008
	30	1.59±0.046	$0.00020 \pm 9.7 \times 10^{-5}$	4.57±1.58	$8.8 \times 10^{-5} \pm 5.7 \times 10^{-5}$	0.051±0.0006	0.00029±0.00013
	40	1.751±1.39	$6.4 \times 10^{-5} \pm 3.6 \times 10^{-5}$	0.804±0.40	$5.2 \times 10^{-5} \pm 3.4 \times 10^{-5}$	0.00268±0.0003	0.00010±0.0009
	50	0.844±0.46	$6.41 \times 10^{-5} \pm 1.9 \times 10^{-5}$	0.595±0.17	$1.81 \times 10^{-5} \pm 7.06 \times 10^{-5}$	0.00018±0.00013	0.00035±0.00012
Nigrospora sphaerica	0.0	0.237±0.061	$0.00016 \pm 2.9 \times 10^{-5}$	0.109±0.04	$4.62 \times 10^{-5} \pm 3.1 \times 10^{-5}$	$0.00014 \pm 6.3 \times 10^{-5}$	$6.4 \times 10^{-5} \pm 3.6 \times 10^{-5}$
	10	0.362±0.06	0.00052±0.0004	4.29±3.41	$0.00014 \pm 7.1 \times 10^{-5}$	0.011±0.0015	0.00014±0.0001
	20	0.322±0.14	$0.00020 \pm 6.9 \times 10^{-5}$	0.352±0.314	$5.41 \times 10^{-5} \pm 1.1 \times 10^{-5}$	0.0014±0.0022	0.00017±0.0001
	30	24.10±19.13	0.0156±0.011	0.436±0.032	$0.00021 \pm 6.09 \times 10^{-5}$	0.011±0.0076	0.00090±0.00036
	40	61.25±29.19	0.0241±0.011	2.134±0.22	$0.00011 \pm 6.8 \times 10^{-5}$	0.0028±0.0005	0.00037±0.00031
	50	21.83±8.98	0.00037±0.00024	2.21±1.67	$0.00012 \pm 1.3 \times 10^{-5}$	0.028±0.017	0.00051±0.00018

All values are mean ± S.D of four replicates

For eliciting the expression of inophyllum P in suspension cultures of leaf callus, culture filtrate of both fungi were effective. Maximum 0.051 mg % (962.26 times) and 0.028 mg % (200 times) inophyllum P was expressed in cultures elicited with 30 and 50 % CF of *Phoma species* and *N. sphaerica* respectively. All the concentration levels of CF of both fungi tried, expression of inopyllum P was increased. However this expression did not follow defined pattern. There was no direct relationship between levels of CF and increase in inophyllum P expression (Table 7.4).

On expression of calophyllolide, elicitation with CF of *Phoma species* had negative influence. At all tested concentration levels of CF of *Phoma species*, expression of calophyllolide was decreased. This decreased in calophyllolide was not proportional to concentration level of CF used. On the contrary, CF of *N. sphaerica* had positive influence. All tested concentrations enhanced the expression of calophyllolide. Maximum 0.00090 mg % (14.06 times) calophyllolide was expressed in cultures elicited with 30 % CF of *N. sphaerica.* CF of *N. sphaerica* in the range 10 – 50 % expressed calophyllolide in the range 0.00014 – 0.00090 mg % (2.18 - 14.06 times) (Table 7.4).

Overall expression pattern of dipyranocoumarins in suspension cultures of leaf callus elicited with CF of both fungi suggested that CF of both of these fungi had maximum eliciting effect on expression of inophyllums, A, B, C and P and little influence on expression of inophyllum D and calophyllolide. For maximum expression of inophyllums A and B in suspension cultures of leaf callus, culture filtrates of *N. sphaerica* was found best suited. 40 % CF of *N. sphaerica* induced maximum 258.43 times inophyllum A and 150 times inophyllum B expression. Maximum expressions of inophyllums C (176.2 times) and P (962.26 times) were achieved when cultures were elicited with 20 % and 30 % CF of *Phoma species* respectively. CF of both of the fungi had negligible influence on expression of inophyllum D. 20 % CF of *Phoma species* resulted maximum 1.25 times and 30 % CF of *N. sphaerica* resulted in maximum 4.54 times inophyllum D expression. *Phoma species* had no positive influence on expression of calophyllolide whereas 30% CF of *N. sphaerica* induced 14.06 times calophyllolide expression. Thus in comparison, CF of *N. sphaerica* as biotic elicitor was more effective for induced expression of inophyllums A, B, D and calophyllolide while CF of *Phoma species* was effective for the expression of inophyllums C and P.

7.3.1.7. Effects of fungal culture filtrates as biotic elicitors on dipyranocoumarins' expression in suspension culture of nodal / internodal callus:

Table 7.5 shows the results for effect of CF of both fungi as biotic elicitors on dipyranocoumarins expression in suspension cultures of nodal / internodal callus. In suspension

cultures of nodal / internodal callus also, biomass did not increase when cultures were elicited with CF of both fungi. In elicited cultures, CF of both fungi was found to enhance inophyllum A expression. CF of *Phoma species* in the range 10 – 50 % enhanced inophyllum A expression in the range 0.19 – 41.89 mg % (3.57 – 787 times) and CF of *N. sphaerica* in the same range enhanced inophyllum A expression in the range 0.133 – 51.07 mg % (1.46 – 561.20 times). Maximum 41.89 mg % (787 times) and 51.07 mg % (561.20 times) inophyllum A was expressed on elicitation of cultures with 30 % CF of *Phoma species* and 40 % CF of *N. sphaerica* respectively (Table 7.5).

Levels of expression of inophyllum B in suspension cultures of both callus types elicited with CF of both fungi were almost similar (Table 7.4 and 7.5). In CF elicited suspension cultures of nodal / internodal callus, maximum 0.0066 mg % (84.61 times) and 0.038 mg % (25.33 times) inophyllum B was expressed on eliciting the cultures with 30 % CF of both, *Phoma species* and *N. sphaerica* respectively. Lower concentration range of CF of *Phoma species* (10.-30 %) enhanced inophyllum B expression in the range 0.0001 – 0.0066 mg % (1.28- 84.61 times) and all tested concentration range of CF of *N. sphaerica* enhanced inophyllum B expression in the range 0.0025- 0.038 mg %(1.66- 25.33 times) (Table 7.5).

For eliciting the expression of inophyllum C, CF of *N. sphaerica* was found better suited than CF of *Phoma species*. 30 % CF of both fungi resulted in maximum expression of inophyllum C, but level of expression with *N. sphaerica* (9.94 mg%) was higher than the level of expression with *Phoma species* (4.22 mg %). 30 % CF of *N. sphaerica* resulted 120.77 times increase in inophyllum C expression while 30 % CF of *Phoma species* resulted 13.39 times increase in inophyllum C expression (Table 7.5). In a tested concentration range of CF (10-50 %), *Phoma species* increased inophyllum C expression in the range 0.94 – 4.22 mg % (2.98- 13.39 times) and *N. sphaerica* increased inophyllum C expression in the range 0.55 – 9.94 mg % (6.68 - 120.77 times) (Table 7.5).

On elicitation with CF of *Phoma species*, expression pattern of inophyllum D was least influenced. Although all tested concentrations of CF of *Phoma species* enhanced inophyllum D expression in the range 3.2×10^{-5} – 5.2×10^{-5} mg % (1.14 – 1.85 times), the level of the increase was not considerably high. Only 20 – 30 % CF of *N. sphaerica* had maximum eliciting effect and increased inophyllum D expression in the range 0.00013 – 0.0099 mg % (1.82 – 139.24 times). 10 and 50 % CF *N. sphaerica* had negative influence on inophyllum D expression. Maximum 0.0099 mg % (139.24 times) inophyllum D was expressed in cultures elicited with 20 % CF of *N. sphaerica.* 10 % CF of *Phoma species* resulted maximum 5.2×10^{-5} mg % (1.85 times) inophyllum D expression (Table 7.5).

Table 7.5. Effect of fungal culture filtrate as biotic elicitors on dipyranocoumarins' expression in suspension cultures of nodal / internodal callus.

Fungal Spp.	Culture filtrate % volume (v/v).	Dipyranocoumarins					
		Inophyllum A	Inophyllum B	Inophyllum C	Inophyllum D	Inophyllum P	Calophyllolide
Phoma spp.	0.0	0.0532±0.013	$7.8 \times 10^{-5} \pm 0.03 \times 10^{-5}$	0.315±0.281	$2.8 \times 10^{-5} \pm 1.52 \times 10^{-5}$	$3.64 \times 10^{-5} \pm 4.8 \times 10^{-6}$	$3 \times 10^{-5} \pm 6.5 \times 10^{-6}$
	10	6.520±1.33	$0.0001 \pm 6.4 \times 10^{-5}$	1.003±0.38	$5.2 \times 10^{-5} \pm 2.5 \times 10^{-5}$	0.00064±0.00041	$4.3 \times 10^{-5} \pm 1.2 \times 10^{-5}$
	20	16.96±12.38	0.00017±0.00011	1.028±0.80	$3.2 \times 10^{-5} \pm 9.5 \times 10^{-6}$	0.0014±0.0002	0.00013±0.0001
	30	41.89±33.07	0.0066±0.0012	4.22±3.16	$3.3 \times 10^{-5} \pm 3.8 \times 10^{-6}$	0.0168±0.0016	0.0002±0.0001
	40	0.367±0.23	$7.3 \times 10^{-5} \pm 1.32 \times 10^{-5}$	3.67±3.47	$4 \times 10^{-5} \pm 3.1 \times 10^{-5}$	$0.00011 \pm 7.19 \times 10^{-5}$	$5.07 \times 10^{-5} \pm 3.5 \times 10^{-5}$
	50	0.19±0.11	$6.8 \times 10^{-5} \pm 5.8 \times 10^{-5}$	0.944±0.363	$3.6 \times 10^{-5} \pm 2.02 \times 10^{-5}$	0.00042±0.0003	$6.2 \times 10^{-5} \pm 2.8 \times 10^{-5}$
Nigrospora sphaerica	0.0	0.091±0.030	0.0015±0.001	0.0823±0.05	$7.11 \times 10^{-5} \pm 3.3 \times 10^{-5}$	$8.08 \times 10^{-5} \pm 4.5 \times 10^{-5}$	$2.74 \times 10^{-5} \pm 2.9 \times 10^{-5}$
	10	23.19±16.75	0.0053±0.001	0.55±0.029	$5.2 \times 10^{-5} \pm 3.05 \times 10^{-5}$	0.00037±0.00020	$4.5 \times 10^{-5} \pm 6.5 \times 10^{-6}$
	20	0.187±0.08	0.0146±0.028	0.633±0.089	0.0099±0.001	0.0177±0.0019	$8.05 \times 10^{-5} \pm 5.1 \times 10^{-5}$
	30	0.273±0.17	0.038±0.0279	9.94±6.86	0.00013±0.0001	0.024±0.02	$0.00011 \pm 5.6 \times 10^{-5}$
	40	51.07±33.99	0.00256±0.0004	5.16±0.78	0.00030±0.00032	0.0342±0.027	0.00040±0.0002
	50	0.133±0.06	0.0025±0.0001	6.36±1.62	$6.3 \times 10^{-5} \pm 2.5 \times 10^{-5}$	0.0031±0.0005	$3.1 \times 10^{-5} \pm 1.5 \times 10^{-5}$

All values are mean ± S.D of four replicates

Expression of inophyllum P was positively influenced by elicitation with CF of both fungi. At tested concentration range of CF, *Phoma species* increased inophyllum P expression in the range 0.00011 – 0.0168 mg % (3.02 – 461.53 times); whereas *N. sphaerica* at the same tested concentration range increased inophyllum P expression in the range 0.00037 – 0.034 mg % (4.57 – 420.79 times). 30 % CF of *Phoma species* and 40 % CF of *N. sphaerica* were found to be optimum since maximum 0.0168 mg % (461.53 times) and 0.034 mg % (420.79 times) inophyllum P was expressed with these concentration of CF respectively.

In cultures elicited with CF of both fungi, expression pattern of calophyllolide was erratic. Only 20 and 30 % CF of *Phoma species* and 30 and 40 % CF of *N. sphaerica* resulted considerably high expression of calophyllolide. Remaining concentrations of CF of both fungi also increased the calophyllolide expression, but the level of expression was very low and negligible. Maximum 0.0002 mg % (6.66 times) calophyllolide was expressed in cultures elicited with 30 % CF of *Phoma species* whereas, maximum 0.0004 mg % (1.66 times) calophyllolide was expressed in cultures elicited with 40 % CF of *N. sphaerica.*

Overall it appears that in suspension cultures of nodal / internodal callus, CF of *Phoma species* was most effective and resulted in enhanced expression of most of the dipyranocoumarins (inophyllums A, B, D, P and calophyllolide) under study whereas CF of *N. sphaerica* was found to be effective for expression of only inophyllum C. 30 % CF of *Phoma species* was found to be most effective concentration since highest 787 times inophyllum A, 84.61 times inophyllum B, 461.53 times inophyllum P and 6.66 times calophyllolide expressions were resulted when cultures were elicited with 30 % CF of *Phoma species.* For highest expression of inophyllum D (139.24 time) required 20 % CF of *Phoma species*. For maximum expression of inophyllum C, 30 % CF of *N. sphaerica* was best suited concentration and increased 120.77 times inophyllum C expression.

The overall expression pattern of dipyranocoumarins in suspension cultures of both callus types resulting from biotic elicitation with DCP and CF of both fungi revealed that suspension cultures of nodal / internodal callus and DCP of *Phoma species* are the most optimum conditions for highest dipyranocoumarins expression. Highest inophyllums B, C and D were expressed in suspension cultures of nodal / internodal callus elicited with DCP of *Phoma species* whereas for highest inophyllum A, elicitation of suspension cultures of nodal / internodal callus with CF of *Phoma species* was best suited condition. Highest expression of inophyllum P also resulted in suspension cultures nodal / internodal callus when cultures were elicited with DCP of *N. sphaerica.* Calophyllolide was the only dipyranocoumarin whose maximum expression was estimated in elicited suspension cultures of leaf callus. For eliciting the expression of calophyllolide, DCP of *Phoma species* was most suited.

Although this was the first study directed at the biotic elicitation of suspension cultures of *C. inophyllum* for the increased expression of dipyranocoumarins, biotic elicitation was successfully employed for the production of many secondary metabolites from different plant species. In *Arabidopsis thaliana*, the production of camelexin, indole glucosinolates was elicited using *Erwinia carotovora* as biotic elicitor (Brader et al., 2001) and in *Bidens pilosa* cell cultures, fungal culture filtrate elicited the production of Phenylheptaryn (DiCosmo and Misawa, 1995). In *Catharanthus roseus* cell cultures, fungal elicitor was successfully used for the production of indole alkaloids. In *C. roseus* cell cultures, *Pythium aphanidermatum* and *Trichoderma viridae* were used as elicitors for the production of N-acetyl-tryptamine (Eilert et al., 1986) and ajmalicine production (Namdeo et al., 2002). Puig et al., (1995) reported that indole alkaloid production was increased from 3 mg/g to 9 mg/g in *C. roseus* cell cultures when cultures were elicited with crude extract of *Penicillin species* while whereas Aoyagi et al., (2006) used *Alteromonas macleodii* for the production of 5'-phosphodiesterage in *C. roseus* cell cultures. Cells of *Datura stramonium* were effectively elicited with the biotic elicitors from *Phytophthora megasperma* and enhanced tropane alkaloid production from 0.85 mg/g to 4.27 mg/g DW (Aziz et al., 2003). These authors also reported that using *Rhizopus arrhizus* as biotic elicitors, diosgenin production in cell cultures of *Dioscorea deltoida* was enhanced from 134 mg/L to 230 mg/L. Sanguinarine production was stimulated in cell cultures of *Papaver bracteatum* when cultures were elicited with *Dendryphion*. Sanguinarine production was enhanced from 50 µg/g to 450 µg/g (Cline and Coscia, 1988). Production of dopamine and sanguinarine were simultaneously enhanced when cell cultures of *Sanguinaria canadensis* were elicited with *Verticillium dahaliae*. Sanguinarine production was increased from 3 µg/g to 12 µg/g and dopamine production was raised from 3 mg/g to 15 mg/g (132). Over the years, studies have been directed toward understanding the signaling pathways involved in elicitor responses. These studied suggested that elicitor responses might have been mediated by number of events at molecular and cellular levels. Binding of elicitor to the receptors located in plasma membrane and activation of G-protein might have been the first step in mediating elicitor response. G-protein then might have stimulated adenylyl cyclase (AC) and phospholipase C (PLC) which might have led to increased levels of second messenger (camp, DAG, IP_3) and the activation of their target kinases. This might have also led to change in cytoplasmic Ca^{2+} levels (Vasconsuelo and Boland, 2007). These events at molecular level might have then stimulated the transcription of the enzymes of synthetic pathways of secondary metabolites. In the present study and reports mentioned above, such events might have been involved during elicitations events and led to increased production of secondary metabolites.

7.3.1.8. Statistical analysis (Two Way ANOVA):

Two way statistical analysis for the expression pattern of dipyranocoumarins in suspension cultures both callus types elicited with CF of both fungi revealed that suspension type (type of suspension) was not significantly different for all dipyranocoumarins (Table 7.6). Statistically, elicitation medium (culture filtrate type) for all dipyranocoumarins except calophyllolide was significantly different at 1% level. The interaction between these two factors for inophyllums C, D and P was significant at 1 %, for inophyllum B at 5%, whereas for inophyllum A and Calophyllolide, this interaction was non significant (Table 7.6).

