DEVELOPMENT OF PLANT TISSUE CULTURE OF *PLUMBAGO ROSEA, LINN.* FOR ENHANCED PRODUCTION OF SECONDARY METABOLITES

THESIS SUBMITTED TO THE
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BY

JAYA S.

BIOCHEMICAL PROCESSING AND WASTEWATER TECHNOLOGY
REGIONAL RESEARCH LABORATORY (CSIR)
THIRUVANANTHAPURAM - 695 019
INDIA

AUGUST, 1999

To my Parents

☎ OFF 7171852
RES 7171471

Dr. S.V. RAMA KRISHNA
Group Leader
Biochemical & Environmental Engineering

भारतीय रासायनिक प्रौद्योगिकी संस्थान
तारनाका, हैदराबाद 500 007 भारत
Indian Institute of Chemical Technology
Tarnaka, Hyderabad-500 007 India

Certificate

This is to certify that the thesis entitled "***Development of plant tissue culture of Plumbago rosea Linn. for enhanced production of secondary metabolites***" submitted by **Miss. Jaya.S** in fulfillment of the requirement for the **Ph.D**. degree in Biotechnology of the Cochin University of Science and Technology is an authentic record of research carried out by her under my supervision and guidance and that no part of thereof has been presented before for any other degree.

S V Ramakrishna

Dr. S.V Ramakrishna
(Supervising Guide)

Telex 0425-7061 IICT IN Fax 040-7173757 • 7173387 • 7173229 Telegram RESEARCH, Hyderabad.

DECLARATION

The thesis entitled "**Development of Plant Tissue Culture of *Plumbago rosea*, *Linn.* for enhanced Production of Secondary Metabolites**" is the result of investigations carried out by me at the Biochemical Processing and Wastewater Treatment Division of Regional Research Laboratory, Trivandrum, under the supervision of Dr. S. V Ramakrishna, Scientist, Indian Institute of Chemical Technology, Hyderabad and the same has not been submitted elsewhere for a degree.

Jaya. S

JAYA. S

Place: Trivandrum

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JAYA. S

CONTENTS

ABBREVIATIONS

MS- Murashige and Skoog

SH- Schenk and Hildebrandt

B_5 – Gamborg

2,4-D- 2,4-Dichlorophenoxy acetic acid

NAA-Naphthalene 3-acetic acid

IAA- Indole 3-acetic acid

BAP-Benzyl aminopurine

DMSO – Dimethyl sulfoxide

CTAB- Cetyl trimethyl amino bromide

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CHAPTER I

INTRODUCTION

Plants have been the subjects of Man's curiosity and purpose since time immemorial. Higher plants are recognized as unique sources of a great variety of commercially important compounds such as pharmaceuticals, agrochemicals, flavours, dyes and fragrances.[1,2] Many of these commercially valuable products come under the general category of 'secondary metabolite' [3-5] Secondary metabolites have an eco-chemical function; they often represent as chemical adapters to environmental stress or serve as chemical defense of the plant against microorganisms, insect predators or even other plants.[6,7] Usually, in plant secondary metabolites are accumulated in smaller quantities than primary metabolites. Alkaloids, phenyl propanoids and terpenoids are the predominant class of compounds found in plants.

1.1 **Diversity of chemicals of economic importance**

Natural substances are employed either directly or indirectly by a large number of industries and plant derived compounds figure prominently in several of these. Examples of commercially useful plant secondary metabolites are nicotine, pyrethrins and rotenone, which are used as pesticides, and certain steroids and alkaloids, which are used in drug manufacturing by the pharmaceutical industry [8] The steroids and alkaloids include steroid diosgenins, the anticancer monoterpenoid indole alkaloids (vincristine and vinblastine), belladonna alkaloids (for

examples, atropine, hyoscyamine, and scopolamine), opium alkaloids (codeine, morphine and papaverine), antimalarial cinchonine alkaloids (quinine,quinidine,and cinchonine), reserpine and Digitalis glycosides.[9-13]

Economically and commercially important plant-derived enzymes include papain and chymopapain of *Carica papaya*, Bromelain (protein digester and milk-clotting enzymes) from pineapple and malt extract from Barley which contains amylolytic enzymes.

Plant-derived products are the raw materials of many more industries such as food, beverages, rubber, cosmetics etc. Tannin extracted from the bark and wood of trees are a main ingredient of dyes, ink and medicines.

Secondary plant metabolites often valued at several dollars to several thousand dollars per pound. For example, purified opium alkaloids are valued in the range of 400 to 600 dollars per pound. While rare volatile oils such as rose oil are often valued at over 2,000 to 3,000 per Kg. The anticancer, Catharanthus alkaloids have a wholesale value of about 5,000 dollars per gram.[1]

1.1.1 Medicinal Plants

In recent years, there has been a resurgence of interest in the plant kingdom as a source of many drugs.[14-17] Extensive search for novel biologically active compounds from plants resulted in the discovery of many 'wonderful' drugs against devastating diseases such as AIDS, tumors, heart diseases etc. The alkaloid taxol, isolated from several species of Taxus viz. *T brevifolia, Nutt., T buccata Barren Var barroni, T cuspida Sieb and Zucc.*, and *T. wallichiana.*, is extremely effective against breast and ovarian cancer.[18] Campothecin, isolated from

Camptotheca accuminata DC.[19] and related alkaloids widely used in China for the treatment of liver cancer, leukemia and gastric cancer has been subjected to clinical trials in United states and the People's Republic of China. Commercially another most important antineoplastic drugs are the vincristine and vinblastine; isolated from the leaves of *Catharanthus roseus (L). G. Don.*[20] The successful clinical application of these compounds in the treatment of leukemia and Hodgkin's disease has accorded special importance to this group of alkaloids and their related derivatives.

Several plant-derived compounds exhibiting significant anti-HIV activity are isolated from higher plants used in traditional medicine.[21] Gossypol, a dimeric sesquiterpene aldehyde obtained from the cottonseed plant, Gossypium and other species reported to have good activity against HIV-infected blood cells. Glycyrrhizin, a leanane-type triterpenes diglucuronide extracted from *Glycyrrhiza glabra*, has been known for over a decade to inhibit the growth and cytopathology of several DNA and RNA viruses, and to irreversibly inactivate herpes simplex virus particles. Medicinal preparations containing Trichosanthin, a protein obtained from the roots of chinese medicinal plant, *Trichosanthes kirilowii*, have been used for centuries to induce abortions and to treat trophoblastic diseases.[22] Trichosanthin has recently entered clinical trials at centre in United States and is being used on AIDS patients with advanced HIV disease.[23]

There are over 250,000 higher plants on the earth, and it is estimated that as many as 90% of these plants have not been subjected to any form of scientific, phytochemical and/or biological screening. It can