Table 7.6 Two way ANOVA for dipyranocoumarins expression resuting from elicitation with culture filtrates of fungi in suspension cultures of both callus types

Two way ANOVA	Inophyllum A			Inophyllum B			Inophyllum C		
	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty
S.E.	3.315	NS	NS	0.0015	NS	0.0019	0.440	NS	0.529
C.D.	21.20	NS	NS	0.0125	NS	0.0177	3.970	NS	5.615
Significance	**	NS	NS	**	NS	*	**	NS	**
	Inophyllum D			Inophyllum P			Calophyllolide		
	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty
S.E.	0.00025	NS	0.00014	0.00156	NS	0.0020	NS	0.00018	NS
C.D.	0.0029	NS	0.0041	0.0140	NS	0.0198	NS	0.00021	NS
Significance	**	NS	**	**	NS	**	NS	**	NS

Med- Medium containing hormone, Sus type- Suspension type, Med X Sus type - Interaction between medium and Suspension type. (**) Significant at 1% level; (*) Significant at 5% level; NS- Non significant at 1% & 5% level S. E. – Standard Error.

7.3.2. Abiotic elicitation:

7.3.2.1. Effects of $CaCl_2$ as abiotic elicitors on biomass growth and dipyranocoumarins' expression in suspension culture of leaf callus:

When plants are exposed to different stresses, they cope with these stimuli by initiating the cascade of reactions. The analyses of signal transduction cascade involved in stress response of plant have revealed that the increase in cytoplasmic calcium concentration serves as second messenger to mediate defense responses (White et al., 2003). In plants, calcium is an ubiquitous signal that mediates the regulation of many cellular processes by different stimuli like elicitation. There is evidence that the action of many elicitors involves the change in intracellular calcium status. Change in intracellular calcium status can determine the level of expression of secondary metabolites. Considering these roles of calcium, experiments were conducted to study the effects of calcium on biomass growth and expression pattern of dipyranocoumarins. Table 7.7 shows the results for effects of $CaCl_2$ as abiotic elicitor on biomass growth and dipyranocoumarins'

expression in suspension cultures of leaf callus. All the biotic (previously discussed) and abiotic elicitors except $CaCl_2$ used in this study did not enhance the biomass growth. Incorporation of $CaCl_2$ in the range 1.0 –4.0 mM enhanced the biomass growth in the range 1.4 – 16.70 % (1.31 – 15.72 times). Maximum 16.70 % (15.72 times) biomass growth was resulted in cultures supplemented with 3.0 mM $CaCl_2$. This biomass growth was almost more than 15 times the growth resulted in control medium (without $CaCl_2$). However, this growth of biomass did not enhance the expression of all the dipyranocoumarins under study. With this increase in biomass, expression of inophyllums A, B, P and calophyllolide was decreased (Table 7.7) while expression of inophyllums C and D was increased. On supplementation of $CaCl_2$ in the range 1.0 – 4.0 mM, inophyllums C was enhanced in the range 0.44 – 1.92 mg % (20 – 87.27 times) and inophyllums D was slightly enhanced in the range 3.8×10^{-5} – 6.6×10^{-5} mg % (3.58 – 6.22 times). Maximum 1.92 mg % (87.27 times) inophyllums C and 6.6×10^{-5} mg % (6.22 times) inophyllum D was expressed in cultures supplemented with 2.0 and 1.0 mM $CaCl_2$ respectively. The expression of other dipyranocoumarins was reduced. This trend of expression indicated the inverse relationship between biomass growth resulting from effect of $CaCl_2$ and expression of inophyllums A, B, P and calophyllolide (Table 7.7). Similar type of inverse relationship was also observed between biomass growth resulting from hormonal influence and expression level of some of the dipyranocoumarins (discussed in chapter 6).

7.3.2.2. Effects of $CaCl_2$ as abiotic elicitors on biomass growth and dipyranocoumarins' expression in suspension culture of nodal / internodal callus:

Results for effects of $CaCl_2$ as abiotic elicitors in suspension cultures of nodal / internodal callus have been given in Table 7.8. Like in suspension cultures of leaf callus, $CaCl_2$ also resulted in biomass growth in suspension cultures of nodal / internodal callus. When media were supplemented with $CaCl_2$ in the range 1.0 – 4.0 mM, biomass was enhanced in the range 2.05 – 33.47 % (2.16 – 35.26 times). This biomass growth was higher than the biomass growth resulted in suspension cultures of leaf callus.

Expression pattern resulting from elicitation with $CaCl_2$ was little erratic. For expression of inophyllum A, only 1.0 mM $CaCl_2$ was optimum and resulted in maximum 2.51 mg % inophyllum A expression. This expression of inophyllum A was 1.5 times the expression resulted in control medium. Elicitation of cultures with more than 1.0 mM $CaCl_2$ decreased the inophyllum A expression. $CaCl_2$ was not found suitable for eliciting the expression of inophyllums B, C and D. With all the tested concentrations range of $CaCl_2$, expression of inophyllums B, C and D was decreased. The negative effect of $CaCl_2$ on inophyllum B expression was also observed in suspension cultures of leaf callus.

Table 7.7 Effect of $CaCl_2$ on biomass growth and expression of dipyranocoumarins in suspension cultures of leaf callus.

CaCl2 mM	Biomass	Dipyranocoumarins (mg/100 g elicited biomass FW)					
		Inophyllum A	Inophyllum B	Inophyllum C	Inophyllum D	Inophyllum P	Calophyllolide
0.0	1.062±0.61	0.693±0.23	0.00025±0.00016	0.022±0.004	1.06 $x10^{-5}$±3.1 $x10^{-6}$	0.0030±0.0014	0.00122±0.00103
1.0	1.484±0.65	0.147±0.012	8.9 $x10^{-5}$±5.5 $x10^{-5}$	0.449±0.07	6.6 $x10^{-5}$±1.8 $x10^{-5}$	0.00215±0.0013	0.00080±0.00042
2.0	8.565±2.78	0.410±0.15	0.00017±6.9 $x10^{-5}$	1.925±0.29	6.5 $x10^{-5}$±2.8 $x10^{-5}$	0.00014±0.00011	4.0 $x10^{-5}$±2.5 $x10^{-5}$
3.0	16.70±7.09	0.255±0.108	0.00012±0.0001	0.560±0.45	3.9 $x10^{-5}$±1.4 $x10^{-5}$	0.00013±0.0001	6.1 $x10^{-5}$±2.08 $x10^{-5}$
4.0	4.73±2.60	0.435±0.36	0.00020±6 $x10^{-5}$	1.111±0.212	3.8 $x10^{-5}$±5.1 $x10^{-5}$	0.00011±0.0001	0.00065±0.00018

All values are mean ± S.D of four replicates

Table 7.8 Effect of $CaCl_2$ on biomass growth and expression of dipyranocoumarins in suspension cultures of nodal internodal callus.

CaCl2 mM	Biomass	Dipyranocoumarins (mg/100 g elicited biomass FW)					
		Inophyllum A	Inophyllum B	Inophyllum C	Inophyllum D	Inophyllum P	Calophyllolide
0.0	0.494±0.12	1.666±0.37	0.011±0.002	1.576±0.289	0.004±0.0007	0.0015±0.0010	0.00238±0.0041
1.0	31.74±10.81	2.51±1.46	0.0008±0.0001	0.022±0.006	2.05 $x10^{-5}$±1.2 $x10^{-5}$	0.00336±0.004	0.00036±0.00023
2.0	33.47±9.20	0.500±0.03	0.000177±0.0002	0.293±0.049	0.0020±0.004	0.00255±0.005	0.0036±0.0034
3.0	15.24±8.20	0.999±0.12	0.00024±0.0003	0.0339±0.0027	0.0022±0.0021	0.00254±0.004	0.0025±0.0008
4.0	2.05±0.97	0.315±0.11	0.0030±0.0010	0.762±0.81	4.6 $x10^{-5}$±1.9 $x10^{-5}$	0.00085±0.0001	0.00139±0.00025

All values are mean ± S.D of four replicates

$CaCl_2$ was observed to have eliciting effect on the expression of inophyllum P and calophyllolide. Elicitation of cultures with 1.0 – 3.0 mM $CaCl_2$ enhanced the inophyllum P expression while 2.0 and 3.0 mM $CaCl_2$ enhanced the expression of calophyllolide. Maximum 0.00336 mg % (2.24 times) inophyllum P and 0.0036 mg % (1.51 times) calophyllolide were estimated in cultures elicited with 1.0 and 2.0 mM $CaCl_2$ respectively (Table 7.8).

Recently many reports have suggested that $CaCl_2$ induces the increase in secondary metabolites. Nakoa et al., (1999) reported that supplementation of $CaCl_2$ in the medium induced the increase in flavanol contents in suspension cultures of *Polygonum hydropiper*. But in the present study, $CaCl_2$ was found to have least eliciting effects on expression of dipyranocoumarins. However, calcium was found to promote biomass growth. The inverse relationship between biomass growth and dipyranocoumarins' expression further supports the similar conclusion made in last chapter (Chapter 6). Calcium is well known essential plant nutrient. Divalent cation of calcium i.e. Ca^{2+} is involved in structural roles in cell wall and membranes as a counter-cation for organic and inorganic anions in the vacuole, and as the intracellular messenger in cytosol (Marschner, 1995). Ca^{2+} plays an important role in cell cycle and is also required at almost all stages of plant growth and development, playing a fundamental role in regulating polar growth of cells and tissues (Song et al., 2008). One of these roles of calcium or their combinations might have involved in the biomass enhancing effect of $CaCl_2$ in suspension cultures of *C. inophyllum.*

7.3.2.3. Statistical analysis (Two Way ANOVA):

Table 7.9 Two way ANOVA for dipyranocoumarins' expression resulting from elicitation with $CaCl_2$ in suspension cultures of both callus types.

Two way ANOVA	Inophyllum A			Inophyllum B			Inophyllum C		
	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty
S.E.	NS	NS	NS	NS	NS	NS	NS	NS	NS
C.D.	NS	NS	NS	NS	NS	NS	NS	NS	NS
Significance	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Inophyllum D			Inophyllum P			Calophyllolide		
Two way ANOVA	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty
S.E.	NS	NS	NS	NS	NS	NS	NS	0.00075	NS
C.D.	NS	NS	NS	NS	NS	NS	NS	0.00126	NS
Significance	NS	NS	NS	NS	NS	NS	NS	*	NS

	Biomass		
Two way ANOVA	E. Med.	Sus. Type	E. Med X Su.Ty
S.E.	2.35	5.92	3.95
C.D.	7.967	5.039	11.268
Significance	**	**	**

Med- Medium containing hormone, Sus type- Suspension type, Med X Sus type - Interaction between medium and Suspension type. (**) Significant at 1% level; (*) Significant at 5% level; NS- Non significant at 1% & 5% level S. E. – Standard Error.

Statistically, for all the dipyranocoumarins, elicitation medium, suspension type and their interactions were non significant. Only for calophyllolide, suspension type was significant at 5% level. For biomass growth, elicitation medium, suspension type and their interactions were significantly different at 1 % level (Table 7.9).

7.3.2.4. Effect of copper as abiotic elicitor on dipyranocoumarins expression:

In attempts to elicit the expression of dipyranocoumarins, salts of heavy metals were used as abiotic elicitors. Table 7.10 shows the effects of copper on dipyranocoumarins expression in suspension cultures of both callus types. HPLC analyses of elicited cultures revealed that copper had positive influence on expression of most of the dipyranocoumarins under study.

At all the tested concentration of $CuSO_4$, expression of inophyllum A was increased in suspension cultures of nodal / internodal callus, while only lower concentrations of $CuSO_4$ (0.1 – 5.0 mM) in suspension cultures of leaf callus induced inophyllum A expression. In suspension cultures of leaf callus, $CuSO_4$ at higher than 10 and 20 mM reduced inophyllum A expression. Compared to the expression in control medium, maximum 18.88 and 19.54 mg % (30.06 and 39.87 times) inophyllum A was expressed when suspension cultures of leaf and nodal / internodal callus were elicited with 0.5 and 1.0 mM $CuSO_4$ respectively. $CuSO_4$ in the range 0.1 – 5.0 mM in suspension cultures of leaf callus enhanced inophyllum A expression in the range 0.91 – 18.88 mg % (1.44 –30.06 times). In suspension cultures of nodal / internodal callus, $CuSO_4$ in the tested concentration range enhanced inophyllum A expression in the range 0.53 – 19.54 mg % (1.08 – 39.87 times) (Table 7.10).

$CuSO_4$ as abiotic elicitor was not effective for induced expression of inophyllum B in suspension cultures of leaf callus. At the tested range of $CuSO_4$, inophyllum B expression was decreased showing negative influence of $CuSO_4$ on inophyllum B expression in suspension cultures of leaf callus. In suspension cultures of nodal / internodal callus, only low concentrations of $CuSO_4$ showed enhanced expression of inophyllum B while higher concentrations had no effect on inophyllum B expression. Rather high concentration slightly reduced the inophyllum B expression. Maximum 0.00011 mg % (2.48 times) inophyllum B was expressed on eliciting the suspension cultures of nodal/ internodal callus with 0.5 mM $CuSO_4$ (Table 7.10).

Table 7.10 Effects of copper (heavy metals) as abiotic elicitor on dipyranocoumarins' expression in suspension cultures of both callus types.

Suspension type	Cocn. Of elicitors (mM)	Dipyranocoumarins (mg/100 g elicited biomass FW).					
		Inophyllum A	Inophyllum B	Inophyllum C	Inophyllum D	Inophyllum P	Calophyllolide
Suspension culture of leaf callus	0.0	0.628±0.21	0.0037±0.002	2.58±1.3	$7.2 \times 10^{-6} \pm 3.8 \times 10^{-6}$	$0.00010 \pm 9.5 \times 10^{-5}$	$4.8 \times 10^{-6} \pm 3.3 \times 10^{-6}$
	0.1	11.23±5.08	$3.17 \times 10^{-5} \pm 8.3 \times 10^{-6}$	0.33±0.17	0.00060 ± 0.0004	$5.4 \times 10^{-5} \pm 3.3 \times 10^{-5}$	$2.8 \times 10^{-5} \pm 8.1 \times 10^{-6}$
	0.5	18.88±8.15	$0.00011 \pm 7.3 \times 10^{-5}$	1.33±0.9	0.00159 ± 0.0008	$6.5 \times 10^{-5} \pm 1.9 \times 10^{-5}$	$3.1 \times 10^{-5} \pm 1.8 \times 10^{-5}$
	1.0	2.87±1.98	$7.02 \times 10^{-5} \pm 2.6 \times 10^{-5}$	4.70±2.05	0.0043 ± 0.001	$6.6 \times 10^{-5} \pm 2.5 \times 10^{-5}$	0.00075±0.0006
	5.0	0.91±0.35	$0.00015 \pm 5.3 \times 10^{-5}$	0.30±0.21	0.00041 ± 0.00011	$0.00010 \pm 5.6 \times 10^{-5}$	$8.02 \times 10^{-5} \pm 5.4 \times 10^{-5}$
	10	0.45±0.3	0.00041±0.0002	0.026±0.004	0.014±0.009	0.0155±0.007	$7.5 \times 10^{-5} \pm 4.1 \times 10^{-5}$
	20	0.122±0.01	$0.00010 \pm 5.3 \times 10^{-5}$	0.028±0.006	0.012±0.011	0.0159±0.012	$3.9 \times 10^{-5} \pm 2.1 \times 10^{-5}$
Suspension culture of nodal /internodal callus	0.0	0.49±0.12	$4.42 \times 10^{-5} \pm 2.18 \times 10^{-5}$	0.287±0.137	$1.61 \times 10^{-5} \pm 7.22 \times 10^{-6}$	$7.70 \times 10^{-5} \pm 4.33 \times 10^{-5}$	$1.97 \times 10^{-5} \pm 1.15 \times 10^{-5}$
	0.1	1.24±0.99	$0.00010 \pm 1.53 \times 10^{-5}$	3.86±1.96	$4.21 \times 10^{-5} \pm 2.43 \times 10^{-5}$	$9.08 \times 10^{-5} \pm 4.53 \times 10^{-5}$	$4.9 \times 10^{-5} \pm 3.2 \times 10^{-5}$
	0.5	10.203±7.635	$0.00011 \pm 1 \times 10^{-5}$	6.062±2.41	$3.75 \times 10^{-5} \pm 1.35 \times 10^{-5}$	$7 \times 10^{-5} \pm 8.03 \times 10^{-6}$	$5.2 \times 10^{-5} \pm 3.1 \times 10^{-5}$
	1.0	19.544±9.142	$4.35 \times 10^{-5} \pm 1.98 \times 10^{-5}$	34.74±14.02	$2.08 \times 10^{-5} \pm 4.45 \times 10^{-6}$	$3.2 \times 10^{-5} \pm 2.3 \times 10^{-5}$	$3.57 \times 10^{-5} \pm 1.75 \times 10^{-5}$
	5.0	0.607±0.589	$4.21 \times 10^{-5} \pm 2.11 \times 10^{-5}$	6.01±2.47	$1.63 \times 10^{-5} \pm 1.22 \times 10^{-5}$	$5.18 \times 10^{-5} \pm 2.71 \times 10^{-5}$	$4.49 \times 10^{-5} \pm 3.29 \times 10^{-5}$
	10	0.536±0.302	$4.08 \times 10^{-5} \pm 3.2 \times 10^{-5}$	9.032±6.23	$3.81 \times 10^{-5} \pm 1.91 \times 10^{-5}$	$6.24 \times 10^{-5} \pm 2.05 \times 10^{-5}$	$5.28 \times 10^{-5} \pm 3.75 \times 10^{-5}$
	20	0.964±0.809	$3.51 \times 10^{-5} \pm 1.47 \times 10^{-5}$	2.58±1.77	$2.80 \times 10^{-5} \pm 1.18 \times 10^{-5}$	$7.35 \times 10^{-5} \pm 3 \times 10^{-5}$	$3.67 \times 10^{-5} \pm 1.74 \times 10^{-5}$

All values are mean ± S.D of four replicates

In suspension cultures of leaf callus, eliciting the culture with only 1.0 mM $CuSO_4$ induced the expression of inophyllum C while rest of the concentrations of $CuSO_4$ had negative influence and decreased inophyllum C expression. Maximum 4.70 mg % (1.82 times**)** inophyllum C was expressed in cultures elicited with 1.0 mM $CuSO_4$. On the contrary, $CuSO_4$ at all tested concentration levels in suspension cultures of nodal / internodal callus induced inophyllum C expression in the range 2.58 – 34.74 mg% (8.98 – 121.04 times). Maximum 34.74 mg% (121.04 times) inophyllum C was expressed in cultures elicited with 1.0 mM $CuSO_4$. This expression pattern indicated that for enhanced inophyllum C expression, elicitation of the cultures with 1.0 mM $CuSO_4$ was optimum (Table 7.10).

Inophyllum D expression in suspension cultures of leaf callus was increased with $CuSO_4$ at all the tested concentrations. 10 mM $CuSO_4$ was found best to induce maximum inophyllum D expression in suspension cultures of leaf callus and resulted in maximum 0.014 mg % (1944.44 times) inophyllum D. Elicitation of suspension cultures of leaf callus with $CuSO_4$ at the tested concentration range induced inophyllum D expression in the range 0.0004 – 0.014 mg % (55.55 - 1944.44 times). In suspension cultures of nodal / internodal callus also, elicitation with $CuSO_4$ at all tested concentrations induced inophyllum D expression. However this expression level was very low. Compared to expression in control medium ($1.61x10^{-5}$), $CuSO_4$ at all tested concentrations increased expression level in the range $1.63\ x10^{-5}$ – $4.2\ x10^{-5}$ (1.01 – 2.60 times). Maximum $4.2x10^{-5}$ mg % (2.60 times) inophyllum D expression was resulted on elicitation with 0.1 mM $CuSO_4$ (Table 7.10).

Expression patterns of inophyllum P and calophyllolide were almost similar to the expression pattern of inophyllum D. In suspension cultures of leaf callus, maximum 0.015 mg % (150 times) inophyllum P expression was resulted in cultures elicited with high concentrations of $CuSO_4$ i.e.10 and 20 mM. In suspension of nodal/ internodal callus, like expression of inophyllum D, expression of inophyllum P was also enhanced, but the level of expression was low. Maximum $9.08\ x10^{-5}$ mg % (1.17 times) inophyllum P was expressed in cultures elicited with 0.1 mM $CuSO_4$ (Table 7.10).

Not all the tested concentrations of $CuSO_4$ had positive influence on the expression pattern of calophyllolide in suspension cultures of both callus types. Only1.0 mM $CuSO_4$ enhanced the calophyllolide expression to maximum 0.00075 mg % (156.25 times) in suspension cultures of leaf callus. Low concentration of $CuSO_4$ reduced calophyllolide expression. In suspension cultures of nodal / internodal callus, expression pattern of calophyllolide was almost comparable to the expression pattern of inophyllum D. At all tested concentration range of $CuSO_4$, expression of calophyllolide was enhanced, but the level of induction was low i.e. (1.81 – 2.63 times). Maximum

5.28 x 10_{-5} mg % calophyllolide was expressed in cultures elicited with 10 mM $CuSO_4$ (Table 7.10).

As was the case with the effect of biotic elicitor discussed earlier, the expression pattern was not directly or indirectly proportional to concentrations of $CuSO_4$ used. Overall, in this study elevated level of $CuSO_4$ as abiotic elicitors was noted to have influence on expression pattern of most of the dipyranocoumarins under study. Previously many workers have reported the use of copper as abiotic elicitor to stimulate the production of many secondary metabolites. Cu^{2+} along with Cd^{2+} was used as abiotic elicitors in transformed roots of *Atropa belladona* that enhanced the production of tropane alkaloid (Lee et al., 1998). Kuruyanagi et al., (1988) studied the phytoalexin formation in $CuSO_4$ elicited hairy root cultures of *Hyocyamus albus* and found that $CuSO_4$ effectively increased phytoalexin production. $CuSO_4$ was found to be most effective abiotic elicitor for withaferin A synthesis in transformed callus cultures of *Withania somnifera* (Baldi et al., 2008) and for induction of rishitin in hairy roots cultures of Jimson weed (Whitehead et al., 1992). Fang et al. (1999) studied the effect of $CuSO_4$ on anthocyanin production in Ohelo cell cultures and reported that copper was positive stimulant of anthocyanin production. Increased concentrations of $CuSO_4$ also stimulated betacyanin synthesis in suspension cultured cells of *Portulaca* (Bhuiyan and Adachi, 2003). In the present study it was noted that inophyllum A and C were the dipyranocoumarins whose expression was most influenced by $CuSO_4$. Similar effect of copper on coumarins synthesis was noted in Sunflower. Gutierrez et al., (1995), reported that copper was the most effective abiotic elicitor of coumarin symthesis.

7.3.2.5. Effect of cadmium as abiotic elicitors on dipyranocoumarins expression:

As a defense mechanism against the heavy metals, plants induce synthesis of many secondary metabolites. Cadmium is one of the heavy metals that induce stress in plants. The effects of cadmium on secondary metabolism have been widely studied and reported. At cellular level, Cd induces changes in lipid composition, the activity of enzymes associated with membrane and distribution of macro and micronutrients. Because Cd as abiotic elicitor was shown to induce the synthesis of many secondary metabolites, Cd was also used as abiotic elicitor in the present study. $CdCl_2$ was incorporated as a source of Cd. Table 7.11 shows the results for the effect of Cd on dipyranocoumarins' expression in suspension cultures of both callus types.

Elicitation of suspension cultures of both callus types with $CdCl_2$ positively influenced the expression of inophyllum A. $CdCl_2$ at lower concentrations i.e. 0.5 and 0.1 mM were found optimum for maximum inophyllum A expression in suspension cultures of leaf and nodal /internodal callus respectively. Compared to the expression of inophyllum A in unelicited suspension cultures of leaf and nodal internodal callus (0.0044 and 0.0072 mg % respectively), 0.5

mM $CdCl_2$ resulted 1.27 mg % (288 times) and 0.1 mM $CdCl_2$ resulted 7.07 mg % (981 times) enhanced expression of inophyllum A in suspension cultures of leaf and nodal / internodal callus respectively. At the tested concentration range of $CdCl_2$, inophyllum A expression in suspension cultures of leaf and nodal / internodal callus was increased in the range 0.084 – 1.27 mg % (19 – 288 times) and 0.067 – 7.07 mg % (9.30- 981 times) respectively (Table 7.11).

For enhanced expression of inophyllum B in suspension cultures of both callus types, Cd as abiotic elicitor was noted to have positive influence. In suspension culture of leaf callus, compared to the expression in unelicited cultures ($1.7x10^{-5}$ mg %), maximum 300 times (0.0051 mg %) inophyllum B expression was enhanced when cultures were elicited with 0.5 mM $CdCl_2$. In suspension cultures of nodal internodal callus, unelicited cultures expressed maximum $2.5x10^{-5}$ mg % inophyllum B which was increased to 124.5 times (0.0032 mg %) when cultures were elicited with 1.0 mM $CdCl_2$. All the tested concentration range of $CdCl_2$ enhanced inophyllum B expression in the range $8.3x10^{-5}$ – 0.0051 mg % (4.88 - 300 times) and 7.04 $x10^{-5}$ – 0.0032 mg % (2.73 – 124.5 times) in suspension cultures of leaf and nodal / internodal callus respectively (Table 7.11).

$CdCl_2$ at lower concentrations (0.1 – 1.0 mM) in suspension cultures of leaf callus was noted to enhance the expression of inophyllum C while higher concentrations reduced the expression. At 0.1 – 1.0 mM $CdCl_2$, expression of inophyllum C was enhanced in the range 0.081 – 0.766 mg % (1.3 – 12.17 times). Maximum 0.766 mg % (12.17 times) inophyllum C was expressed on eliciting the cultures with 0.5 mM $CdCl_2$. In suspension cultures of nodal / internodal callus, all the tested concentrations of $CdCl_2$ increased inophyllum C expression in the range 0.033 – 0.44 mg % (9.7 – 129.4 times). Highest 0.44 mg % (129.4 times) inophyllum C was expressed in cultures elicited with 5.0 mM $CdCl_2$ (Table 7.11).

Similar to the expression of inophyllum C, expression of inophyllum D in suspension cultures of leaf callus was also increased on eliciting the cultures with low concentrations of $CdCl_2$. Only 0.1 – 5.0 mM $CdCl_2$ increased inophyllum D expression in the range 0.00032 – 0.0152 mg % (5.28 – 251 times). 1.0 mM $CdCl_2$ induced the maximum 251 times expression (0.0152 mg %) of inophyllum D. In suspension cultures of nodal / internodal callus, $CdCl_2$ at all tested concentrations induced the expression of inophyllum D in the range 2.55 $x10^{-5}$ – 0.0039 mg % (1.23 – 188.4 times). $CdCl_2$ at low concentration (0.5 – 5.0 mM) was found best suited since these three concentrations of $CdCl_2$ induced inophyllum D expression in the range 0.00034 – 0.0039 mg % (10.86 – 188.4 times). Maximum 0.0039 mg % (188.4 times) inophyllum D was expressed in cultures elicited with 1.0 mM $CdCl_2$ (Table 7.11).

Table 7.11 Effects of cadmium (heavy metals) as abiotic elicitor on dipyranocoumarins' expression in suspension cultures of both callus types.

Suspension type	Cocn. Of elicitors (mM)	Dipyranocoumarins (mg/100 g elicited biomass FW).					
		Inophyllum A	Inophyllum B	Inophyllum C	Inophyllum D	Inophyllum P	Calophyllolide
Suspension culture of leaf callus	0.0	0.0044±0.001	$1.7 \times 10^{-5} \pm 6.6 \times 10^{-6}$	0.0629±0.04	$6.05 \times 10^{-5} \pm 1.7 \times 10^{-5}$	$0.00010 \pm 5.9 \times 10^{-5}$	$7.05 \times 10^{-5} \pm 1.3$
	0.1	0.267±0.13	0.00029±0.0002	0.081±0.05	0.00032±0.0002	0.0035±0.003	0.022±0.014
	0.5	1.27±0.18	0.0051±0.003	0.766±0.63	0.0026±0.002	0.034±0.02	0.0240±0.007
	1.0	0.268±0.197	0.00023±0.00016	0.252±0.169	0.0152±0.007	0.00018±0.0001	0.028±0.02
	5.0	0.118±0.036	0.0013±0.0001	0.0454±0.026	0.0037±0.003	0.00013±0.0001	0.019±0.01
	10	0.084±0.043	$8.3 \times 10^{-5} \pm 3.1 \times 10^{-5}$	0.0384±0.014	$3.25 \times 10^{-5} \pm 2.2 \times 10^{-5}$	0.00018±0.0002	$9.8 \times 10^{-5} \pm 6.3 \times 10^{-5}$
	20	0.166±0.11	0.00017±0.0001	0.026±0.020	$5.5 \times 10^{-5} \pm 4.6 \times 10^{-5}$	$5.3 \times 10^{-5} \pm 3.1 \times 10^{-5}$	$2.9 \times 10^{-5} \pm 1.2 \times 10^{-5}$
Suspension culture of nodal /internodal callus	0.0	0.0072±0.0022	$2.57 \times 10^{-5} \pm 8.15 \times 10^{-6}$	0.0034±0.003	$2.07 \times 10^{-5} \pm 6.39 \times 10^{-6}$	$7.23 \times 10^{-5} \pm 2.62 \times 10^{-5}$	$4.21 \times 10^{-5} \pm 2.82 \times 10^{-5}$
	0.1	7.07±4.19	0.00033±0.00030	0.248±0.167	$3.13 \times 10^{-5} \pm 1.43 \times 10^{-5}$	0.00066±0.00061	$0.00010 \pm 9.29 \times 10^{-5}$
	0.5	1.356±0.96	$7.04 \times 10^{-5} \pm 5.8 \times 10^{-5}$	0.033±0.013	0.0034±0.0025	0.019±0.015	$2.08 \times 10^{-5} \pm 1.11 \times 10^{-5}$
	1.0	0.159±0.11	0.0032±0.0024	0.435±0.41	0.0039±0.0032	$6.98 \times 10^{-5} \pm 2.76 \times 10^{-5}$	$7.21 \times 10^{-5} \pm 3.84 \times 10^{-5}$
	5.0	0.067±0.034	$9.5 \times 10^{-5} \pm 5.8 \times 10^{-5}$	0.44±0.33	0.00034±0.00010	$4.94 \times 10^{-5} \pm 8.07 \times 10^{-6}$	0.026±0.0026
	10	0.075±0.051	$0.00011 \pm 9.29 \times 10^{-5}$	0.041±0.034	$2.55 \times 10^{-5} \pm 1.73 \times 10^{-5}$	$5.36 \times 10^{-5} \pm 2.20$	0.410±0.35
	20	0.115±0.06	0.00030±0.00017	0.109±0.103	$5.69 \times 10^{-5} \pm 1.42 \times 10^{-5}$	0.0009±0.0008	0.024±0.006

All values are mean ± S.D of four replicates

$CdCl_2$ was noted to positively influence the expression of inophyllum P in suspension cultures of both callus types. Low concentrations of $CdCl_2$ i.e. 0.1 and 0.5 mM were found to be most suited since maximum inophyllum P was estimated in cultures elicited with these two concentrations. 0.5 mM $CdCl_2$ resulted highest 0.034 mg % and 0.019 mg % (340 and 262 times) inophyllum P in suspension cultures of leaf and nodal / internodal callus respectively (Table 7.11).

Low concentrations of $CdCl_2$ in suspension cultures of leaf callus and high concentrations of $CdCl_2$ in suspension cultures of nodal / internodal callus were found optimum for maximum induction of calophyllolide expression. In suspension culture of leaf callus, 20 mM $CdCl_2$ reduced calophyllolide expression while $CdCl_2$ in the range 0.1 – 10 mM induced calophyllolide expression in the range 9.8 $x10^{-5}$ – 0.028 mg % (1.39 – 397.16 times). Maximum 0.028 mg % (397.16 times) calophyllolide was expressed when cultures were elicited with 1.0 mM $CdCl_2$. On the contrary, higher concentrations of $CdCl_2$ i.e. 5.0 – 20 mM were found optimum in suspension cultures of nodal / internodal callus and induced calophyllolide in the range 0.024 – 0.410 mg % (570 – 9738 times). Maximum 0.410 mg % (9738 times) calophyllolide was expressed in cultures elicited with 10 mM $CdCl_2$ (Table 7.11).

In the present study, cadmium as abiotic elicitors in the cell suspension cultures of both callus types did not result in biomass growth. Cadmium is known to be toxic to plants and was reported to have inhibitory effect on growth of plants. Cadmium was studied for its effect on growth of the pea plant and it was reported that cadmium produced a significant inhibition of the growth and alteration in the nutrient status in leaf (Sandalio et al., 2001). Cadmium and copper were investigated for their effects at cellular level on membrane and it was concluded that both of these heavy metals induced changes in membrane lipids in tomato (Ouariti et al., 1997). It was hypothesized that Cd_2^+ stress could trigger H_2O_2 biosynthesis by inducing NADPH oxidase activity and antioxidant enzymes and subsequent oxidative burst and generation of reactive oxygen species (Olmas et al., 2003). Similar to the effects of copper, cadmium was also observed to influence the expression pattern of most of the dipyranocoumarins under study. The effect of cadmium on growth of callus mass and synthesis of ajmalicine was studied in *Catharanthus roseus* (Zheng and Wu, 2004). Zheng and colleagues reported that biomass growth was not resulted in cultures treated with 0.6 mM or more Cd. They also reported that treatment of the cultures with cadmium also enhanced the ajmalicine yield. In general, Cd in plants reduces growth due to suppression of the elongation growth- rate of the cells (Sanita di Toppi and Gabriella, 1999). These physiological actions of Cd might have contributed to its negative influence on biomass growth in this study. Lee et al., (1998) studied the effect of Cd on tropane alkaloid in transformed roots of *Atropa belladona* and reported that Cd treatment increased the excretion of tropane alkaloid into the medium. Cd was

also reported to elicit the production of some secondary metabolites such as sesquiterpenoid in transformed root cultures of *Datura stramonium* (Furze et al., 1991).

7.3.2.6. Effect of Chromium as abiotic elicitors on dipyranocoumarins expression:

Chromium (Cr) is the seventh most abundant metal in the earth's crust and known to induce stress in plants. Number of the studies with Cr at high and low concentrations revealed severe phytotoxic effects of the metal on plant growth and metabolism, leading to severe oxidative damage to cells (Sanita di Toppi et al., 2002; Panda et al., 2003; Choudhury and Panda, 2005; Panda and Choudhury, 2005b). Among the different effects Cr phytotoxicity are inhibition of seed germination and seedling growth, disruption of nutrient and water balance, degradation of the pigments, decrease in the activity of antioxidant enzymes and induction of the formation of electron-dense material in cells (Poschenrieder et al., 1991; Corradi et al., 1993; Barcelo´ and Poschenrieder, 1997; Panda et al., 2003; Choudhury and Panda, 2005; Panda and Choudhury, 2005b). In addition to these effects, Cr induces reactive oxygen species (ROS) that results in oxidative damage (Panda and Choudhury, 2005b; Choudhury and Panda, 2005). Because chromium is known to induce stress to plant, it was used for stress induction in the present study. To study the effect of chromium on expression pattern of dipyranocoumarins, chromium was incorporated as abiotic elicitor in suspension culture of both callus types. Table 7.12 shows the results for the effects of chromium on dipyranocoumarins' expression in suspension cultures of both callus types.

In suspension cultures of leaf callus, chromium at lower concentrations (0.1 – 5.0 mM) was found to reduce the inophyllum A expression. However, higher concentrations of chromium showed positive influence and enhanced inophyllum A expression. 10 mM Cr was the optimum concentration and resulted in 100.28 mg % inophyllum A. This was 84.26 times the expression in control medium. In suspension cultures of nodal / internodal callus, Cr at all tested concentrations induced inophyllum A expression. When cultures were elicited with Cr in the tested range 0.1 – 20 mM, inophyllum A was expressed in the range 0.078 – 0.657 mg % (28.88 – 243.33 times). 1.0 mM Cr induced highest 0.657 mg % (243.33 times) inophyllum A (Table 7.12).