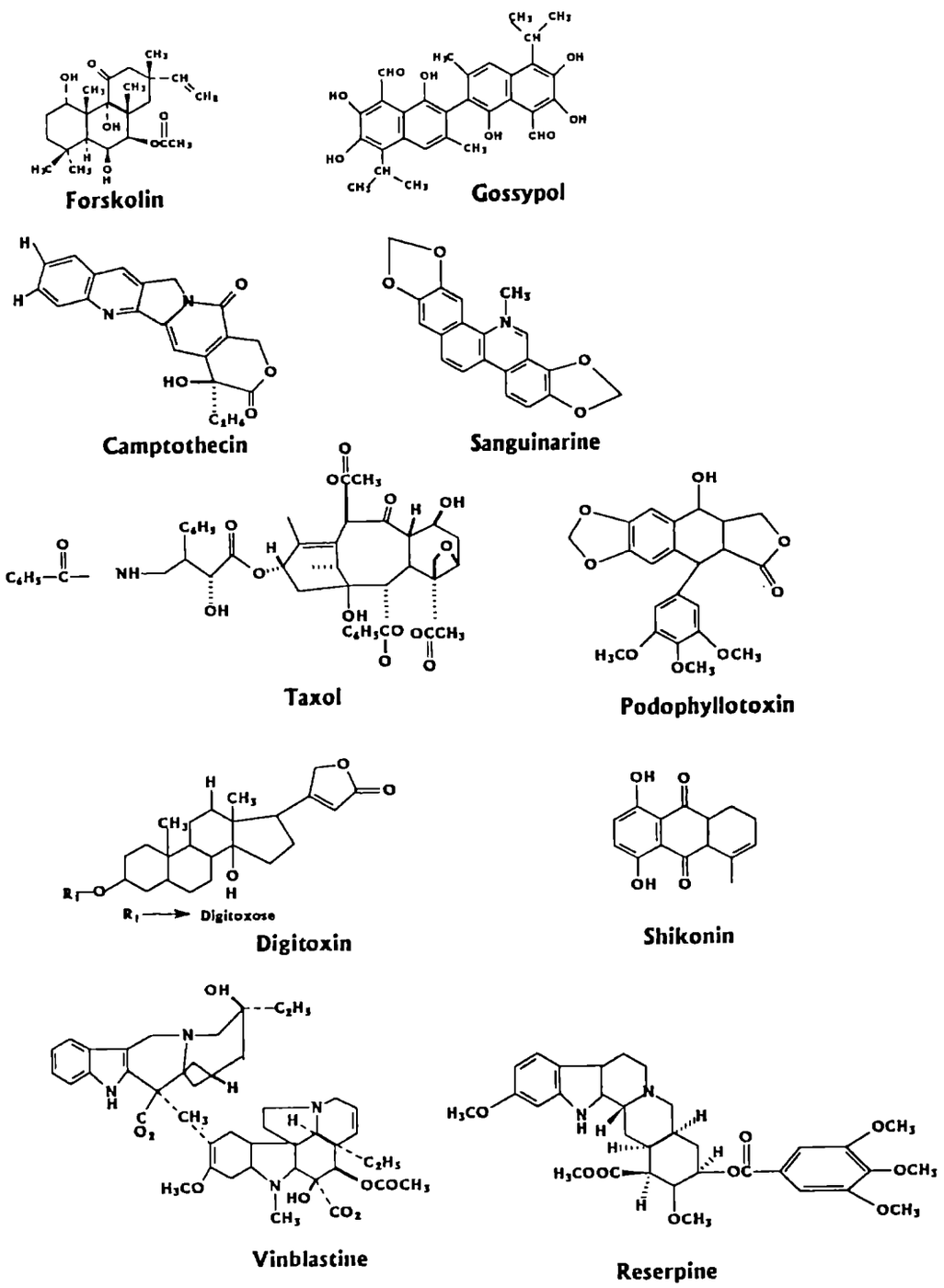


Fig.1.1. Chemical structure of few commercially valuable secondary metabolites from plants

be assumed that as more diverse plants are examined, novel agents will continue to make an important contribution in drug discovery efforts towards the cure of diseases.

1.1.2 Agrochemical

Extracts of plants have been used as insecticides since the time of ancient civilization. Presently, more than 2,000 plant species have reported to have insecticide properties. Because of low cost of production, rapid degradability and less toxicity to mammals, bio-pesticides are using widely than chemical pesticides.

The most economically important of the natural plant compounds used in commercial insect control are the pyrethrins, obtained from the flower of *Chrysanthemum cinerariaefolium*.[9] Rotenone and rotenoids have long been used as insecticides and piscicides. Roots of many plants of *Leguminosae* (*Derris, Lonchocarpus, and Tephrosia* sps.) are the sources of these compounds. The insecticidal use of nicotine alkaloids of tobacco dates back to 1,600's. One of the most important plants used as source of agrochemical is the neem tree (*Azadirachta indica*) which contains an array of terpenoids having insecticide properties.[24]

Several more compounds from higher plants including terpenoids phenylpropanoids, , quinones, coumarins, flavanoids, tannins, cyanogenic glycosides, essential oils and flavors are potent agrochemicals, may provide models for new synthetic insecticides. Other notable plant species that contain secondary metabolites with potential use as commercial insecticide include *Acorus calamus, Ocimum basilicum, Artemisia annua, Ageratum houstonianum, Tagetus patula,* etc. Natural

plant chemicals will undoubtedly play a significant role in the future of pest control.

1.2 **Plant tissue culture as alternative source of secondary metabolites**

Plants are recognized as the most remarkable chemical factories on earth. However, the environmental and geopolitical instabilities make it impossible to acquire plant-derived products constantly. Alternatively, the structural complexities of bioactive plant metabolites have precluded in many cases the use of organic synthesis for their commercial production. As the demand for natural products grows, the increasing cost and scarcity of high quality phytochemicals have made application of plant biotechnology for the plant-based chemicals.

Plant cell culture refers to cellular mass that is derived from an explant of plant tissue grown under aseptic conditions on a solid or liquid medium. Plant tissue culture originated at the turn of this century with the Haberlandt's work on *Tredescantia*.[25] The achievement of White in the year 1934[26] by developing tomato root cultures on defined nutrient medium, is a milestone in plant tissue culture technology. Even though, the utility of plant tissue culture was visualized in the early 1940's but J. B. Routin and L. G. Nickel[27] took the first patent on plant secondary metabolite in 1956 (US Patent 2,747,334, 1956). The concept was based on the well-developed use of microorganism for fermentation. Many other investigators have further developed the art of plant tissue culture and the real break through in this area came in the early 70's when some of the basic aspects of cellular metabolism and differentiation were studied in great detail. Tabata et al. (1976)[28] reported that in *Lithospermum erythrorrhizon* cell cultures, metabolite synthesis occurred

in highly specialized cells. Fujita et al. (1981)[29] selected a highly productive cell line of *L. erythrorrhizon* and developed a two-stage culture system. In 1983, Mitsui petrochemical marketed the first commercial plant cell line producing shikonin from *L. erythrorrhizon* cell suspension cultures. The cell line developed at Mitsui can accumulate over 20% of shikonin on a dry weight basis, which is 10 times higher than the field grown plants. At present, there are a number of reports on cell cultures producing concentrations of chemical products that are equal to or greater than those found in the whole plant. In some cases, yields of 18 % to 25 % of the cell dry weight have been reported, for example, rosmarinicacid, anthraquinones and shikonin.[30,31] More over, with *in vitro* culture, it may be possible to produce new active compounds or to convert low value substances to high value compounds by 'biotransformation' [32,33]

The *in vitro* approach for production of useful plant chemicals has several attractive features. Plant cells have relatively shear stress resistance and can grow on simple defined culture media. Production of plant chemicals in bioreactors can be more efficient space–wise than many acres of field-grown plants. Plant cells can be subjected to selective pressures that favours the growth of highly productive cell lines and results could be optimized in less time than in conventional breeding and selection.