Cr as abiotic elicitor for induction of inophyllum B expression in suspension cultures of leaf callus had negative influence. Cr at all tested concentrations reduced the expression of inophyllum B. In suspension cultures of nodal/ internodal callus, low concentrations (0.1 – 0.5 mM) of Cr showed negative influence and reduced inophyllum B expression, whereas, Cr at high concentrations (1.0- 20 mM) positively influenced inophyllum B expression and enhanced in the range 0.00015 – 0.037 mg% (1.15 – 284.61 times). Highest 0.037 mg % (284.61 times) inophyllum B was expressed when cultures were elicited with 1.0 mM Cr (Table 7.12).

Cr was also noted to positively influence the expression of inophyllum C in suspension cultures of both callus types. At tested concentration range, inophyllum C was expressed in the range 0.036 – 0.064 mg % (6.79 – 12.07 times) and 0.0178 – 0.616 mg % (2.96 – 102.66 times) in suspension cultures of leaf and nodal / internodal callus respectively. Maximum 0.064 mg % (12.07 times) and 0.616 mg % (102.66 times) inophyllum C was expressed in suspension cultures of leaf and nodal / internodal callus when cultures were elicited with 10 and 0.5 mM Cr respectively (Table 7.12).

On the expression of inophyllum D, Cr at the tested concentration range had negative influence in suspension cultures of leaf callus. On the contrary, inophyllum D expression was increased when suspension cultures of nodal / internodal callus were elicited with Cr at tested concentration range. This increase in the inophyllum D expression was in the range 2.67×10^{-5} – 0.029 mg % (1.93 – 2230 times). Maximum 0.029 mg % (2230 times) inophyllum D was expressed when cultures were elicited with 1.0 mM Cr (Table 7.12).

Cr as abiotic elicitor was also found to induce the expression of inophyllum P and showed enhanced expression of inophyllum P in both suspension types. In suspension cultures of leaf callus, Cr at tested concentration range enhanced inophyllum P expression in the range 7.7×10^{-5} – 0.0004 mg % (1.11 – 5.79 times). 10 mM Cr resulted in the maximum 0.0004 mg % (5.79 times) inophyllum P expression. Pattern of inophyllum P expression in suspension cultures of nodal / internodal callus was little erratic. Although Cr at all tested concentration range enhanced the expression of inophyllum P (5.1×10^{-5} – 0.0019 mg %), Cr in the range 0.5 – 5.0 mM was found to be best suited. In the concentration range 0.5 – 5.0 mM Cr, inophyllum P was expressed in the range 0.00012 – 0.0019 mg % (6.34 – 100.52 times). Maximum 0.0019 mg % (100.52 times) inophyllum P was expressed when cultures were elicited with 0.5 mM Cr (Table 7.12).

Although the expression of calophyllolide was increased in all suspension cultures of leaf callus elicited with Cr at all tested concentrations, 1.0 and 5.0 mM Cr was best suited and resulted maximum 0.001 mg % (23.25 times) and 0.00433 mg % (100.69 times) calophyllolide respectively. In suspension cultures of nodal / internodal callus, only 0.5 and 1.0 mM Cr resulted enhanced expression of calophyllolide. Highest 0.0007 mg % (7.52 times) calophyllolide was expressed in cultures elicited with 0.5 mM Cr (Table 7.12).

Chromium as abiotic elicitor was studied for its effect on production of flavonoids in callus cultures and cell suspension cultures of *Ononis arvensis* (Tumova and Blazkova, 2002). Tumova and Blazkova showed that Cr positively influenced the formation of flavonoids and reported that Cr increased 98 % flavonoids in callus cultures and 100 % flavonoids in cell suspension cultures. Similar to the effect of copper and cadmium, chromium also influenced the expression pattern of the most of the dipyranocoumarins under study.

Table 7.12 Effects of chromium (heavy metals) as abiotic elicitor on dipyranocoumarins' expression in suspension cultures of both callus types.

Suspension type	Cocn. Of elicitors (mM)	Dipyranocoumarins (mg/100 g elicited biomass FW).					
		Inophyllum A	Inophyllum B	Inophyllum C	Inophyllum D	Inophyllum P	Calophyllolide
Suspension culture of leaf callus	0.0	1.19±0.73	0.00016±7.4 $x10^{-5}$	0.0053±0.001	4.4 $x10^{-5}$±2.8 $x10^{-5}$	6.9 $x10^{-5}$±3.6 $x10^{-5}$	4.3 $x10^{-5}$±2.5 $x10^{-5}$
	0.1	0.36±0.27	0.00011±2.2 $x10^{-5}$	0.036±0.01	2.6 $x10^{-5}$±1.5 $x10^{-5}$	0.000155±0.00013	7.5 $x10^{-5}$±9.8 $x10^{-5}$
	0.5	0.225±0.11	8.9 $x10^{-5}$±3.1 $x10^{-5}$	0.054±0.023	2.2 $x10^{-5}$±1.5 $x10^{-5}$	0.00026±0.00012	6.9 $x10^{-5}$±3.3 $x10^{-5}$
	1.0	0.37±0.2	0.00012±1.2 $x10^{-5}$	0.0385±0.016	4.39 $x10^{-5}$±2.2 $x10^{-5}$	0.000119±8.4 $x10^{-5}$	0.001±0.0001
	5.0	0.674±0.53	0.00013±0.00012	0.043±0.01	3.7 $x10^{-5}$±8.9 $x10^{-6}$	0.00018±0.0001	0.00433±0.0054
	10	100.28±26.26	0.00011±3.4 $x10^{-5}$	0.064±0.05	2.5 $x10^{-5}$±1.25 $x10^{-5}$	0.0004±0.00013	5.04 $x10^{-5}$±3.9 $x10^{-5}$
	20	52.81±7.90	0.00012±5.8 $x10^{-5}$	0.0485±0.015	2.7 $x10^{-5}$±1.6 $x10^{-5}$	7.7 $x10^{-5}$±2.6 $x10^{-5}$	5.15 $x10^{-5}$±2.8 $x10^{-5}$
Suspension culture of nodal /internodal callus	0.0	0.0027±0.0019	0.00013±9.6 $x10^{-5}$	0.006±0.002	1.38 $x10^{-5}$±1.08 $x10^{-5}$	1.89 $x10^{-5}$±6.2 $x10^{-6}$	9.3 $x10^{-5}$±2.3 $x10^{-5}$
	0.1	0.078±0.017	0.00011±8.4 $x10^{-5}$	0.0308±0.01	5.7 $x10^{-5}$±2.04 $x10^{-5}$	5.18 $x10^{-5}$±2.4 $x10^{-5}$	5.08 $x10^{-5}$±1.7 $x10^{-5}$
	0.5	0.416±0.36	0.00010±9.6 $x10^{-5}$	0.616±0.52	0.00032±0.0002	0.0019±0.001	0.0007±0.0001
	1.0	0.657±0.47	0.0037±0.002	0.0178±0.005	0.029±0.0058	0.00012±0.0001	0.00010±1.8 $x10^{-5}$
	5.0	0.123±0.09	0.00015±9.4 $x10^{-5}$	0.058±0.04	2.67 $x10^{-5}$±1.3 $x10^{-5}$	0.0005±0.0003	5.2 $x10^{-5}$±1.5 $x10^{-5}$
	10	0.102±0.03	0.00015±6.3 $x10^{-5}$	0.049±0.024	3.7 $x10^{-5}$±8.3 $x10^{-6}$	7.1 $x10^{-5}$±3.01 $x10^{-5}$	3.72 $x10^{-5}$±1.59 $x10^{-5}$
	20	0.174±0.11	0.00024±7.2 $x10^{-5}$	0.079±0.043	4.8 $x10^{-5}$±1.6 $x10^{-5}$	0.00015±0.0001	6.3 $x10^{-5}$±3.4 $x10^{-5}$

All values are mean ± S.D of four replicates

The overall expression pattern of dipyranocoumarins in suspension cultures of both callus types resulting from abiotic elicitation (with calcium, copper, cadmium and chromium) indicated that for the maximum expression of inophyllums A, cadmium as abiotic elicitor was best suited in suspension cultures of both callus types. 0.1 mM Cd enhanced maximum 981 times inophyllum A expression in suspension cultures of nodal/ internodal callus and 0.5 mM Cd enhanced 288 times inophyllum B expression in suspension cultures of leaf callus. For highest expression of inophyllum B, cadmium in suspension cultures of leaf callus and chromium in suspension cultures of nodal / internodal callus were best suited. 0.5 mM cadmium in suspension cultures of leaf callus and 1.0 chromium in suspension cultures of nodal / internodal callus expressed highest 300 and 284.61 times inophyllum B expression respectively. As abiotic elicitor, $CaCl_2$ was noted to have no positive influence on most of the dipyranocoumarins under study. Only 2.0 mM $CaCl_2$ induced the expression of inophyllum C to maximum 87.27 times in suspension cultures of leaf callus. Expression pattern of the rest of the dipyranocoumarins was either not influenced or had negligible influence. For the highest expression of inophyllum D in suspension culture of leaf callus, Cu was found most suitable abiotic elicitor. 10 mM Cu enhanced maximum 1944.44 times inophyllum D expression. In suspension cultures of nodal / internodal callus, maximum 2230 times inophyllum D was expressed in cultures elicited with 1.0 mM Cr. Among all the heavy metals as abiotic elicitors tested in this study, Cd was noted best for eliciting the expression of inophyllum P and calophyllolide in suspension cultures of both callus types. In suspension cultures of leaf callus, 0.5 mM and 1.0 mM Cd resulted maximum 340 times inophyllum P and 397.16 times calophyllolide expression respectively. In suspension cultures of nodal / internodal callus, 0.5 mM and 10.0 mM Cd elicited inophyllum P and calophyllolide expression to maximum 262 and 9738 times respectively. This expression of calophyllolide recorded in this study was the highest so far.

7.3.2.7. Statistical analysis (Two Way ANOVA):

Two way ANOVA was performed for the data of effect of heavy metals (Cu. Cd. Cr) on expression of dipyranocoumarins in suspension cultures of both callus types. Table 7.13 shows the results of two way ANOVA. Two way ANOVA revealed that for inophyllums A, C and D, elicitation medium, suspension type and their interactions were significantly different at 1 % level (p=001).

Table 7.13. Two way ANOVA for dipyranocoumarins expression resulting from elicitation with heavy metals in suspension cultures of both callus types.

Two way ANOVA	Inophyllum A			Inophyllum B			Inophyllum C		
	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty
S.E.	2.175	3.462	2.696	0.00014	NS	0.00018	0.770	1.167	0.861
C.D.	6.522	2.012	9.223	0.0018	NS	0.0025	3.270	1.009	4.625
Significance	**	**	**	**	NS	**	**	**	**
Two way ANOVA	Inophyllum D			Inophyllum P			Calophyllolide		
	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty	E. Med.	Sus. Type	E. Med X Su.Ty
S.E.	0.00050	0.00099	0.00050	0.00112	NS	0.0011	0.0077	0.0082	0.0097
C.D.	0.0037	0.0011	0.0053	0.0077	NS	0.0109	0.0721	0.0168	0.102
Significance	**	**	**	**	NS	**	**	*	**

Med- Medium containing hormone, Sus type- Suspension type, Med X Sus type - Interaction between medium and Suspension type. (**) Significant at 1% level; (*) Significant at 5% level; NS- Non significant at 1% & 5% level S. E. – Standard Error.

For calophyllolide, suspension type was significantly different at 5% level (p=005) and elicitation medium and interaction between suspension type and elicitation medium was significantly different at 1% level. For inophyllums B and P, suspension type was non significant and interaction between suspension type and elicitation medium was significantly different at 1% level.

7.4. CONCLUSIONS:

The results on isolation of fungi endophytic to *C. inophyllum*, their identification and use as biotic elicitors in suspension cultures of both callus types have been described. The effect of dried cell powder and culture filtrate of the endophytic fungi as biotic elicitors and calcium, copper, cadmium and chromium as abiotic elicitor on expression pattern of dipyranocoumarins have studied and described. From leaf of *C. inophyllum*, four endophytic fungi were isolated and identified as *Nigrospora sphaerica* (Sacc.) Mason**.,** *Phoma Species, Cladosporium cladosporioides* (Fresen.) de Vries and *Aspergillus sydowi* (Bain. & Sart.) Thom. & Church. DCP and CF of *Nigrospora sphaerica* and *Phoma Species* were used as biotic elicitor for inducing the expression of dipyranocoumarins.

The overall expression pattern resulting from biotic elicitation revealed that DCP of *Phoma species* as biotic elicitor in suspension cultures of nodal / internodal callus were the best suited condition for maximum expression of inophyllums B, C and D while for the expression of inophyllum A, CF of *Phoma species* in suspension cultures of nodal / internodal callus was most suitable. For highest expression of inophyllum P and calophyllolide, DCP of *N. sphaerica* and

Phoma species in suspension cultures of nodal / internodal and leaf callus respectively were the best suited conditions.

Cadmium in comparison to calcium, copper, and chromium was noted to be most suitable as abiotic elicitor. Except for inophyllum D, cadmium positively influenced the expression of inophyllums A, B, C, P and calophyllolide. Using cadmium as elicitor, highest expressions of inophyllums A, B and P were achieved in suspension cultures of leaf callus whereas highest expressions of inophyllum C and calophyllolide were achieved in suspension cultures of nodal/ internodal callus. Maximum inophyllum D expression was resulted when chromium as abiotic elicitor was used in suspension cultures of leaf callus.

When expression patterns resulting from biotic and abiotic elicitations were compared, biotic elicitation was the best suited for induction of the maximum expressions of inophyllums A, B, C and inophyllum P, while for the maximum expression of inophyllum D, abiotic elicitation was the most suitable condition. The level of elicited expression of calophyllolide resulting from biotic and abiotic elicitors was almost similar. Based on these results, it can be concluded that biotic elicitation of suspension cultures of nodal/ internodal callus of *C. inophyllum* was best suited condition for eliciting the expression of dipyranocoumarins.

Part of this work (abiotic elicitation) has been communicated to journal Plant Cell Report

K.D. Pawar and S. R. Thengane.
Influence of abiotic elicitation on expression pattern of dipyranocoumarins in suspension cultures of *Calophyllum inophyllum* L.
(Communicated to Plant Cell Report).

Manuscript on biotic elicitation is under preparation

SUMMARY

Summary:

C. inophyllum is medicinally important tree species and several classes of secondary metabolites like xanthones, triterpenes and coumarins were isolated and characterized. This plant is a source of anti HIV inophyllums B and P, anti cancerous inophyllums A, C. D and calophyllolide. Inophyllum C and calophyllolide were also shown to have antimicrobial and cytotoxic activity. Inophyllums B and P (IC_{50}, 38nM and 130 nM, respectively) act as Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) and inhibit the activity of reverse transcriptase Type-1 enzyme of HIV-1 by the virtue of their ability to bind irreversibly at the non-substrate binding, allosteric site. *C. lanigerum,* a potential source of NNRTIs "calanolides" has not been reported from India so far, while *C. inophyllum,* a source of another NNRTIs "inophyllums" (dipyranocoumarins) grows at several locations along The Western Ghats of India. As an alternative to calanolides, inophyllums isolated from *C. inophyllum* can be used in anti retroviral therapies. This plant is also a source of good quality timber that can be used in boat and ship making because of which, it is cut fairly on large scale. Due to its large scale cutting for obtaining timber, this plant species has been included in IUCN's red list of threatened species and needs to be conserved.

Presently, many other natural products are derived by extracting and isolating solely from massive quantities of whole plant parts. Often the source plants are either wild or cultivated in tropical or subtropical and geographically remote areas which are subject to political instability, drought, disease and changing land use patterns and other environmental factors. In addition, the long cultivation periods between planting and extraction make selection of high-yielding plants difficult, thus resulting in expensive drugs. Cultivation periods may range from several months to decades. In spite of these difficulties and costs, the extraction of medicinally important secondary metabolites from cultivated plants or plants in the wild continues because of lack of credible alternatives. Clearly, the development of alternative and complimentary methods to whole plant extraction for the production of clinically important secondary metabolites is an issue of considerable socioeconomic importance. These facts have generated great interest in the use of plant cell culture technologies for the production of inophyllums like pharmaceuticals and other plant derived secondary metabolites. Indeed, the plant cell culture technology is now sufficiently advanced to allow for large quantities of relatively homogeneous, undifferentiated cells to be produced. When compared to whole plant extraction, plant cell and tissue culture systems are complementary and may provide competitive metabolite production systems.

The present study "*In vitro* production of secondary metabolites from cultured cells/ tissues and molecular characterization of *Calophyllum inophyllum*" was taken up with the following objectives:

1. To develop and validate HPLC method for analysis of dipyranocoumarins in *in vitro* growing cultured cells / tissues and *in vivo* growing plant materials.
2. To study the chemo diversity and genomic diversity in *C. inophyllum.*
3. To establish micro propagation protocol for *C. inophyllum*.
4. To establish callus cultures using different media, hormones and explants for studying the *in vitro* expression pattern of dipyranocoumarins.
5. To establish cell suspension culture for increased biomass and dipyranocoumarins, expression.
6. To study the effects of different biotic and abiotic elicitors on *in vitro* expression of dipyranocoumarins.

HPLC method for the analysis of dipyranocoumarins was developed and validated. Normal phase HPLC method with µ Porasil column and ethyl acetate and petroleum ether as a mobile phase was best suited for maximum base line separation and quantifications of dipyranocoumarins. As per the results of HPLC method validation (like linearity, precision, recovery, LOD, and LOQ) method was acceptable and was used for determinations of dipyarnocoumarins in seed, callus cultures and cell suspension cultures.

Chemical diversity study revealed that seeds from locations Harne and Kalavali were estimated with highest dipyranocoumarins, total dipyranocoumarins and yield of acetone extracts. Based on these results, it was concluded that plants from these two locations were elite in terms of highest dipyranocoumarins, total dipyranocoumarins and yield of acetone extracts. Plants from these locations were collected for further *in vitro* experiments.