1.2.1 Products from plant tissue culture

Plant cells grown in culture produce a wide range of molecular species. Plant cell suspension cultures are regarded as a potentially suitable system for producing phytochemicals at an industrial level.

Alkaloids are the predominant group of compounds reported from plant cell cultures. The subject has been dominated by the studies on the indole alkaloid of *C. roseus*, some of which are important antitumor agents. Cell cultures of *Catharanthus roseus* is an important source of monomeric and dimeric indole alkaloids viz. ajmalicine, serpentine, vincristine and vinblastine. Zenk et al. (1977)[34] reported indole alkaloids, serpentine and ajmalicine from cell suspension cultures of *C. roseus.* Hirata et al. (1987)[35] and Scott et al. (1980)[36] reported two more indole alkaloids, vindoline and catharanthine from multiple shoot culture and cell suspension culture of *C. roseus*, respectively. The most important dimeric indole alkaloid, vinblastine was obtained from callus culture with differentiated roots (Miura et al. 1987).[37] Asada et al. (1989)[38] enhanced the production of ajmalicine in *C. roseus* by immobilization, elicitation and *in situ* product recovery. Jung et al. (1992)[39] improved the catharanthine productivity in hairy root culture of *C. roseus* by manipulating medium composition. Indole alkaloids such as vomilenine have been isolatd in good yields (1.6% D. M.) from *Rauwolfia serpentina* and other speices.[40]

Callus, root and adventitious shoot cultures of *Cephaelis ipecacuanha* have been shown to be able to produce two main pharmacologically active alkaloids, cephaeline and emetine, which are used as expectorant, emetic and amoebacide.[41] Cephaeline was the main product in the adventitious roots as well as in callus cultures though intact plant contained almost the same levels of emetine and cephaeline in its roots.[42] It indicates the different capability of alkaloid biosynthesis between *in vitro* culture and the intact plant.

Cinchona ledgeriana cell culture produce a large variety of quinoline alkaloids, of which quinine and quinidine are commercially most important. Crown gall cultures developed after infection with *Agrobacterium tumefaciens* accumulated 6.7mg/l of chinchona alkaloids.[43] Hairy root culture of Cinchona Species produced a maximum of 50μg alkaloid per gram fresh weight after 45 days.[44] *In vitro* cultures of *Digitalis lanata* is a main source of cardiac glycosides. However, the commercially more valuable form of digitoxin and β-methyl digitoxin were obtained from differentiated cultures.[45] The anticancer compound, podophyllotoxin was obtained from callus and cell suspension cultures of *Podophyllum hexandrum*.[46,47] The production of podophyllotoxin derivatives has been reported from root and cell suspension cultures of *Linum flavum*.[48,49]

Liverworts are known to produce a large variety of bioactive terpenes. The *in vitro* culture of *Fossombronia pusilla* accumulated diterpenedialdehyde perrottetianal A, B and 8-hydroxyperrottetianal A. santonin for the first time as constituents of a bryophyte.[50]

Napthoquinones are extracted from the cell cultures of a no. of higher plants viz. *Ebenaceae, Droseraceae, Plumbaginaceae, Balsaminae*, *Juglandaceae* and *Boraginaceae*.[51] Fujita et al. (1981) reported the production of shikonin, a naphthoquinone from the cell suspension culture of *Lithospermum erythrorrhizon*.[29]

Table 1.1 Recent examples of production of useful secondary metabolites by *in vitro* cultures

PRODUCT	PLANT	CULTURE	REFERENCES
Indole alkaloids			
Vinblastine	*Catharanthus roseus*	shoot	[35]
Catharanthine	*C. roseus*	hairy root	[39]
Ajmalicine	*C. roseus*	hairy root	[39]
Pleiocarpamine	*Amsonia elliptica*	hairy root	[52]
Quinoline	*Cinchona ledgeriana*	Crown gall	[43]
Alkaloid	*C. pubescens*	hairy root	[44]
Berberine	*Thalictrum minus*	cell suspension	[53]
	Coptis japonica	immobilizd cell	[54]
Hyoscyamine	*Hyoscyamus muticus*	hairy root	[55]
Emetine, cephaeline	*Cephaelis ipecacuanha*	callus and root	[42]
Linalool	*Mentha citrata*	hairy root	[56]
Limonene	*Pelargonium fragrans*	callus with shoot	[57]
Chrysanthemic-acid	*Chrysanthemum cinerariaefolium*	callus	[58]
Amarogentin	*Swertia japonica*	hairy root	[59]
Artemisinin	*Artemisia annua*	hairy root	[60]
Santonin	*Fossombronia pusilla*	callus	[50]
Hernandulcin	*Lippia dulcis*	shoot, hairy root	[61,62]

Sclareol	*Salvia sclarea*	cell suspension	[63]
Digitoxin	*Digitalis lanata*	cell suspension,	[64,65]
		hairy root	[45]
Camptothecin	*Camptotheca accuminata*	callus	[19]
Podophyllotoxin	*Podophyllum hexandrum*		[46,47]
	Linum flavum		[48,49]
Shikonin	*Lithospermum erythrorrhizon*		[29]

1.2.2 **Novel compounds from cell culture**

Often, plant cell culture exhibits variability named 'somaclonal variation' by Larkin and scorcroft,[66] which may sometimes leads to the production of novel compounds. An example of unexpected metabolism in plant tissue culture was the isolation of three isomeric paniculides from shoot cultures of *Andrographis paniculata.*[67] Very unusual metabolites can accumulate in plant tissue culture e.g. the novel disulfide from *Ricinus communis*,[68] the stress protein osmotin and its polypeptide precursors from Nicotiana tabacum[69] and polypeptides that chelate heavy metals from *Lycopersicon esculentum.*[70] Feeding of tryptophan to *Cinchona sps.* led to the formation of alkaloids with the β-carboline skeleton that had not been reported in this genus.[71]