DNA extraction protocol yielded good quality and quantities of DNAs from *C. inophyllum* leaves. PCR conditions optimized were the optimum for reproducible and clear amplifications pattern. ISSR markers successfully identified the variations (38. 68%) among the 20 trees. Clustering in dendrogram and PCA based on ISSR variation grouped 20 locations in three groups and indicated correlation between geographical position and genetic differentiation. Principle component Analysis (PCA) based on ISSR and chemical diversity of dipyranocoumarins in 13 locations also grouped 13 locations. This grouping indicated the correlation of chemical variation (dipyranocoumarins' content) with genetic variation and ecological factors.

Micropropagation protocol using seedling explants was developed. For seed germination, WPM and WPM with BAP showed good and fast germination. Presoaking in SDW and/ or GA_3 (0.058µM) hastened the germination process. WPM supplemented with BAP (2.22-44.0µM) and TDZ (0.91- 4.54 µM) induced the multiple shoots from decapitated seedling explants. Higher concentrations of BAP (44.0µM) or lower concentration of TDZ were optimum for multiple

shoot induction and induced almost equal number of multiple shoots. Statistically TDZ was significantly better than BAP for multiple shoot induction. WPM with BAP (2.22 μM) was optimum for elongation of multiple shoots whereas stunted shoots induced on WPM with BAP and TDZ elongated well on half strength WPM without any growth regulators. For rooting, shoots of more than 4.0 cm responded well and half strength and /or full strength WPM supplemented with IBA (2.46-24.60 μM) alone or in combination with BAP (2.22 μM) was found to be optimum. Rooted shoots were acclimatized best in greenhouse condition when planted in a sterile mixture of soil, cocoa peat and sand in proportion 1:2:1 which was optimum. Only minimum 3-5 months old and acclimatized plantlets were able to survive in a field conditions when planted in garden soil and farm yard manure (1:1).

Callus induction protocol was developed using WPM basal medium and seed, leaf and nodal/internodal explants. Combinations of 2, 4-D, kinetin and BAP were not suitable for callus induction, whereas combinations of IBA, NAA, picloram and BAP in media were most effective in inducing callus. Induction of calluses from leaf and nodal/ internodal explants took place ten days earlier as compared to induction from seed explants. Frequency of callus inductions from seed explants on medium incorporated with IBA alone and with BAP was almost same to that of frequency achieved by incorporating NAA alone and with BAP in the medium. IBA 19.60 μM along with BAP 4.44 μM and NAA 10.74 μM along with BAP 8.88 μM were optimum for callus induction from seed explants. All concentrations of picloram along with BAP consistently resulted in higher frequencies of callus induction from leaf and nodal/ internodal explants. Picloram 24.84 μM with BAP 8.88 μM was the optimum for callus induction from these explants i.e. leaf and nodal/ internodal explants. Supplementation of only IBA in the medium was as good as supplementation of picloram and BAP for callus induction from nodal/ internodal explants. Highest anti HIV inophyllum B (40.59 %) was expressed in callus induced from seed explants on medium containing 9.80μM IBA, while highest inophyllum P (141.35 mg %) was estimated in seed callus induced on medium containing IBA 9.80 μM along with BAP 4.44 μM.

Two types of suspension cultures viz. suspension cultures of leaf callus and nodal internodal callus were initiated. The effects of hormone IBA, picloram**,** BAP and medium components such as sucrose, nitrate, sulphate and vitamins on biomass growth and expression pattern of dipyranocoumarins in suspension cultures of leaf and nodal / internodal callus was studied. In suspension cultures of both callus types, incubation period for 50 days was optimum for maximum biomass growth and expression of dipyranocoumarins. Among the hormones IBA, picloram and BAP studied in suspension cultures of both callus types, IBA 19.60 μM resulted maximum 54.60% (3.6 times) biomass growth in suspension cultures of leaf callus whereas IBA 14.70 μM + BAP 4.44 μM resulted maximum 59.27 % (4.95 times) biomass growth in suspension

cultures of nodal / internodal callus. Inverse relationship between biomass growth and expression of inophyllums B, D, and calophyllolide was observed in suspension cultures of leaf callus. In suspension cultures of nodal/internodal callus also, inverse relationship between biomass growth and expression pattern of inophyllums D, P, and calophyllolide was noted. Variation in sucrose did not result in biomass growth in suspension cultures of both callus types. In suspension cultures of leaf callus, sucrose was found to have little influence on expression pattern on inophyllums A and C. In suspension culture of nodal / internodal callus, sucrose was observed to have major influence on expression of inophyllum A and inophyllum C. Expression patterns of other dipyranocoumarins in suspension cultures of both callus types were not influenced by variation in initial sucrose concentrations. Variation in total nitrate did not enhance biomass growth, did increase the expression of inophyllums A and C and had little influence on expression pattern of other dipyranocoumarins (inophyllums B, D, P and calophyllolide). On the contrary, variation in total sulphate was found to increase biomass growth in suspension cultures of both callus types. One fold increase in sulphate concentration resulted maximum 61.22% (2.42 times) biomass growth in suspension cultures in leaf callus whereas, maximum 38.23 % (6.2 times) biomass was increased on supplementation of three fold sulphate in suspension cultures of nodal / internodal callus. Like nitrate and sucrose, variation in vitamins did not increase biomass in suspension cultures of both callus types, had maximum influence on expression of inophyllums A, B and C and had little influence on expression of other dipyranocoumarins. Maximum 234 times inophyllum B expression was increased on supplementation of 3 fold vitamins in suspension cultures of nodal / internodal callus.

From leaf of *C. inophyllum,* four endophytic fungi were isolated and identified as *Nigrospora sphaerica* (Sacc.) Mason., *Phoma Species, Cladosporium cladosporioides* (Fresen.) de Vries and *Aspergillus sydowi* (Bain. and Sart.) Thom. and Church. Dried cell powder (DCP) and culture filtrate (CF) of *Nigrospora sphaerica* and *Phoma Species* were used as biotic elicitor for inducing the expression of dipyranocoumarins. The overall expression pattern resulting from biotic elicitation revealed that DCP of *Phoma species* as biotic elicitor in suspension cultures of nodal / internodal callus were best suited conditions for maximum expression of inophyllums B, C and D while for the expression of inophyllum A, CF of *Phoma species* in suspension cultures of nodal / internodal was most suitable. For highest expression of inophyllum P and calophyllolide, DCP of *N. sphaerica* and *Phoma species* in suspension cultures of nodal / internodal and leaf callus respectively were best suited conditions.

Cadmium in comparison to calcium, copper, and chromium was noted to be most suitable as abiotic elicitor. Except inophyllum D, cadmium positively influenced the expression of inophyllums A, B, C, P and calophyllolide. Using cadmium as elicitor, highest expressions of

inophyllums A, B and P were achieved in suspension cultures of leaf callus whereas highest expressions of inophyllum C and calophyllolide were achieved in suspension cultures of nodal/ internodal callus. Maximum inophyllum D expression was resulted when chromium as abiotic elicitor was used in suspension cultures of leaf callus.

Conclusion of the present work:

Production of secondary metabolites by *in vitro* techniques from important medicinal plants has been in practice since long time. The foremost prerequisite in such studies is the selection of elite chemotypes as starting material whose identification is based on chemodiversity studies. In the present work, quantitative HPLC analysis with molecular ISSR marker analysis has helped in identification of elite plants. *In vitro* propagation protocol was developed using sterile seedling explants. *C inophyllum*, being the littoral species produces large number of fruits, but the frequencies of natural regeneration is very low. Initiation of *in vitro* cultures had difficulties of contamination. In view of these, protocol described in the present study holds the merits for *in vitro* conservation of *C. inophyllum*. Response However frequencies of multiplication, elongation and rooting depended on combinations and concentrations hormones. Till date, *in vitro* production of dipyranocoumarins in *C. inophyllum* has not been reported so far. This is the first report for dipyranocoumarins' *in vitro* production through callus cultures using WPM basal medium and seed, leaf and nodal / internodal explants. The frequencies of callus induction and contents of each dipyranocoumarins depended on hormone combinations and concentrations. Maximum callus induction response and expression of anti HIV inophyllums B and P were achieved in seed callus. However the production of biomass is also a very important criteria for commercial application. In the view of this, for further enhancing the expression of dipyranocoumarins, cell suspension culture and elicitation techniques were used. Although Leaf and nodal / internodal callus expressed comparatively low levels of dipyranocoumarins, they were best growing and suitable for initiating suspension cultures. Manipulating the hormonal regimen and medium components like nitrate, sulphate and vitamins were the effective strategy for enhancing the expression of dipyranocoumarins. Elicitation with heavy metals as well as endophytic fungi further enhanced the dipyranocoumarins' expression. In comparison, biotic elicitation with fungi endophytic to *C. inophyllum* was effective approach for eliciting the dipyranocoumarins' expression.

Based on the results of suspension cultures and elicitation incorporated in this thesis, suitable production strategies and optimum production medium for each of the inophyllums can be formulated. However further experimentations will necessary to optimum production media for commercial application.

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AUTHOR'S PUBLICATION

Micropropagation of Indian laurel (*Calophyllum inophyllum*), a source of anti-HIV compounds

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An efficient protocol for *in vitro* micropropagtion of *Calophyllum inophyllum* (Linn.), an evergreen littoral tree, through multiple shoot formation from seed explants was developed. *In vitro* germination of the seeds was standardized on Woody Plant Medium (WPM) hormone free and/or supplemented with 6-benzylaminopurine (BAP; 2.22 μM) and on half or full strength MS medium. Multiple shoot formation was achieved on WPM supplemented with BAP (2.22–44.00 μM) and thidiazuron (TDZ; 0.91–4.54 μM) from the decapitated seedling explants. The maximum multiple shoots, 20.9 per explant were induced on TDZ (0.91 μM) after two subcultures. Elongated shoots of size >4.0 cm were obtained on all media combinations with an average of 2.2–8.7 per explant. Elongation of the stunted shoots induced on BAP and TDZ was done on half strength WPM without any growth hormones. The elongated shoots on half WPM and/or full strength WPM supplemented with indole-3-butyric acid (2.46–24.60 μM) alone or in combination with BAP (2.22 μM) resulted in 52% rooting with 1–5 roots per rooted plant. The micropropagated plants were acclimatized successfully with 77% survival rate after five weeks. These plants were planted in the institute campus for *ex situ* conservation, where 72% plants are showing good growth and development.

Keywords: *Calophyllum inophyllum*, *ex situ* conservation, micropropagation, seed.

CALOPHYLLUM inophyllum Linn. [Guttifereae (Clusiaceae)], commonly known as 'Indian laurel' or 'Alexandrian laurel' is a broad leaved evergreen tree (Figure 1 *a*) occurring as a littoral species along the beach crests, although sometimes occurring inland[1]. It is known to have cancer chemopreventive agents[2], coumarins and xanthones with antimicrobial activity[3]. The oil has various medicinal uses in rheumatism, skin diseases, joint pains and haemorrhage[4–6]. The aqueous extracts of the root bark and leaves are used as a cicatrisant, and those of the fruit have analgesic properties and are used in treatment of wounds and herpes[7]. Oil is also used as luminant, lubricant, for soapmaking, etc. The timber is used for beams, furniture, railway carriages and shipbuilding[8]. *Calophyllum* species are gaining importance as a source of anti-HIV medicines. The inophyllums and (+)-calanolide isolated from *C. inophyllum* L. and *C. lanigerum* Miq[9–13] showed strong activity against human immunodeficiency virus type-1 (HIV-1). *C. inophyllum* possesses potential threat due to decline in the population because of various biotic[14] and abiotic factors. The fruit being a drupe, has a hard endocarp with long dormancy period and low rate of germination. Being a littoral species, the seeds are taken away in the tidal water thereby limiting the propagation rate. Conventional propagation via vegetative cuttings is not practised due to difficulty in rooting[15] in almost all species of *Calophyllum* and immediate protective measures are essential for the continued existence of the genus[16]. Tissue culture technology would be a useful tool for overcoming these limitations and accelerate mass propagation of this important medicinal tree.

Here, we report a reliable method for *in vitro* multiplication and micropropagation of *C. inophyllum* using mature seed as explant, followed by successful plantlet growth, development and *ex situ* conservation.

Mature fruits of *C. inophyllum* Linn. (Figure 1 *b*) were collected from Harne village (17°49′63″N, 73°05′65″E; 2 m altitude) near Dapoli, Ratnagiri along the coast of Maharashtra, in the fruiting season during the second week of March 2003.

For *in vitro* germination, mature fruits were decoated mechanically to remove the hard, stony endocarp and the seeds (Figure 1 *c*) were washed thoroughly with liquid detergent solution (Labolene 0.1%, v/v; Qualigens, India) as surfactant for 5 min and then washed with tap water (× 5), followed by washing with antiseptic (Savlon 10%, v/v; Johnson and Johnson Ltd, India). The seeds were then rinsed thoroughly with double distilled-water (DDW) (× 3) and treated with insoluble polyvinylpyrrolidone (0.1%, w/v; Sigma, USA) for 30 min and later by antifungal agent (Bavistin, 1% w/v; BASF, India) for 30 min. Seeds were again rinsed with DDW (× 5). All further treatments were carried out under sterile conditions in a laminar airflow chamber. The seeds were rinsed in ethyl alcohol (70%, v/v; Merck, India) for 15–20 s, washed with sterile distilled water (SDW) (× 3), followed by mercuric chloride (0.1%, w/v; Qualigens, India) for 5 min and finally washed thoroughly with SDW (× 7). Seeds were presoaked in SDW and/or gibberellic acid (GA_3; 0.058 μM) and/or heated at 35°C and then presoaked in GA_3 (0.058 μM), for 24 h prior to inoculation. For germination, four types of media were tested, viz. hormone-free Woody Plant Medium[17] (WPM) and/or supplemented with 6-benzylaminopurine (BAP; 2.22 μM) and half strength and full strength Murashige and Skoog[18] (MS) medium without any growth regulators. All the media were supplemented with sucrose (2%, w/v) and pH was adjusted to 5.6–5.8 with 0.1 N NaOH solution. All the media were gelled with 0.80% agar (w/v, Qualigens). Growth regulators were incorporated into the media prior to autoclaving. The media were autoclaved at 1.05 kg cm^{-2} and 121°C for 20 min before use. One seed was inoculated per glass cul-

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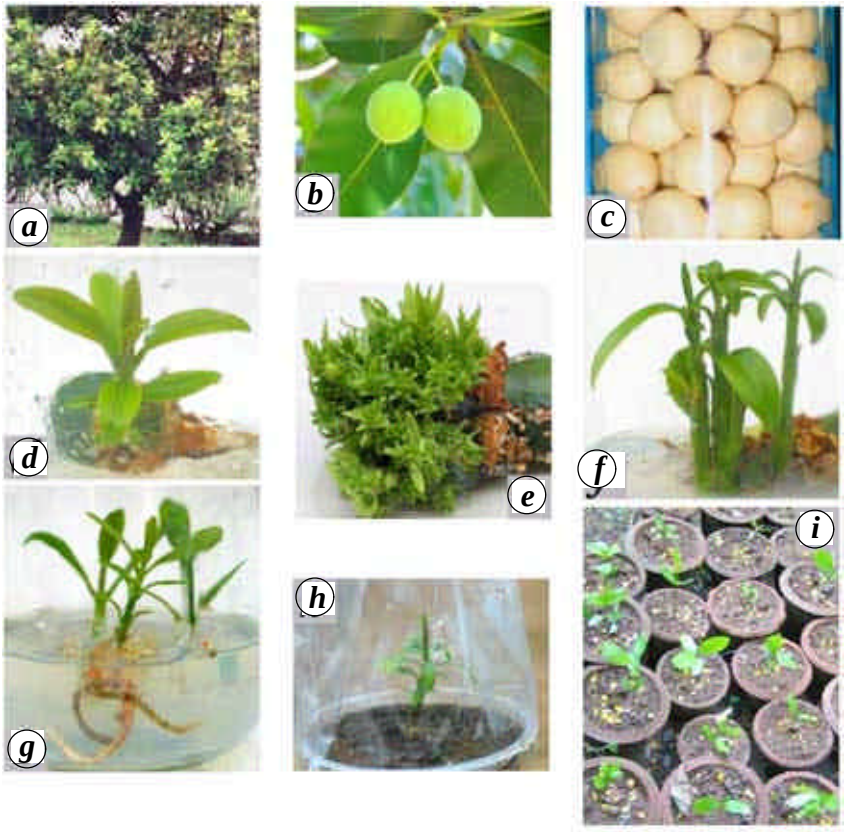

Figure 1. ***a***, Tree of *Calophyllum inophyllum* Linn. at Harne. ***b***, Globose drupe fruits. ***c***, Seeds removed after breaking the hard, stony endocarp. ***d***, *In vitro* germinating seed on WPM full strength medium (45 days after inoculation). ***e***, Proliferation of multiple shoots from a single decapitated seedling after 7 weeks of culture on WPM medium containing 0.91 μM TDZ. ***f***, Elongated multiple shoots of size >4.0 cm. ***g***, Well-rooted plantlets prior to transfer to potting mixture. ***h***, Acclimatized plant 3 weeks after transfer to greenhouse. ***i***, *In vitro* propagated plantlets 4 months after transfer to the pots.

ture bottle (Laxbro, India), with 50 seeds per treatment. The cultures were incubated at 25 ± 1°C under cool white fluorescent light (16/8 h photoperiod, 35 μmol m^{-2} s^{-1}; Philips, India). The frequency of seed germination was scored after 25 days based on five replications for each experiment.