1.3 **Biotransformation**

Plant cell cultures possess considerable biochemical ability to transform foreign substrates administered exogenously.[32,33] Biotransformation by plant cell cultures have been served as an important

tool for the structural modification of several molecules. Furuya *et al.* reported the transformation of tabersonine into lochnericine and its hydroxyl compounds by *Catharanthus roseus* culture.[72] Vanek *et al.* reported the biotransformation of 2-(4-methoxy benzyl)-1-cyclo-hexanone to its glycoside by the cell culture of *Dioscorea deltoidea.*[73] Other examples are, the conversion of reticuline into codeinone by *Papaver somniferum* cultures[74], geraniol, carvone and nerol into 5-β-hydroxy neodihydrocarceol and 5-α-hydroxy carvone by *C. roseus* cultures[75], digitoxigenin into digoxin by *Panax ginseng* cells[76] and paclitaxel into 2-debenzoyltaxol and baccatin III by cell suspension cultures of *Eucalyptus perriniana.*[77] Two other biotransformations of interest are the degradation of linolenic acid to hexanals by cell culture of apple tree[78] and the spectacular capacity of shoot culture of *Rauwolfia serpentina*[79] that had been optimized for glucoalkaloid production to glycosylate hydroquinone to form arbutin, which was formed at a level (24g L^{-1} in seven days) claimed to be the highest for a natural product produced by cell culture. The most impressive biotransformation was reported from *Digitalis lanata* cultures in which digitoxin and β-methyl digoxin were converted into more useful 12-hydroxy derivatives digoxin and β-methyl digoxin and the system has been scaled up to 200 L stage fermenter.[80] The more commonly observed chemical reactions in plant cell culture are—oxidation, reduction, hydrolysis, esterification, glycosylation, epoxidation, isomerization, etc.

1.4 Major strategies for the improvement of secondary metabolite production

1.4.1 Hairy root culture

The hairy root disease affecting a wide range of dicotyledonous species is caused by a gram negative soil bacterium *Agrobacterium rhizogenes*.[81,82] When this phytopathogen infects a plant one or both of two lengths of transfer-DNA (TL and TR) from the bacterial Ri (root-inducing) plasmid is firmly inserted into the genome of the infected plant cell.[83] The integrated genes appear to alter the production and sensitivity of auxins, resulting in the proliferation of fast-growing adventitious roots at the host wound site (Gelvin, 1990).[84] The T-DNA of the RI-plasmid also carries genes for unusual aminoacid called 'opines'[85], the production of which serves to confirm that transformation has taken place. Fast growing hairy roots can grow in medium devoid of any plant hormones. The potential of hairy root cultures for the production of plant chemicals have been confirmed by many research groups and a wide variety of compounds have been reported, spanning well over hundred species of higher plants.[86]

1.4.2 Elicitation

In recent years, there have been a number of reports on the elicitation of secondary metabolites in cell cultures by certain molecules called 'elicitors' [87,88] It may be either from biotic or abiotic sources. Biotic elicitor molecules include the cell wall components of fungi, bacteria or even plant materials, glyco-proteins, glucan polymers, low molecular weight organic acids and enzymes. Darvill and Albersheim[89] have reported a number of elicitors from various sources. Majerus *et al.*

(1985) cited that elicitor molecule act as signal molecules which interact with cell surface receptors and phosphoinositide derived messenger molecules stimulating a variety of cellular responses.[90] A number of plant cell cultures have been elicited to produce the secondary metabolites much higher than unelicited cells (see Table 4.1).

1.4.3 Immobilized culture

The technique of immobilization has attempted in plant tissue culture system and has received much attention as a product enhancing strategy (Brodelius, 1984, 1988).[91,92] It has the advantage over the use of fermenter vessels with free cells in suspension that large quantity of cells can be handled in packed columns of cells in a protected environment (Witcher et al. 1983; Asada and Shuler, 1989).[93,94] The feed back inhibition of toxins or products is minimized in immobilized conditions. Use of columns of immobilized cells may permit easy recycling of medium. Some important examples of products accumulated in immobilized plant system has been given in Table 5.1.

1.4.4.Genetic engineering

Recently there has been much speculation on the application of the techniques of genetic engineering to enhance the production of secondary metabolites in plant tissue culture. [95,96] Hashimoto et al. (1993)[97] introduced the gene for hyoscyamine 6β-hydroxylase from *Hyoscyamus niger* into the cells of *Atropa belladonna* resulted in the level of production of scopolamine five-fold greater than non-transformed cells. The bacterial endotoxin gene from *Bacillus thuringiensis* have been cloned and expressed in tobacco plants (Adang, *et al.* 1986)[98] *Agrobacterium* mediated genetic transformation has made tremendous

change in the plant's basic ability to produce secondary metabolites.[99-102] Yet, a third general technique is the introduction of antisense genes [103,104], which can block the expression of unwanted mRNA.[105]

1.5. Studies on basic metabolism

Plant tissue culture has provided source tissue for numerous studies on the mechanism of secondary product biosynthesis using either direct feeding techniques, cell-free extracts or purified enzymes. Ochoa-Alejo and Gomez-peralta, (1993) studied the functionality of the capsaicinoid biosynthetic pathway in callus culture of *Capsicum annum*.[106] Nair *et al.* (1994) characterized the tetra hydroberberine oxidase, the enzyme involved in the final step of berberine synthesis, from *Coscinium fenestratum* cell cultures.[107] The response of plants to microbial attack has been studied by feeding elicitor molecules to cell cultures of host plants.[108-110] Stoadler and Zenk, (1993) have purified two enzymes, N-methyl transferase and a cytochrome P-450 dependent oxidase involved in intermolecular coupling to dimeric berberine skeleton.[111]

Use of antibody techniques and c-DNA probes for hyoscyamine 6β-hydroxylase which catalyses the first step in the conversion of hyoscyamine into scopolamine-proved that the enzyme was expressed in the pericyle of the roots.[112]

1.6.Objectives of present work

India has a long tradition for the use of plant and plant products in the field of medicine. Versatile medicinal use of *Plumbago Sps*, have been known in India since the time of Charaka and Susruta.[113-115] The genus Plumbago includes about 10 species[116], namely, *P. auriculata, P. coerulea, P. europea, P pearsonii, P pulchella, P. rosea, P, scandans, P.*

tristis, P wissi, and P zeylanica. P auriculata, P rosea and *P zeylanica* are found growing in India. Among them *P zeylanica* is grown widely in tropical and subtropical regions of Asia, Australia and Africa and hence, more extensive studies on this species have been carried out. Morphologically, *P rosea* is distinctive with red petals and *P. auriculata* with blue petals, while all others have white petals. Several compounds have been isolated from these plant.

Recently, Dinda *et. al.* have published a review article on the naphthoquinone and its analogs isolated from the leaf, root and aerial parts of different Plumbago Sps.[117] (Table 1.2). Of which, Plumbagin (2-methyl, 5-hydroxy 1,4-naphthoquinone) is the major compound with a number of biological activities. Earlier studies on *P. rosea* Linn. have reported the isolation and identification of several naphthoquinones, flavanoids, anthocyanidins, tannins and B-sitosterol from various plant parts such as leaf, root and aerial parts. In addition to plumbagin, several important naphthoquinones such as droserone, isoshinanolone, 2-methyl naphthazarin, elliptinone, chitanone, chitranone, 3,3'-biplumbagin, plumbazeylanone etc. were isolated from theses plants. (Dinda *et al.* 1997).

The properties of plumbagin have been summarized in the Table 1.3.

Table 1.2 Occurrence of naphthoquinones and other compounds isolated from *Plumbago Sps.*

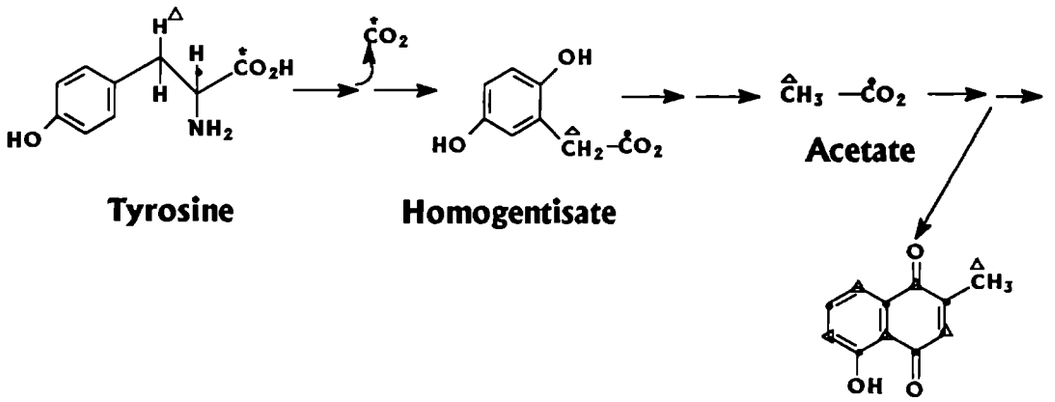
Compound	Species	References
Plumbagin	*P. rosea*	Gunaherath *et al.* 1983[123]
	P zeylanica	
Epi-isoshinanolone	*P scandens*	Bhattacharya & Carvalho, 1986[124]
Zeylanone	*P zeylanica*	Sankaram *et al.*, 1979[125]
Isozeylanone	*P zeylanica*	Dinda *et al.*, 1995[126]
1,2 (3)-Tetrahydro-3,3'-Biplumbagin Droserone	*P zeylanica*	Gunaherath *et al.*, 1983[123]
Dihydronaphtho-quinone	*P zeylanica*	Dinda and Saha, 1986[127]
Dihydroflavonol	*P rosea*	Dinda *et al.*, 1994[128]
Roseanone	*P rosea*	Dinda *et al.*, 1995[129]
Elliptinone	*P zeylanica*	Sankaram *et al.*, 1976[130]
3,3'-Biplumbagin	*P zeylanica*	Sindhu *et al.*, 1971[131]
Chitranone	*P zeylanica*	Gunaherath *et al.*, 1988[132]
Chitanone	*P zeylanica*	Dinda *et al.*, 1989[133]
Plumbazeylanone	*P zeylanica*	Antarkar *et al.*, 1980[134]

Table 1.3 Medicinal and other properties of Plumbagin

Uses	References
Antimicrobial	Lima *et al.*, 1968[135];Durga *et al.*, 1990[136]; Fuji *et al.*1992[137]; Lakshmi *et al.*,1987[138]; Ray and Majumdar, 1976[139]
Antileishmanial	Craft et al., 1985[140]
Anti cancer	Krishnaswamy& Purushothaman,1980[141]; Chandrasekharan *et al.*, 1982[142]; Krehar, *et al.*, 1990[143]
Antifertility, Abortifacient	Bhargava, 1984[144]; Bhargava & Dixit, 1985[145]
Anticoagulant	Santhakumari, *et al.*, 1978[146]
Bronchial infection	Denoel, 1949[147]
Antifeedant	Antarkar, *et al.*, 1980[134] Gujar, 1990[148]
Insecticidal	Kubo *et al.*, 1983[149]
Molluscicidal	Marston *et al.*, 1984[150]; Marston & Hostettmann, 1985[151]
Adult sterilant effect	Joshi *et al.*, 1988[152]
Larval mortality effect	Ghosh *et al* 1994[153]
Cardiotonin	Itoigawa *et al.*, 1991[154]
Antinematodes	Fetterer and Fleming, 1991[155]
Antimutagenic	Farr *et al.*, 1985[156];Durga, *et al.*, 1992[157]

1.6.1 Biogenesis of plumbagin

Regarding the biogenesis of naphthoquinones, it was suggested that in higher plants, four separate pathways, shikimate, homogentisate, the polyacetate-malonate and the mevalonate pathways might form these metabolites. But, feeding experiments with acetate-1-^{14}C-2-^{14}C and malonate-2-^{14}C in young shoots of *P europea* suggested that plumbagin and its analogs be produced in higher plants via the polyacetate-malonate pathway. (Scheme A)[118, 119]



SCHEME 1. BIOSYNTHETIC PATHWAY FOR THE INCORPORATION OF TYROSINE INTO PLUMBAGIN

1.6.2 In vitro studies on *P. rosea*

Apart from pharmacological and phytochemical aspects, studies on cell culture of *P rosea* were very less. Plant regeneration either directly or from callus cultures have been reported by Harikrishnan & Molly

(1996) [120] and Kumar and Bhavanandan, (1987).[121,122] Due to totipotency of plant cells and the high frequency of somaclonal variation in cell cultures, selection of highly potent cell lines from callus culture is possible. Also, the cultivation medium and the appropriate ratio of growth regulators are the determining factors of biomass accumulation and secondary metabolite productivity, nutrient media and combination and concentration of growth regulators were standardized. Root being a rich source of plumbagin, hairy root culture of *P. rosea* was also tried to develop from this plant species. More over, the secondary metabolite production in *in vitro* cultures of *P. rosea* has been not much studied earlier. In the present investigations, we were attempted to increase the production of plumbagin using strategies viz. optimization of media components, elicitations, immobilization, etc.

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CHAPTER II

TISSUE CULTURE STUDIES ON *PLUMBAGO ROSEA, LINN.*

INTRODUCTION

Plumbago rosea, Linn., a perennial shrub of *Plumbaginaceae*, is cultivated throughout India for medicinal and ornamental purposes.(Table 1.3) The plant is characterized by bright red flowers in auxillary or terminal racemes. The root of this plant is used as an important indigenous drug in the traditional medicinal systems. Several compounds have been isolated from this plant (Table 1.2). Plumbagin is a natural naphthoquinone showing a broad range of medicinal properties. It has been reported to have anticancer and antibiotic properties. Plumbagin was also reported to be an effective chitin-synthetase inhibitor and, therefore it can be utilized for insect killing in agriculture.