The *in vitro* germinated seedlings were removed from the culture bottles under sterile conditions in a laminar airflow chamber. Decapitation (of shoot and root) was done using sterile surgical blade. The decapitated seedlings (one per culture bottle) were then inoculated on hormone free WPM and/or WPM supplemented with BAP (2.22–44.00 μM) or thidiazuron (TDZ; 0.91–4.54 μM). The culture conditions were similar to the germination experiment. The numbers of multiple shoots induced were scored after every 20 days from the day of inoculation to a period of 60 days. The explants on all the combinations of media were subcultured twice during this period at an interval of 20 days. The multiple shoots induced on BAP (2.22–8.90 μM) and TDZ (0.91–4.54 μM) elongated well on the same medium. The stunted shoots induced on higher concentrations of BAP (13.30–44.00 μM) and some from TDZ (0.91–4.54 μM) were elongated on half strength WPM without any growth regulators. Well-elongated shoots were shifted to half and full strength WPM alone or supplemented with indole-3-butyric acid (IBA; 2.46–24.60 μM), alone or in combination with 2.22 μM BAP for root induction. For all the treatments, a minimum of 50 shoots with three replicates was maintained. The rooted shoots were acclimatized in sterilized mixture of soil, cocoa peat and sand (1 : 2 : 1) under greenhouse conditions for four weeks and later transferred to earthen pots for further growth and development in the nursery. These micropropagated plants were planted after 3–5 months in the institute campus in an attempt for their *ex situ* conservation. Pits of size 50 cm^3 were made at the plantation sites, which were filled with garden soil, and farmyard manure (1 : 1). Plants were carefully transplanted to the pits from the earthen pots; 72% of the plants are showing good growth and development.

Analysis of variance (ANOVA) was done by completely randomized block design (CRBD) using Agrobase 99 software for all the experiments and the angular transformation values were derived according to Snedecor and Cochran[19].

Germination of seeds was observed after 10–15 days of incubation on all media combinations. Without phytohor-

Table 1. Influence of BAP and TDZ on multiple shoot induction from decapitated seedling explant

Medium	Hormone	Concentration (μM)	Average no. of multiple shoots after incubation (days)		
			20	40 S1	60 S2
WPM	–	–	1.2	2.3	4.8
	BAP	2.22	3.6	9.7	12.0*
		4.40	1.3	5.1	8.1
		8.90	2.0	4.9	6.4
		13.30	3.5	6.3	9.8
		22.19	3.8	8.8	12.7*
		44.00	6.9	11.6	13.3*
	TDZ	0.91	6.9	15.0	20.9**
		2.27	4.4	10.1	15.0**
		4.54	3.6	6.6	13.1*

LSD (P = 0.05) = 6.97; LSD (P = 0.01) = 9.24.
*,**Significant at 5% and 1% level respectively; S1, S2, Subcultures one and two.

mones, seed germination on MS basal medium was poor (24–42%) and slow compared to WPM basal medium with/or without hormones, which showed good and faster germination (36–78%).

Presoaking facilitated leaching of phenolics from the seeds, prevented browning and caused swelling of seeds, thereby hastening the germination process by three weeks, compared to the seeds which were not presoaked prior to inoculation (used as control). The best germination 78% (significant at P = 0.01) was observed when seeds were soaked for 24 h prior to inoculation on WPM basal medium without any growth hormones (Figure 1 *d*). However, with increase in presoaking time to 36 or 48 h, there was no further increase in the germination percentage. Moreover, it resulted in contamination of cultures. Therefore, for seedling establishment 24 h presoaked seeds were used. On an average, all seeds germinated within 10–15 days. However, growth was slow and it took about 25–30 days to develop into seedlings of 5–8 cm size. Well-germinated seedlings (size >5.0 cm) were decapitated and inoculated on hormone-free WPM and/or WPM supplemented with BAP (2.22–44.00 μM) or TDZ (0.91–4.54 μM) for multiple shoot induction. The induction of multiple shoots was observed 10–15 days after inoculation. Observations for multiple shoot induction were taken at 20 days intervals up to a period of 60 days (Table 1). More multiple shoots were induced on 2.22, 22.19, and 44.00 μM BAP compared to other concentrations (4.40, 8.90 and 13.30 μM). Low concentration of TDZ (0.91 μM) induced equal number of shoots as that of higher concentration of BAP (44.0 μM) after 20 days of incubation. The same trend of response continued for 60 days. After 60 days of incubation, the maximum number of multiple shoots observed was 20.9 per explant with TDZ (0.91 μM; Figure 1 *e*). All concentrations of BAP induced multiple shoots from 6.4 to 13.3 per explant. ANOVA showed that BAP (2.22, 22.19 and 44.00 μM) and TDZ (4.54 μM) were significant at 5% level, while TDZ (0.91 and 2.27 μM) was found to be significant at 1% level. On the basis of statistical analysis, it appears that TDZ is significantly better than BAP for multiple shoot induction, since it induced greater number of shoots at much lower concentrations.

Though more multiple shoots were induced at high concentrations of BAP, the number of elongated shoots of size greater than 4.0 cm (Figure 1 *f*) was significantly higher at lower concentrations of BAP (2.22 and 4.40 μM). Generally, longer shoots are produced at lower BAP concentrations, whereas more shoots are induced with higher BAP concentration[20]. However, TDZ even at very low concentration (0.91 μM) induced more multiple shoots. Also, the proportion of elongated shoots of size >4.0 cm was significantly higher (4.9–7.2 per explant) compared to BAP (Table 2). The possible reason is its auxin and cytokinin-like activity[21]. Maximum number of elongated shoots, 8.7 per explant (significant at P = 0.01), was noticed on WPM supplemented with BAP (2.22 μM). Transfer of the stunted shoots induced on BAP/TDZ to half strength WPM without any growth hormones showed considerable elongation in about 30–40 days.

The developing shoots of more than 4.0 cm size were excised and used for root induction. These shoots were cultured on half and/or full strength WPM supplemented with IBA (2.46–24.60 μM) alone or in combination with BAP (2.22 μM). Combination of half WPM supplemented with IBA (2.46–24.60 μM) had no effect on root induction. Shoots induced on BAP-containing medium induced rooting in 8–12 days, with 1–5 roots per explant. However, shoots induced on TDZ rooted only after two passages of one-month duration each on half strength WPM supplemented with IBA (2.46–24.60 μM) alone or in combination with BAP (2.22 μM). The high carry-over effect of TDZ has been well documented in the literature[22,23]. In all the combinations, rooting was observed with 14–52% frequency (Table 3; Figure 1 *g*). Maximum rooting (52%) was observed on WPM supplemented with IBA (2.46 μM) alone. On the basis of ANOVA, it was observed that full strength WPM and/or WPM supplemented with IBA (2.46–24.60 μM) was significantly superior to half strength

Table 2. Average number of elongated shoots induced with different hormone concentrations

Medium	Hormone	Concentration (μM)	Average no. of elongated shoots (>4.0 cm) after incubation (days)		
			20	40 S1	60 S2
WPM	–	–	0.4	1.2	2.2
	BAP	2.22	1.8	6.1	8.7**
		4.40	0.8	5.1	6.2*
		8.90	0.6	1.5	4.9
		13.30	1.0	2.1	3.7
		22.19	0.7	2.0	2.7
		44.00	1.3	1.9	2.3
	TDZ	0.91	3.1	6.3	7.2*
		2.27	2.1	5.6	6.4
		4.54	2.7	3.8	4.9

LSD (P = 0.05) = 3.77; LSD (P = 0.01) = 5.00.
*,**Significant at 5% and 1% level respectively; S1, S2, Subcultures one and two.

Table 3. *In vitro* root induction in shoots of size >4.0 cm

Medium	Hormone concentration (μM)		Mean per cent rooting	No. of plantlets obtained	Per cent acclimatization
	IBA	BAP			
Half WPM	–	–	14 (21.59)	10	71.40
	2.46	2.22	40 (39.23)**	32	80.00
	4.90	2.22	34 (35.26)**	26	76.47
	9.80	2.22	27 (31.30)**	22	78.57
	14.70	2.22	23 (28.65)**	20	83.33
	24.60	2.22	18 (24.57)*	14	77.77
WPM	–	–	16 (23.04)	12	75.00
	2.46	–	52 (45.76)**	42	80.76
	4.90	–	41 (39.42)**	36	85.71
	9.80	–	29 (32.15)**	22	73.33
	14.70	–	43 (40.59)**	34	77.27
	24.60	–	41 (39.61)**	30	71.42

LSD (P = 0.05) = 2.85; LSD (P = 0.01) = 3.87.
*,**Significant at 5% and 1% level respectively.
Figures in parenthesis are angular transformation values of percentage of response.

WPM and/or half WPM supplemented with IBA (2.46–24.60 μM) and BAP (2.22 μM).

The rooted plantlets were transferred to sterilized potting mixture of soil, cocoa peat and sand (1 : 2 : 1) and acclimatized in a greenhouse with temperature of 25 ± 2°C, 80% relative humidity, and with 77% survival rate after a period of five weeks (Figure 1 *h*). The well developed and hardened plants after 8 weeks were transferred to earthen pots containing a mixture of garden soil and farmyard manure (1 : 1) for further growth and development (Figure 1 *i*) and finally planted in the institute campus.

Nair and Seeni[15] reported *in vitro* multiplication of *C. apetalum* Willd. using mature tree explants on MS medium supplemented with BAP (8.8 μM). The multiplication ratio was 1 : 2/3. The present work is on *in vitro* propagation of *C. inophyllum* Linn., a tree species with immense medicinal importance (especially in AIDS chemotherapy). Thus efforts have been made for the *ex situ* conservation of this threatened medicinal tree. The above protocol can be used for mass propagation of *C. inophyllum*, since the success rate of vegetative propagation is low and is usually not practised.

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Influence of northeasterly trade winds on intensity of winter bloom in the Northern Arabian Sea

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Chlorophyll and wind pattern retrieved from remote sensing data have been used to study biological activity in the oceanic waters of Northern Arabian Sea (NAS) during February–March 2002–05. Occurrence of algal bloom in these waters during this period was noticed with the help of ship observations in the past. The same was detected from OCEANSAT I/OCM with time series chlorophyll images for January–March 2000. Occurrence of this bloom was later re-confirmed using OCM data in the subsequent years also. The time-series chlorophyll images established that the bloom develops every year during February–March. This period happens to coincide with the presence of northeasterly trade winds over the NAS. Two ship cruises were conducted with the help of research vessels FORV *Sagar Sampada* (SS-212 during 26 February–7 March 2003 and SS-222 during 21 February–11 March 2004) during this period at the bloom site. The aim was species identification of the bloom and to study various environmental parameters associated with the bloom. Two diverse situations in the context of biological activity were observed while collecting *in situ* data in 2003 and 2004. Distribution of the bloom was found uniform over a large area and concentration of phytoplankton was relatively higher in 2003. Compared to this, it was observed during the same period in 2004 that phytoplankton was distributed in scattered and small patches and its concentration was relatively less. Corresponding to this observation, it was noticed from the ship data that wind strength was significantly weaker and the oceanic waters were less turbulent in 2004 compared to the same in 2003. In light of this elementary observation, an attempt was made to observe variations in the wind pattern during 2003 and 2004 using QuikSCAT/SeaWinds scatterometer data. It could be established that occurrence of the bloom as well as the observed inter annual variability in chlorophyll pattern were coupled with prevailing trade winds. It was found that density of surface water increased (inversion) during this period, which could result in convective action and the observed bloom. The vertical density gradient revealed an increasing pattern with increase in wind speed. Moreover, it was observed that response of chlorophyll to acting wind force is delayed by one to two weeks. This led to an important inference that wind can be treated as a precursor to predict

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Pattern of anti-HIV dipyranocoumarin expression in callus cultures of *Calophyllum inophyllum* Linn.

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Abstract

Callus cultures of *Calophyllum inophyllum* were established using seed, nodal/ internodal and leaf explants on WPM basal medium supplemented with indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA), picloram (4-amino-3,5,6-trichloropicolinic acid), and 6-benzylaminopurine (BAP) in different combinations and concentrations with the view to study the influence of hormones on callus induction and the pattern of expression of dipyranocoumarins including anti-HIV, non-nucleoside reverse transcriptase inhibitors inophyllum B and P in callus cultures. 96.01% seed explants, 87.50% nodal/internodal explants and 86.66% leaf explants were converted into calluses when inoculated on WPM supplemented with IBA 4.0 mg l^{-1} along with BAP 1.0 mg l^{-1}, IBA 4.0 mg l^{-1}, and picloram 6.0 mg l^{-1} along with BAP 2.0 mg l^{-1}, respectively. Calluses induced from seed explants were white, friable and irregular whereas nodal/internodal and leaf explants induced dark brown, nodular and compact calluses. In order to facilitate the rapid quantitative analysis of dipyranocoumarins under study, a novel HPLC method capable of separating all six dipyranocoumarins in a single isocratic run has been optimized. Quantitative HPLC analysis of callus extracts revealed that highest inophyllum B (40.59 mg 100 g callus^{-1}) was expressed in callus induced from seed explant on medium containing 2.0 mg l^{-1} indole-3-butyric acid, while highest inophyllum P (141.35 mg 100 g callus^{-1}) was estimated in seed callus induced on medium containing 2.0 mg l^{-1} indole-3-butyric acid along with BAP 1.0 mg l^{-1}.

Keywords: Woody plant medium; *Calophyllum inophyllum*; Callus cultures; Dipyranocoumarins; Inophyllum; NNRTIs; HPLC

1. Introduction

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are the group of compounds that inhibit the activity of reverse transcriptase Type-1 enzyme of HIV-1 by the virtue of their ability to bind irreversibly at the non-substrate binding, allosteric site (De Clercq, 2000). Few of such compounds namely calanolides A and B (IC_{50} 20 and 15 μM, respectively) were isolated from *Calophyllum lanigerum* (Kashman et al., 1992) and inophyllum B and P (IC_{50} 38 nM and 130 nM, respectively) were isolated from *Calophyllum inophyllum* Linn *clusiaceae,* (Patil et al., 1993). Beside anti-HIV-1 inophyllum B and inophyllum P, few other dipyranocoumarins viz. inophyllum A, inophyllum C, inophyllum D and calophyllolide isolated from *C. inophyllum* have been reported to exhibit cancer chemopreventive activity against Epstein-Barr Virus early antigen activation induced by 12-*O*-tetradecanoylphorbol-13-acetate in Raji cells (Itoigawa et al., 2001), while inophyllum C and calophyllolide were also shown to have antimicrobial and cytotoxic activity (Yimdjo et al., 2004).

Chemistry of *C. inophyllum* has been extensively studied and several compounds belonging to different classes have been isolated. Xanthones like caloxanthones A, B and D from root bark and caloxanthone E from root heartwood (Munekazu et al., 1994), jacareubin, 1,7-dihydroxyxanthone, 1,5,6-trihydroxyxanthone, 1,6-dihydroxy-5-methoxyxanthones (buchanaxanthone), 6-desoxyjacareubin from heartwood were isolated (Faik and Locksley, 1971). Caloxanthones A, B, macluraxanthone and 1,5-dihydroxyxanthone were reported to have

Abbreviations: WPM, woody Plant Medium; IBA, indole-3-butyric acid; BAP, 6-benzyl amino purine; NAA, α-naphthalene acetic acid; PVP, poly vinyl pyrrolidone; HPLC, high performance liquid chromatography; NNRTIs, non-nucleoside reverse transcriptase inhibitors; R_T, retention time

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cytotoxic and antimicrobial activities (Yimdjo et al., 2004), while antioxidant, radical scavenging activities of caloxanthone and macluraxanthone were also tested (Haraguchi et al., 2006). Among the triterpenes reported from *C. inophyllum* are friedelin, friedelan-3-β-ol (Kumar et al., 1976). Properties like antiseptics, astringents, expectorants, diuretics and purgatives of ethanolic extracts of fresh leaves of *C. inophyllum* were attributed to the presence of pair of new epimers named as inophynone, isoinophynone along with some known constituents like friedelin, canophyllol and canophyllic acid (Muhammad et al., 1999). However, dipyranocoumarins are the most important group of bioactive molecules isolated from *C. inophyllum*. Eleven compounds of inophyllum class were isolated from *C. inophyllum* and were described together with the SAR (structure–activity relationship) of these novel anti-HIV compounds (Patil et al., 1993).

Plant *C. lanigerum* from which potential NNRTI calanolide was isolated has not been reported so far from India. As an alternative to calanolides A and B, inophyllums especially B and P can be used in antiretroviral therapies. Moreover there are no reports on *in vitro* production of anti-HIV dipyranocoumarins in callus cultures. *C. inophyllum,* popularly known as Indian Laurel, grows along The Western Ghats of India and was also reported from Malaysia, Australia, Fiji, and North America. As no attempts have been made to study the pattern of expression of bioactive constituents from cultured cells or tissues of *C. inophyllum*, in this paper we report for the first time the pattern of expression of dipyranocoumarins including anti-HIV-1 inophyllum B and P along with inophyllum C, D, A and calophyllolide in callus cultures raised from seed, leaf and nodal/internodal sectors of *in vitro* grown seedlings as explants on WPM (Woody Plant Medium, Lloyd and Mc.Cown, 1980) basal medium supplemented with indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA), picloram (4-amino-3,5,6-trichloropicolinic acid) and 6-benzylaminopurine (BAP) in different combinations and concentrations. For the quantitative analysis of dipyranocoumarins in callus cultures, extraction protocol and HPLC method capable of separating all six dipyranocoumarins under study in a single isocratic mode has been optimized. The effect of different hormone combinations and concentrations on callus induction from various explants and the pattern of expression of dipyranocoumarins in these callus cultures have also been discussed.