Presently, the most exploited source of plumbagin is the roots of Plumbago species (*P europea*, *P rosea* and *P. zeylanica*). However, these plants grow quite slowly and the roots suitable for extraction take years to grow.[1] Frequent harvesting of natural population has resulted in the extinction of many of the important plants. Therefore, the search for alternative and more effective source of plumbagin was necessary. One of the possibilities was a synthetic approach, [2-4] but each method published so far has been ineffective from the commercial point of view. Due to low productivity or complicated cultivation, extraction of plumbagin from other sources also proved uneconomical. Tissue culture technique has widely

PLATE 1 : INFLORESCENCE OF *P. ROSEA*

accepted as a tool for the multiplication of medicinal plants.[5,6] It has also been recognized as a source of natural products.[7,8]

The root of *P rosea* is the accepted source of drug in Kerala and is locally known by the vernacular name 'chettikotuveli' The plant is propagated vegetatively by offsets or small cuttings and has never been known to bear fruits.[9] Tissue culture studies have been attempted earlier for micro-propagation of *P rosea* cultures and were raised from young leaf and stem on MS medium supplemented with various concentration of growth hormones.[10-12] Plant regeneration directly from leaves or internodes (Harikrishnan & Molly , 1996)[12] or from callus cultures have been reported (Kumar and Bhavanandan, 1987)[10] Due to totipotency of plant cells and the high frequency of somaclonal variation in cell culture, selection of highly potent cell lines from callus culture is possible.[13] As the cultivation medium and the appropriate ratio of growth regulators are the determining factors of growth and secondary metabolite production, nutrient media and combination and concentration of growth regulators for *P rosea* cultures were standardized. Root being the rich source of plumbagin, hairy root culture of *P rosea* was also tried to develop from this plant species. Growth kinetics and plumbagin productivity of callus culture and hairy root culture were also studied as these factors vary greatly among plant cells grown *in vitro*.

2.2 Materials and methods

Source of plant:

The source plant was procured from Tropical Botanical Garden and Research Institute, Palode, Trivandrum. The plant was grown in a green house at Regional Research Laboratory, Trivandrum, and used for various experimental purposes.

Selection of explants and disinfection:

Young leaves and stems from plant grown for 6 months in green house were selected as explants. The excised plant parts were thoroughly washed with a mild detergent in tap water and then rinsed with distilled water. The remaining procedures were done in a sterilized laminar flow chamber. The explants were first immersed in 70 per cent ethyl alcohol for 30 seconds and then washed with sterilized double distilled water for 3 times for ten minutes each. The explants were then rinsed with 0.01 per cent mercuric chloride for 5 minutes with enough solution. Explants were thoroughly rinsed with sterilized double distilled water for three times so as to remove the mercuric chloride completely. They were cut into pieces of approximately 10mm length or diameter. Each piece was again rinsed with sterile double distilled water and the adhering water content was removed by sterile tissue paper. Each surface sterilized plant material was inoculated into flask containing semi-solid Murashige and Skoog medium (1962)[14], Schenk and Hildebrandt medium(1972)[15] and B_5 medium (1968)[16] under aseptic conditions. Explants were slightly pressed on the surface so that the entire surface would be in proper contact with the medium.

Preparation of culture medium:

Murashige and Skoog (1962), Schenk and Hildbrandt (1972) and Gamborg's or B_5 (1968) were the standard media used for the initiation of callus in *P rosea* cultures. The composition of each of the above-mentioned media is given in Table 2.1, 2.2 and 2.3. Constituents of the media were classified into six categories:

(1) Major Inorganic nutrients

(2) Trace elements

(3) Iron source

(4) Vitamins

(5) Organic supplements (Growth regulators)

(6) Carbon sources

Stock solutions of culture media were prepared by dissolving appropriate quantity of each medium component in double distilled water and kept in refrigerator. When needed, appropriate amount of stock solutions were mixed with required amount of growth regulators, sucrose and myoinositol and made up to the volume with double distilled water. Before autoclaving the medium pH was adjusted to 5.8 (± 0.2) by adding 0.1 N HCl or 0.1 N NaOH. For the preparation of semisolid medium 0.8% agar was added after pH determination. The culture medium was sterilized at 121 °C and one Kg/cm^2 pressure for 20 minutes in an autoclave. The containers with media were removed from the autoclave immediately after sterilization and were kept in the culture rooms.

Preparation of stock solutions of growth regulators:

Auxin:

The stock solutions (mg/l) of various auxins viz. IAA, NAA and 2,4-D were prepared separately by dissolving the appropriate quantity of auxin in 2 ml. of absolute alcohol and gradually diluted to 10 ml. with double distilled water in volumetric flasks. The stock solutions were kept under refrigeration and used within one month.

Cytokinins:

The stock solutions (mg/l) of cytokinin viz. Benzyl Adenine and Kinetin were first dissolved in 2 ml. of 0.1 N sodium hydroxide solution and then diluted with double distilled water to 10 ml in volumetric flasks.

Table 2.1 Composition and preparation of Murashige and Skoog medium

Constituents	Molarity in the medium	Concentration of stock solution (mg/litre)	Volume of stock per litre of medium (ml)	Storage of stock solution
Major Inorganic nutrients				
NH_4NO_3	2.06×10^{-2}	33,000		
KNO_3	1.88×10^{-2}	38,000		
$Ca\ Cl_2.2H_2O$	3.00×10^{-3}	8,800	50	+4 °C
$MgSO_4.7H_2O$	1.50×10^{-3}	7,400		
KH_2PO_4	1.25×10^{-3}	3,400		
Trace elements				
KI	5.00×10^{-6}	166		
H_3BO_3	1.0×10^{-4}	1,240		
$MnSO_4.4\ H_2O$	9.99×10^{-5}	4,460		
$ZNSO_4.5H_2O$	2.99×10^{-5}	1,720	5	+4 °C
$Na_2MoO_4.2\ H_2O$	1.0×10^{-6}	50		
$CuSO_4.\ 5H_2O$	1.0×10^{-7}	5		
$CoCl_2.\ 6H_2O$	1.0×10^{-7}	5		
Iron source				
$FeSO_4.\ 7\ H_2O$	1.00×10^{-4}	5,560	5	+4 °C
$Na_2EDTA.\ 2H_2O$	1.0×10^{-4}	7,460		
organic supplement	4.90×10^{-4}	20,000		
Myo-inositol	4.66×10^{-6}	100		
Nicotinic acid	2.40×10^{-6}	100		-20 °C
Pyridoxine-HCl	3.00×10^{-7}	100		
ThiamineHCl	3.00×10^{-5}	400		
Glycine	8.80×10^{-2}	–	Add as solid (30g/l)	
Carbon source				
Sucrose				

Table 2.2 Modified Schenk and Hildebrandt medium-composition and preparation

Constituent	Molarity in the medium	Concentration of stock solution (mg/litre)	Vol. of stock per litre medium (ml)	Storage of stock solution
Major inorganic constituents				
KNO_3	2.5×10^{-2}	101,000	25	
$MgSO_4.7H_20$	1.5×10^{-3}	24,640	15	
$NH_4H_2PO_4$	2.5×10^{-3}	11,500	25	+4 °C
$CaCl_2.2H_2O$	1.5×10^{-3}	14,680	15	
Trace elements				
$MnSO_4.4H_2O$	5.9×10^{-5}	1,320		
H_3BO_3	1.3×10^{-4}	500		
$ZnSO_4.7H_2O$	3.5×10^{-6}	100		
KI	6.0×10^{-6}	100	10	+4 °C
$CuSO_4.5H_2O$	8.0×10^{-7}	20		
$Na_2MoO_4.2H_2O$	4.1×10^{-7}	10		
$CoCl_2.6H_2O$	4.2×10^{-7}	10		
Iron source				
$FeSO_4.7H_2O$	5.4×10^{-5}	1500	10	+4 °C
Na_2EDTA	5.4×10^{-5}	2,000		
Organic supplement				
Thiamine HCl	1.5×10^{-5}	500		
Nicotinic acid	4.1×10^{5}	500	10	-20 °C
Pyridoxine HCl	2.4×10^{-6}	50		
Myoinositol	5.6×10^{-3}	–	Add as solid (1g/l)	
Carbon source				
Sucrose	8.8×10^{-2}	–	Add as solid (30g/l)	

Table 2.3 Gamborg's (B5) medium. Composition and preparation

Constituents	Molarity in medium	Concentration of stock solution (mg/l)	Vol. of stock per litre of medium (ml)	Storage of stock solution
Major Inorganic constituents				
KNO_3	2.5×10^{-2}	25,275	100	
$NaH_2PO_4.H_2O$	1.1×10^{-3}	3,000	50	
$Mg\ SO_4.7H_2O$	1×10^{-3}	2,465	100	+4 °C
$CaCl_2.2H_2O$	1.02×10^{-3}	1,500	100	
$(NH_4)_2SO_4$	1.014×10^{-3}	26,800	50	
Trace elements				
KI	4.5×10^{-6}	150		
H_3BO_3	4.85×10^{-5}	600		
$MnSO_4.H_2O$	5.91×10^{-5}	2,000		
$ZnSO_4.7H_2O$	6.9×10^{-6}	400	5	+4 °C
$CoCl_2.2H_2O$	1.0×10^{-7}	5		
$CuSO_4.\ 5H_2O$	1.0×10^{-7}	5		
$Na_2MoO_4.2H_2O$	1.0×10^{-6}	50		
Iron source				
$FeSO_4.7H_2O$	9.99×10^{-5}	5,560	5	+4 °C
$Na_2EDTA2H_2O$	1.0×10^{-4}	7,460		
Organic supplement				
Myoinositol	4.9×10^{-4}	20,000		
Nicotinic acid	9.32×10^{-6}	200		
Pyridoxine HCl	4.8×10^{-6}	200	5	-20 °C
Thiamine HCl	3.00×10^{-7}	2,000		
Carbon source				
Sucrose	5.87×10^{-2}	–	Add as solid 20g/l	

Different concentrations and combinations of auxin and cytokinin used for different experiments and its details were given in corresponding tables.

Measurement of growth of cultured cells:

The growth of cells in callus cultures and cell suspension cultures were estimated by determining the fresh weight and dry weight of the tissue. For fresh weight determination, the cells were separated from the culture medium and washed twice with culture medium and excess medium was removed with sterilized tissue paper. The cells were weighed in a Metler analytical balance. Cells which were used for further experiments were taken in pre-weighed flasks and then measured the weight of the flasks together with added cells. The dry weight of the cells were determined after freeze drying the tissues and then followed the same procedure as in the case of callus culture samples using Lyophilizer (HETOSIC) at –40 °C for 3 hours.

Growth of cells in suspension cultures was determined by separating the cells from the culture medium by filtration with Whatman No. 1 filter paper using Millipore filter units. Tissues were washed twice with sterilized culture medium and then followed the same procedure as described in the case of callus tissues.

Development of hairy root culture

Bacterial culture:

Agrobacterium rhizogens 15834 was used for hairy root induction. The bacterium was maintained on YEB agar medium containing 1 g/L Mannitol, o.1 g/l NaCl, 0.5 g/l Glutamine, 0.2g/l KH2PO4, 0.02g/l yeast extract and 1.5 g/l Agar. Prior to inoculation Bacteria were sub-

cultured on YEB liquid medium and cultured for 1 day in the dark on a rotary shaker at 80 rpm.

A slight incision was made at tip of the plant-lets with a sterile forceps. Bacterial broth was then applied over the wounded site. Plant-lets were kept under dark for 3 days in ½ strength MS basal medium containing 2 g/l agar. The infected plant-lets, after being rinsed with sterile distilled water for 3 times, were transferred to full strength solid MS basal medium containing 1 g/l Carbencillin (Himedia) and again kept under darkness.

After about 3 three weeks, plant-lets having hairy roots at the infected sites were sub-cultured again in MS solid basal medium containing Carbencillin 250mg/l. Before transferring to the solid medium and the plant-lets were rinsed with MS liquid medium containing the antibiotics. The bacteria were completely removed after 4 th subculturing.

The axenic hairy roots were cut off and inoculated in hormone-free MS liquid with 3% sucrose and cultured at 28°C on a rotary shaker at 80 rpm in the dark. They were sub-cultured every 14 days. The cultures were harvested in four replicates. Fresh weight was determined after completely removing the medium by using tissue paper. Hairy roots were lyophilized and then determined the dry weight.

Growth kinetics

Growth kinetics of *P rosea* was studied by calculating the kinetic parameters such as specific growth rate and generation time during the growth cycle of callus and cell suspension cultures (Stanier, *et al*).[17]

(a) Specific growth rate (μ)

Specific growth rate (μ) is an index of the rate of growth during the logarithmic phase of growth, which is calculated by the equation,

$$\mu = \frac{\log (X_t / X_o)\, 2.303}{t}$$

where 'X_o' is the initial biomass of logarithmic phase and 'X_t' is the biomass at time 't'

(b) Generation time (t_g)

Generation time is the period required for a cell to undergo division or to complete one cell cycle. It is calculated from the equation',

$$t_g = 0.693 / \mu$$

Extraction and analysis of plumbagin

Cells were separated from the culture medium by filtering under vacuum with Millipore filter unit. Cells were washed thoroughly with distilled water for twice. Fresh weight of the biomass was determined and freeze dried. After lyophilization dry weight of the biomass was determined.

Metabolites from the dried biomass was extracted with methanol (100%) in soxhlet apparatus for 3 hours (Crouch et al., 1990).[18] The

methanol fraction was separated from the plant material by filtration through Whatman No.1 filter paper. The filtrate was reduced to dryness under vacuum at temperature below 40°C, suspended in distilled water and washed twice with petroleum ether and diethyl ether. Then, extracted with chloroform thrice and evaporated to dryness under vacuum resuspended in 3ml. of 80% HPLC methanol for analysis.

Alkaloid from the medium was extracted with chloroform thrice as described above. Chloroform phase was collected and evaporated under vacuum. The dried sample was dissolved in 3ml. HPLC grade methanol and used for analysis.