2. Materials and methods

2.1. Materials

For induction of callus cultures, mature fruits of *C. inophyllum* were collected from Harne village (17°49′63″N, 73°05′65″E; 2 m altitude) near Dapoli, Dist-Ratnagiri, Maharashtra, India in the months of May–July. For surface sterilization of explants, liquid detergent solution Labolene and surface sterilant $HgCl_2$ from Qualigens Fine Chemical Division (Mumbai, India) were used. Fungicide Benomyl was procured from E.I.D. Parry Ltd. (Chennai, India) while sucrose and agar–agar were purchased from Hi Media Laboratories Ltd. (Mumbai, India). Laboratory grade chloroform and acetone were purchased from Merck Ltd. (Mumbai, India), while HPLC grade petroleum ether (60–80 °C) was purchased from Spectrochem Pvt. Ltd. (Mumbai, India) and HPLC grade ethyl acetate was obtained from Qualigens Fine Chemical Division, (Mumbai, India). Phytohormones IBA, NAA, Picloram, BAP and polyvinylpyrrolidone insoluble were procured from Sigma–Aldrich Chemicals (St. Louis, USA). For HPLC analysis, standard samples of inophyllum A, B, C, D, P and calophyllolide were isolated from the seed kernel of *C. inophyllum* by using different chromatographic techniques and identified by detailed spectroscopic studies (UV, IR, H NMR, 13C NMR and EIMS) at Division of Organic Chemistry Technology, National Chemical Laboratory, Pune, India.

2.2. Induction of callus cultures

Mature, air dried fruits of *C. inophyllum* were decoated to take out the seeds from its hard shell. Seeds were washed thoroughly and sequentially first with liquid detergent solution Labolene, followed by antiseptic Savlon (10%, v/v). Thereafter, seeds were pretreated with PVP (insoluble; 0.5%, w/v) for 1/2 h and fungicide Benomyl (0.5%, w/v) for 1/2 h. After fungicide treatment and thorough washing with sterile distilled water for 4–5 times, seeds were surfaced sterilized with 70% ethanol (v/v) followed by $HgCl_2$ (0.1%, w/v) in laminar air flow chamber. Surfaced sterilized whole seeds were inoculated on hormones free, agar solidified WPM basal medium with 2% sucrose in larger culture vessels to raise sterile seedlings. For the callus induction from seeds, prior to inoculation, mature, surface sterilized seeds were cut into four to six pieces to get 3 cm × 3 cm size initial explants. Pieces of leaves and nodal/internodal sectors of 2–3 months old sterile seedlings were used for induction of callus cultures. Leaves were cut into 3 cm × 3 cm pieces and were injured with sterile blade, while, green stems of *in vitro* grown seedlings were cut into circular slices or discs before placing onto a media in Petri dishes. All three types of explants were inoculated on agar solidified (0.7%, w/v) WPM basal medium with 2% sucrose and incorporated with IBA (1.0–4.0 mg l^{-1}), IBA + BAP (1.0–4.0 mg l^{-1} + 1.0 mg l^{-1}), NAA (2.0–8.0 mg l^{-1}), NAA + BAP (2.0–8.0 mg l^{-1} + 2.0 mg l^{-1}) and Picloram + BAP (2.0–8.0 mg l^{-1} + 2.0 mg l^{-1}). All the cultures were incubated at 25 ± 2 °C under cool white fluorescent light (16/8 photoperiod, 35 μmol m^{-2} s^{-1}; Philips, India). Cultures were maintained by sub culturing and transferring after every 45 days on their respective fresh medium containing same hormone combinations and concentrations.

2.3. Extraction of callus masses and sample preparation for HPLC analysis

Two-month old callus masses induced from seed, leaf and nodal/internodal explants were used for extraction and HPLC analysis. One gram (fw) of callus masses were crushed and suspended in acetone (4 ml g $callus^{-1}$) for 6 h. Yellowish, brown colored acetone extracts were filtered off and callus masses were

resuspended in fresh acetone for four more times. From combined acetone extracts, acetone was evaporated under reduced pressure in rotary evaporator to yield yellowish oily extracts. These acetone extracts were then partitioned in a mixture of chloroform and water (1:1). Chloroform soluble portions were separated and evaporated to dryness under reduced pressure and were re-dissolved in 1 ml mobile phase (25% ethyl acetate in petroleum ether, v/v). For HPLC analyses, 15 μl of these extracts were injected.

2.4. HPLC analysis

The HPLC analyses were done in isocratic mode using Perkin-Elmer's series 200 HPLC system equipped with a quaternary gradient pump, an autosampler and a diode array detector using Water's (Milford, MA, USA) μPorasil (3.9 mm ID × 300 mm L; 10 μm) stainless-steel HPLC column. Ethyl acetate and petroleum ether (25:75) was used as mobile phase in a run time of 20 min with flow rate of 1 ml min^{-1} and chromatograms were recorded at 245 nm. The injection volume was 15 μl for each of 1 mg ml^{-1} solution of standard samples of inophyllum B, P, C, A, D and calophyllolide in mobile phase. Peak areas in sample chromatograms of seed, nodal/internodal and leaf callus extracts were compared with the reference chromatograms of standards to evaluate the differences in the expression of dipyranocoumarins. TotalChrom Navigator software was used to process the chromatographic data. External standard method was used for quantification and two way ANOVA was performed on software AgroBase 99.

3. Results and discussions

Among the different basal media like M.S (Murashige and Skoogs, 1962), Gamborgs B5 (1968), White's medium (1939), WPM (Woody Plant Medium, Lloyd and Mc.Cown, 1980) used initially to test the response of explants, WPM medium was found to be most suitable for callus induction and seed germination. For callus induction the media were supplemented with various combinations and concentrations of IBA, NAA, and Picloram and BAP. Initiation of callus induction from seed explants was observed after 45 days of incubation, while in case of nodal/internodal explants, initiation was after 30 days. Callus masses induced from seed explants were initially white, friable and irregular in appearance and turned dark brown colored

Table 1
Influence of different hormone combinations and concentrations on % induction of callus cultures from seed, nodal/internodal and leaf explants of *Calophyllum inophyllum* L.

Medium	Auxins (mg l^{-1})			Cytokinin (mg l^{-1})	Explants		
	IBA	NAA	Picloram	BAP	Seed	Nodal/internodal	Leaf
A1	1.0	–	–	–	87.25	80.83	32.13
A2	2.0	–	–	–	90.49	80.23	43.20
A3	3.0	–	–	–	88.25	73.66	29.20
A4	4.0	–	–	–	79.38	87.50	33.00
A5	1.0	–	–	1.0	79.32	15.35	48.80
A6	2.0	–	–	1.0	80.99	20.87	39.10
A7	3.0	–	–	1.0	80.10	21.66	51.00
A8	4.0	–	–	1.0	96.01	34.00	55.20
B1	–	2.0	–	–	85.73	69.86	54.10
B2	–	4.0	–	–	87.73	73.20	54.80
B3	–	6.0	–	–	79.27	61.68	56.06
B4	–	8.0	–	–	78.47	67.86	62.59
B5	–	2.0	–	2.0	88.66	66.40	66.96
B6	–	4.0	–	2.0	87.08	73.20	54.83
B7	–	6.0	–	2.0	86.39	67.73	28.03
B8	–	8.0	–	2.0	82.00	69.74	26.43
C1	–	–	2.0	2.0	N R	75.63	78.72
C2	–	–	4.0	2.0	N R	78.50	62.47
C3	–	–	6.0	2.0	N R	86.38	86.66
C4	–	–	8.0	2.0	N R	84.33	67.05

Med.	Exp.	Med. × Exp.
S.E.		
2.45 a	11.85 a	3.22 a
3.34 b	3.74 b	3.13 b
±C.D.		
NS a	11.13 a	44.55 a
NS b	NS b	NS b
NS a	a, **	a, **
NS b	NS a	NS b

Each value represents the mean ± S.D. of three replicates. Statistical analysis was performed separately for A1–B8 and C1–C4 medium. a: values for A1–B8; b: values for C1–C8. **: significant at 1% level; *: significant at 5% level; NS: non-significant at 1% and 5% level. NR: no result.

on subsequent subculturing and transferring, whereas, calluses induced from nodal/internodal and leaf explants were dark brown, nodular, compact and remained same throughout their sub culturing and transferring.

Maximum response for callus induction was 96.01% for seed explants, 87.50% for nodal/internodal explants, and 86.66% for leaf explants on WPM medium supplemented with IBA 4.0 mg l^{-1} + BAP 1.0 mg l^{-1}, IBA 4.0 mg l^{-1}, and picloram 6.0 mg l^{-1} + BAP 2.0 mg l^{-1}, respectively (Table 1). Supplementation of IBA in the range of 1.0–4.0 mg l^{-1} alone and in combination with BAP 1.0 mg l^{-1}, induced calluses in the ranges of 79.32–96.01% from seed explants; 15.35–87.50% from nodal/internodal explants and 29.20–55.20% from leaf explants. Addition of Picloram in the range of 2.0–4.0 mg l^{-1} along with BAP 2.0 mg l^{-1} could not induce calluses from seed explants (Table 1).

Maximum 88.66% seed explants, and 66.96% leaf explants formed calluses on supplementation of NAA 2.0 mg l^{-1} along with BAP 2.0 mg l^{-1}, while highest of 73.20% nodal/internodal explants formed calluses on supplementation of NAA 4.0 mg l^{-1} alone or along with BAP 2.0 mg l^{-1}. Upon supplementation of NAA in the range of 2.0–8.0 mg l^{-1} alone and in combination with BAP 2.0 mg l^{-1}, callus inductions from seed, nodal/internodal and leaf explants were recorded in the ranges of 78.47–88.66%, 61.68–73.20%, and 26.43–66.96%, respectively. Almost same highest callus induction responses of 86.38% for nodal/internodal explants and 86.66% for leaf explants were achieved when supplemented with picloram 6.0 mg l^{-1} along with BAP 2.0 mg l^{-1}. Variation in picloram concentrations in the range of 2.0–8.0 mg l^{-1} keeping BAP 2.0 mg l^{-1} constant could not give significantly different callus induction response. Significantly different callus induction responses were recorded for all three explants and their interactions with the different media combinations were significantly different (Table 1).

Sequential extraction of callus samples with acetone and mixture of chloroform and water ensured the maximum extractions of dipyranocoumarins under study; while HPLC method developed with mixture of petroleum ether and ethyl acetate as a mobile phase and μPorasil column for quantitative analysis ensured the maximum separation of dipyranocoumarins (Figs. 7–9). Upon loading 15 μl of each of 1 mg ml^{-1} solution of standard samples of dipyranocoumarins, inophyllum B, R_T 9.17 min (Fig. 1); inophyllum P, R_T 7.35 min (Fig. 2); calophyllolide, R_T 5.00 min (Fig. 3); inophyllum C, R_T 8.06 min (Fig. 4); inophyllum A, R_T 13.30 min (Fig. 5) and inophyllum D, R_T 8.93 min (Fig. 6) were eluted.

For the expression of dipyranocoumarins including anti-HIV-1 inophyllum B and P in callus cultures, calluses induced from seed explants on medium range A1–A6 were best suited. Among A1–A6, media, A2 was most suitable combination, since highest of inophyllum B (40.59 mg 100 g $callus^{-1}$), calophyllolide (45.23 mg 100 g $callus^{-1}$), inophyllum C (142.13 mg 100 g $callus^{-1}$), and inophyllum D (44.72 mg 100 g $callus^{-1}$) were recorded from calluses induced from seed explants on A2 medium (Tables 2 and 3). Highest inophyllum P (141 mg 100 g $callus^{-1}$) and inophyllum A (73.73 mg 100 g $callus^{-1}$) from

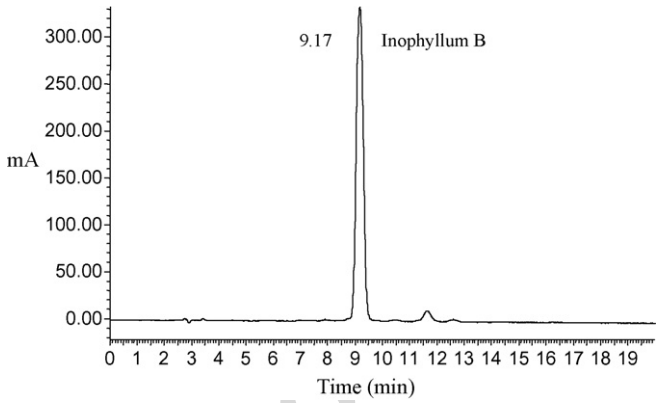

Fig. 1. HPLC chromatogram of inophyllum B (RT 9.17).

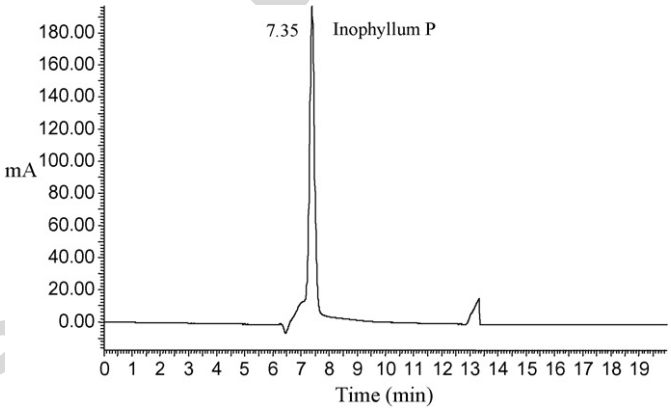

Fig. 2. HPLC chromatogram of inophyllum P (RT 7.35).

seed calluses were recorded on A6 and A1 medium, respectively. Compared to calluses induced from seed explants, calluses induced from nodal/internodal explants expressed much lower quantities of dipyranocoumarins. Although lower in quantities, similar to that of seed calluses, pattern of higher expression from nodal/internodal calluses was also recorded on A1–A6 medium. In nodal/internodal calluses, maximum inophyllum B (13.52 mg 100 g $callus^{-1}$) on A5, inophyllum P (20.43 mg 100 g $callus^{-1}$) on A4, calophyllolide (32.04 mg 100 g $callus^{-1}$) on A1, inophyllum C (8.73 mg 100 g $callus^{-1}$) on A5, inophyllum

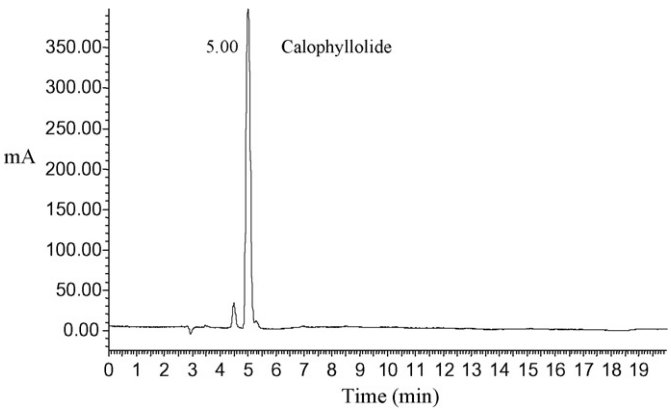

Fig. 3. HPLC chromatogram of calophyllolide (RT 5.00).

Table 2
Quantitative analysis of dipyranocoumarins in callus cultures induced from seed, nodal/internodal and leaf explants of *C. inophyllum* L. (dipyranocoumarin content in mg per 100 g FW of callus mass)

Medium	Inophyllum B			Inophyllum P			Calophyllolide		
	Seed	Nodal/internodal	Leaf	Seed	Nodal/internodal	Leaf	Seed	Nodal/internodal	Leaf
A1	17.855 ± 3.09	4.180 ± 0.056	2.211 ± 0.51	0.297 ± 0.04	5.513 ± 0.92	3.262 ± 0.42	24.809 ± 1.71	32.049 ± 2.99	1.328 ± 0.89
A2	40.596 ± 4.96	8.147 ± 1.01	19.237 ± 1.04	45.836 ± 3.31	10.409 ± 1.78	1.459 ± 0.36	45.237 ± 3.04	19.576 ± 1.90	0.247 ± 0.02
A3	14.953 ± 1.61	1.420 ± 0.22	3.831 ± 0.29	78.149 ± 13.2	9.099 ± 0.78	0.090 ± 0.01	35.363 ± 1.94	8.308 ± 0.78	16.125 ± 1.01
A4	32.037 ± 1.82	1.167 ± 0.88	0.660 ± 0.05	4.991 ± 0.49	20.432 ± 2.44	15.861 ± 5.32	39.675 ± 1.69	4.894 ± 0.37	1.189 ± 0.12
A5	16.484 ± 2.57	13.527 ± 1.28	0.881 ± 0.06	81.858 ± 13.6	2.655 ± 0.22	3.432 ± 0.50	24.906 ± 2.35	24.037 ± 10.67	2.890 ± 0.29
A6	7.234 ± 1.21	3.321 ± 0.32	0.333 ± 0.02	141.350 ± 16.0	5.360 ± 2.35	148.943 ± 23.49	29.409 ± 8.18	5.308 ± 1.89	3.747 ± 0.28
A7	0.388 ± 0.02	0.365 ± 0.04	0.044 ± 0.004	31.618 ± 5.35	1.012 ± 0.03	0.638 ± 0.05	16.028 ± 6.18	3.720 ± 0.26	1.843 ± 0.23
A8	0.402 ± 0.04	0.247 ± 0.03	0.382 ± 0.05	26.675 ± 5.59	1.817 ± 0.15	0.274 ± 0.03	6.719 ± 1.79	3.431 ± 0.37	2.068 ± 0.33
B1	7.314 ± 1.99	0.022 ± 0.009	0.219 ± 0.009	0.363 ± 0.05	0.065 ± 0.012	0.042 ± 0.004	22.450 ± 2.80	1.526 ± 0.82	4.874 ± 0.33
B2	14.806 ± 3.47	0.013 ± 0.001	0.043 ± 0.01	0.009 ± 0.001	0.016 ± 0.008	0.031 ± 0.009	15.102 ± 1.47	1.711 ± 0.73	1.927 ± 0.66
B3	5.884 ± 0.06	0.047 ± 0.002	0.175 ± 0.09	0.037 ± 0.005	0.056 ± 0.02	0.088 ± 0.03	15.881 ± 5.20	0.818 ± 0.05	0.029 ± 0.009
B4	21.676 ± 2.13	0.304 ± 0.03	0.268 ± 0.02	29.143 ± 5.03	0.060 ± 0.03	0.320 ± 0.15	9.596 ± 1.75	0.150 ± 0.05	0.201 ± 0.05
B5	3.170 ± 0.33	0.079 ± 0.009	0.020 ± 0.009	0.017 ± 0.006	0.126 ± 0.04	0.002 ± 0.0007	11.576 ± 1.33	0.030 ± 0.001	1.187 ± 0.09
B6	0.020 ± 0.01	0.043 ± 0.007	0.0467 ± 0.008	0.104 ± 0.01	0.065 ± 0.01	0.966 ± 0.28	27.660 ± 3.65	1.191 ± 0.11	1.256 ± 0.50
B7	5.158 ± 0.49	0.322 ± 0.02	0.224 ± 0.09	0.036 ± 0.002	0.320 ± 0.02	0.589 ± 0.03	45.196 ± 9.99	0.039 ± 0.01	1.246 ± 0.16
B8	0.147 ± 0.04	0.352 ± 0.01	0.063 ± 0.02	0.979 ± 0.07	0.443 ± 0.04	0.525 ± 0.05	35.069 ± 11.80	0.126 ± 0.01	0.583 ± 0.16
C1	–	0.037 ± 0.002	0.126 ± 0.06	–	11.334 ± 1.72	2.633 ± 0.43	–	4.800 ± 1.25	2.555 ± 1.83
C2	–	0.520 ± 0.04	0.662 ± 0.37	–	3.631 ± 0.51	13.956 ± 1.69	–	8.330 ± 1.01	2.870 ± 1.00
C3	–	0.764 ± 0.11	7.612 ± 1.10	–	0.482 ± 0.13	3.092 ± 1.62	–	8.235 ± 4.43	4.100 ± 0.50
C4	–	0.583 ± 0.04	0.523 ± 0.04	–	2.836 ± 1.68	1.078 ± 0.63	–	8.420 ± 0.71	2.932 ± 1.42
Med.	Exp.	Med. × Exp.		Med.	Exp.	Med. × Exp.	Med.	Exp.	Med. × Exp.
S.E.									
0.00148 a	0.0032 a	0.00128 a		0.0061 a	0.007 a	0.047 a	0.0018 a	0.0083 a	0.0026 a
0.00095 b	0.00087 b	0.00089 b		0.0017 b	0.00030 b	0.0017 b	0.00054 b	0.0021 b	0.00092 b
±C.D.									
0.0135 a	0.0058 a	NS a		0.048 a	NS a	NS a	0.014 a	a 0.006 a	0.025 a
N S b	NS b	NS b		NS b	NS b	NS b	NS b	NS b	NS b
a, **	a, **	NS a		a, *	NS a	NS a	a, **	a, **	a, **
NS b	NS b	NS b		NS b	NS b	NS b	NS b	NS b	NS b

Each value represents the mean ± S.D. of three replicates. Statistical analysis was performed separately for A1–B8 and C1–C4 medium. a: values for A1–B8; b: values for C1–C4. **: significant at 1% level; *: significant at 5% level; NS: non-significant at 1% and 5% level.

Table 3
Quantitative analysis of dipyranocoumarins in callus cultures induced from seed, nodal/internodal and leaf explants of *C. inophyllum* L. (dipyranocoumarin content in mg per 100 g FW of callus mass)

Medium	Inophyllum C			Inophyllum A			Inophyllum D		
	Seed	Nodal/internodal	Leaf	Seed	Nodal/internodal	Leaf	Seed	Nodal/internodal	Leaf
A1	41.063 ± 6.09	0.190 ± 0.05	0.182 ± 0.01	73.732 ± 12.76	0.058 ± 0.01	0.335 ± 0.04	3.540 ± 0.41	3.330 ± 0.43	0.141 ± 0.04
A2	142.132 ± 7.2	2.174 ± 1.95	0.050 ± 0.03	59.848 ± 0.12	0.305 ± 0.03	0.052 ± 0.007	44.727 ± 4.71	0.497 ± 0.07	0.026 ± 0.006
A3	52.751 ± 4.63	1.483 ± 1.29	0.050 ± 0.0009	22.621 ± 3.85	1.703 ± 0.27	0.186 ± 0.01	0.102 ± 0.011	0.495 ± 0.08	0.008 ± 0.004
A4	17.665 ± 2.14	0.798 ± 0.12	0.112 ± 0.01	0.257 ± 0.14	0.183 ± 0.01	0.344 ± 0.02	4.069 ± 0.63	0.649 ± 0.05	0.085 ± 0.005
A5	46.098 ± 3.81	8.730 ± 0.96	0.011 ± 0.001	4.762 ± 0.71	0.077 ± 0.02	0.596 ± 0.04	0.175 ± 0.01	0.061 ± 0.01	0.051 ± 0.03
A6	84.340 ± 7.53	0.357 ± 0.05	1.228 ± 0.19	46.414 ± 8.02	0.074 ± 0.05	0.485 ± 0.07	0.561 ± 0.05	0.313 ± 0.02	0.136 ± 0.01
A7	20.450 ± 2.62	0.529 ± 0.07	0.214 ± 0.017	29.510 ± 5.08	0.103 ± 0.01	0.030 ± 0.008	5.833 ± 0.99	0.064 ± 0.005	0.052 ± 0.005
A8	10.989 ± .25	1.317 ± 0.14	0.188 ± 0.02	14.764 ± 1.75	0.181 ± 0.03	0.017 ± 0.004	0.009 ± 0.001	0.012 ± 0.009	0.263 ± 0.09
B1	12.512 ± 1.27	0.011 ± 0.002	1.833 ± 0.20	9.648 ± 0.22	0.391 ± 0.05	6.638 ± 2.02	0.032 ± 0.002	0.014 ± 0.003	0.029 ± 0.007
B2	17.827 ± 5.44	0.070 ± 0.03	0.096 ± 0.001	8.233 ± 1.00	0.112 ± 0.009	2.118 ± 0.30	0.012 ± 0.004	0.015 ± 0.009	0.005 ± 0.0009
B3	11.529 ± 8.82	0.062 ± 0.05	0.708 ± 0.06	13.740 ± 3.47	0.058 ± 0.02	0.069 ± 0.001	0.003 ± 0.0002	0.014 ± 0.005	0.013 ± 0.003
B4	23.667 ± 2.64	0.024 ± 0.004	0.654 ± 0.05	21.187 ± 5.14	0.543 ± 0.04	0.743 ± 0.04	0.065 ± 0.001	0.008 ± 0.003	0.592 ± 0.26
B5	2.017 ± 0.50	0.079 ± 0.01	0.083 ± 0.004	0.040 ± 0.01	0.618 ± 0.05	0.172 ± 0.07	0.002 ± 0.001	0.019 ± 0.009	0.019 ± 0.0005
B6	17.117 ± 0.78	0.605 ± 0.05	0.0047 ± 0.001	8.277 ± 1.14	0.392 ± 0.02	0.029 ± 0.002	0.004 ± 0.0003	0.007 ± 0.002	0.016 ± 0.005
B7	0.035 ± 0.004	0.096 ± 0.07	0.192 ± 0.09	0.026 ± 0.005	0.267 ± 0.12	0.193 ± 0.05	0.011 ± 0.001	0.067 ± 0.02	0.113 ± 0.02
B8	8.233 ± 0.66	0.050 ± 0.02	0.017 ± 0.001	0.029 ± 0.009	0.092 ± 0.007	0.043 ± 0.02	0.002 ± 0.0007	0.074 ± 0.008	0.009 ± 0.003
C1	–	0.117 ± 0.01	0.834 ± 0.10	–	0.048 ± 0.02	0.064 ± 0.008	–	0.256 ± 0.02	0.548 ± 0.008
C2	–	0.101 ± 0.05	1.006 ± 0.43	–	0.663 ± 0.10	0.476 ± 0.098	–	0.482 ± 0.06	0.061 ± 0.005
C3	–	0.342 ± 0.04	0.971 ± 0.42	–	0.062 ± 0.03	0.534 ± 0.19	–	0.553 ± 0.08	0.409 ± 0.14
C4	–	0.218 ± 0.02	0.701 ± 0.07	–	0.185 ± 0.01	0.974 ± 0.08	–	0.152 ± 0.01	0.903 ± 0.12

Med.	Exp.	Med. × Exp.	Med.	Exp.	Med. × Exp.	Med.	Exp.	Med. × Exp.
S.E.								
0.0031 a	0.010 a	0.0036 a	0.0022 a	0.0078 a	0.0027 a	0.00093 a	0.0011 a	0.00093 a
0.00044 b	0.00034 b	0.00013 b	0.00060 b	0.00067 b	0.00056 b	0.00020 b	0.00023 b	0.00019 b
±C.D.								
NS a	0.016 a	NS a	NS a	0.017 a	NS a	0.0086 a	0.0028 a	0.015 a
NS b	0.00063 b	NS b	0.0010 b	0.00071 b	0.0014 b	NS b	NS b	NS b
NS a	a, **	NS a	NS a	a, **	NS a	a, **	a, **	a, **
NS b	b, **	NS b	b, **	b, **	b, **	NS b	NS b	NS b

Each value represents the mean ± S.D. of three replicates. Statistical analysis was performed separately for A1–B8 and C1–C4 medium. a: values for A1–B8; b: values for C1–C4.**: significant at 1% level; *: significant at 5% level; NS: non-significant at 1% and 5% level.

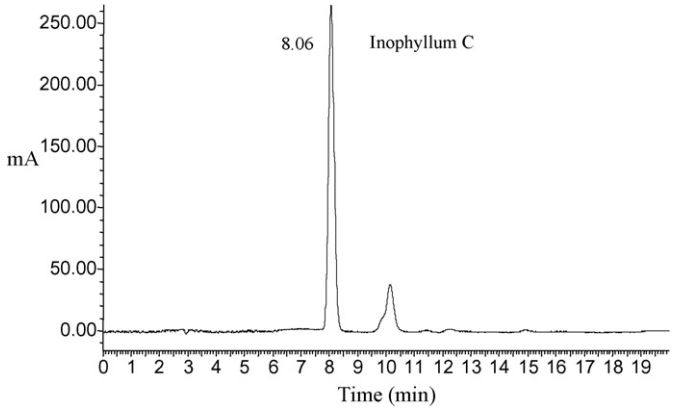

Fig. 4. HPLC chromatogram of inophyllum C (RT 8.06).

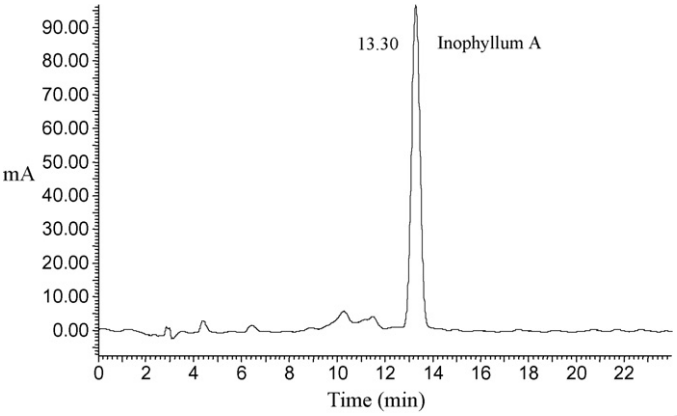

Fig. 5. HPLC chromatogram of inophyllum A (RT 13.30).

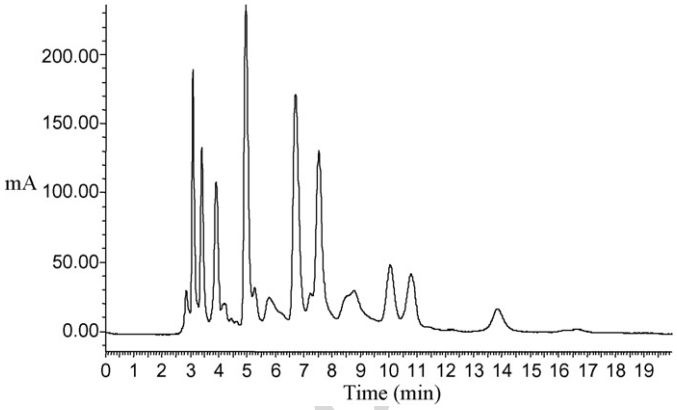

Fig. 7. HPLC profile of callus extracts of seed explant.

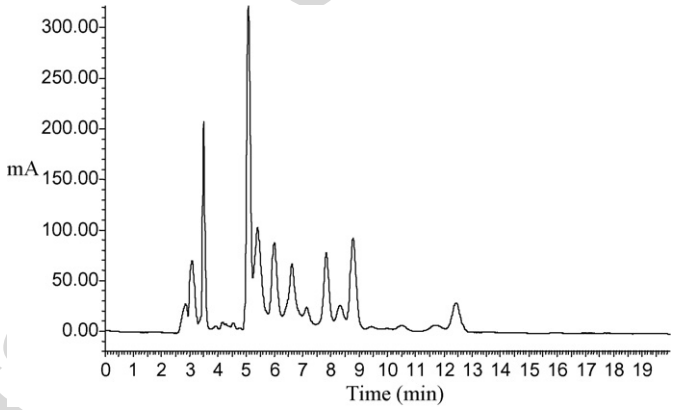

Fig. 8. HPLC profile of callus extracts of nodal/internodal explant.

A (1.70 mg 100 g callus^{-1}) on A3 and inophyllum D (3.3314 mg 100 g callus^{-1}) on A1 were recorded. In nodal/internodal calluses, highest expressions of inophyllum B, and C were recorded on medium combination A5 whereas highest of calophyllolide and inophyllum D were recorded on medium combination A1. Similarly, in leaf calluses, highests of inophyllum B, inophyllum P, and calophyllolide (19.23, 148.93, and 16.12 mg 100 g callus^{-1}, respectively) were estimated on A2, A6, and A3, respectively, whereas highest of inophyllum C (1.83 mg 100 g callus^{-1}), inophyllum A (6.63 mg 100 g callus^{-1}) were estimated on B1, and inophyllum D (0.59 mg 100 g callus^{-1}) on B4 (Table 3). Overall medium combination B1–B8 is less significant or useful for the expression of dipyranocoumarins in callus cultures induced from seed and nodal/internodal explants whereas only maximum inophyllum C and A on B1 and inophyllum D on B4 in leaf calluses were recorded (Table 3).

Medium combinations C1–C4 were not as good as A1–A8, since in any of these combinations, expression

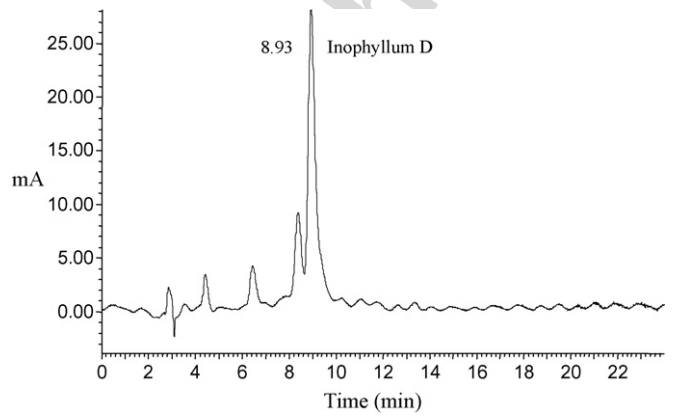

Fig. 6. HPLC chromatogram of inophyllum D (RT 8.93).

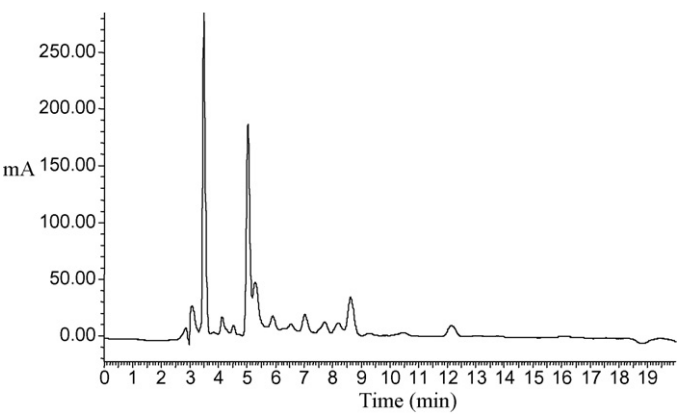

Fig. 9. HPLC profile of callus extracts of leaf explant.

of inophyllum B was not more than in the ranges of 0.03–0.76 mg/100 g callus from nodal/internodal explants, 0.12–7.61 mg 100 g callus^{-1} from leaf explant, inophyllum P, 0.48–11.33 mg 100 g callus^{-1} from nodal/internodal explant, 1.07–13.95 mg 100 g callus^{-1} from leaf explant, calophyllolide, 4.80–8.42 mg 100 g callus^{-1} from nodal/internodal explant, 2.55–4.10 mg 100 g callus^{-1} from leaf explant, inophyllum C, 0.10–0.34 mg 100 g callus^{-1} from nodal/internodal explant, 0.70–1.00 mg 100 g callus^{-1} from leaf explant, inophyllum A, 0.04–0.66 mg 100 g callus^{-1} from nodal/internodal explants, 0.06–0.97 mg 100 g callus^{-1} from leaf explants and inophyllum D, 0.15–0.55 mg 100 g callus^{-1} from nodal/internodal, 0.06–0.90 mg 100 g callus^{-1} from leaf explant. In our chemo diversity study, data collected for contents of inophyllum B and P in leaf and seed tissues collected from different locations along the Western Ghats of India revealed that only 1.55 mg inophyllum B and 0.87 mg inophyllum P 100 g seed tissue^{-1} were estimated, whereas only 0.01 mg inophyllum B and 0.006 mg inophyllum P 100 g leaf tissue^{-1} were estimated (complete data not shown).

Statistically for the production of calophyllolide and inophyllum D, medium combinations A1–B8, all explants and their interaction are significantly different, while only explants were significantly different for inophyllum C and A expression; whereas only explants and media for inophyllum B and only media for inophyllum P were significantly different. Thus establishing callus cultures can be promising method to obtain inophyllums. Moreover contents of inophyllums can be enhanced in callus cultures by medium manipulation, cell suspension culture and elicitation with different biotic as well as abiotic elicitors.

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