Analysis of plumbagin:

The analysis of plumbagin was done according to the procedure of Lee *et al.* (1985).[19] Following solvent extraction, the crude extract dissolved in methanol was loaded onto a reversed phase column (μ-Bondapack C18, 300mm X150 mm I.D.; Shimadzu LC-A 10 AD) connected with an UV detector (SPD 10A) The detection wavelength was 254 nm. The solvent mixture of methanol and water (80:20) at pH 3.5 adjusted with triethyl amine was eluted at a flow rate of 0.8 ml/min. The quantitative analysis was carried out by comparing the peak area of the sample with authentic plumbagin (Sigma).

2.3 Results and Discussion

(a)Induction of callus

Callus cultures of *P rosea* were established by evaluating the suitability of various standard media such as Murashige and Skoog, Schenk

and Hildebrandt and Gamborg's media with the supplementation of different auxins and cytokinins. Table 2.4 represents the percentage of callus induction by various combinations of growth regulators. The data represented on the 35th day of incubation and the percentage was calculated on the basis of the number of explants induced callus formation to the total number of explants inoculated. For callus development, young leaf bits and stem internodes were used. The auxins tried were 2,4-D, NAA and IAA and cytokinins were BAP and Kinetin. The concentrations of cytokinins were used at two levels of 0.3 and 0.5 mg/l and the auxins were varied at 0.5 to 10mg/l. The auxins were individually added and further supplemented with either BA or Kinetin. (Table 2.5 and 2.6) At highest concentration of auxins, no callus induction was observed in any one of the media tried. Most of the explants were inducted to calli development when the auxin concentrations were varied between 0.75 to 2.5 mg/l. Beyond 3 mg/l the callus induction was not very significant. It was noticed that between 1 mg/l to 2.5 mg/l of NAA with 0.3 mg/l BA or K, 100% callus induction was noticed. Below 1.0 mg/l and above 1.25 mg/l of NAA, the callus induction was slightly lower (93 and 95%). At the same levels of auxins and cytokinins in SH medium and B5 medium, have resulted in lower callus induction. Among the auxins, NAA was found to be most effective than 2,4-D and IAA in all the media tried. With 2.0 mg/l NAA in B5 medium, it was observed profuse rooting along with callus formation (Table 2.7). Both BA and Kinetin have found to have same effect on callus formation. All further experiments for callus development were carried out in MS medium with 1.0 mg/l NAA. The induction of callus culture of *P rosea* is shown in Plate 2.

Table 2.4 Response of explants from *P rosea* to various standard medium and growth hormone in callus induction

Auxin # (mg/l)	BAP (mg/l)	Percent of callus induction*								
		MS			SH			B_5		
		I	2	3	1	2	3	1	2	3
0.5	0.3		60							
0.5	0.5	40							30	
0.75	0.3	63	95	30	65	80	40	20	44	43
1.00	0.3	71	100	75	63	80	48	37	57	52
1.25	0.3	74	100	80	68	84	53	33	53	50
1.5	0.3	80	93	84	73	89	60	48	63	67
2.00	0.3	76	81	60	79	86	67	41	54**	54
2.50	0.5	69	70	67	54	65	68	30	27	58
3.00	0.5	71	55		32	40	52			34
4.00	0.5	55	47				38			
5.00	0.5	43	41							
7.50	0.5	23								
10.00	0.5									

Symbols 1,2 and 3 indicate 2,4-D, NAA and IAA, respectively;

Explants used were young leaves and internodes;

One Auxin at a time was used in each medium;

* Percentage was calculated on the basis of total no. of explants induced the callus formation to the total no. of explants inoculated;

** indicates profuse rooting along with callus formation;

Data represents the mean value of 10 independent observations.

Table 2.5 Influence of auxins on growth and plumbagin production in callus cultures of *P rosea*

Auxins (mg/l)	Concn. of auxins (mg/l)	Fresh Wt. (g/culture)	Plumbagin (mg/g D. Wt.)
Basal (B)	Nil	2.135 (±0.23)	1.78 (±0.033)
B + IAA	0.5	1.131 (± 0.23)	0.189 (±0.043)
	0.75	0.960 (± 0.44)	0.180 (± 0.09)
	1.00	1.360 (± 0.37)	0.194 (±0.03)
	1.25	1.476 (±0.11)	0.203 (±0.17)
	1.5	1.541 (±0.33)	0.183 (±0.08)
B + 2,4-D	0.5	1.187 (±0.21)	0.175 (±0.03)
	0.75	1.275 (±0.33)	0.189 (±0.02)
	1.00	1.657 (±0.271)	0.193 (±0.11)
	1.25	1.942 (±0.11)	0.173 (±0.11)
	1.50	2.031 (±0.17)	0.164 (±0.09)
B + NAA	0.50	1.213 (±0.11)	0.183 (±0.03)
	0.75	1.694 (±0.16)	0.174 (±0.03)
	1.00	2.089 (±0.31)	0.219 (±0.03)
	1.25	2.170 (±0.13)	0.203 (±0.041)
	1.50	1.976(±0.27)	0.181 (±0.02)

Basal (B): MS basal medium + sucrose 3% + 0.5 g/l myoinositol
Inoculam:500 ± 30 mg fresh tissue in 40 ml. medium
Incubation period: 20 days at 25 ± 2°C under 14 hour light period
Data represents the average of 5 replicates and data in parenthesis indicate ± SD

Table 2.6 Influence of combination of auxins on growth and plumbagin production in callus cultures of *P rosea*

Sl. No.	Hormones (mg/l)			Fresh Wt. (g/culture)	Plumbagin (mg/g D. Wt.)
	1AA	2,4-D	NAA		
1		0.5	0.5	1.444(±0.11)	0.22 (±0.43)
2	0.5		0.5	2.135 (±0.25)	0.313 (±0.11)
3	0.5	0.5		1.283 (±0.25)	0.191 (±0.23)
4		0.75	0.5	2.006 (±0.33)	0.249 (±0.21)
5	0.5		0.75	3.349 (±0.27)	0.519 (±0.34)
6	0.75		0.5	3.694 (±0.21)	0.797 (±0.19)
7		1.0	0.5	2.626 (±0.15)	0.571 (±0.29)
8	1.0	0.5		2.004 (±0.21)	0.217(±0.17)
9	0.75	1.0		1.590 (±0.15)	0.213(±0.24)
10	1.0		0.5	4.215 (±0.34)	0.892(±0.35)
11	0.5		1.0	3.608 (±0.45)	0.791(±0.41)
12	1.00		0.75	4.163 (±0.28)	0.843(±0.13)
13	1.00		1.00	(2.041 (±0.18)	0.39 (±0.17)

Medium: MS basal medium + sucrose 3% + 0.5 g/l myoinositol + BAP o.3mg/l
Inoculam: 500 ± 30 mg. fresh tissue in 40 ml medium
Incubation period: 20 days at 25 ± 2°C under 14 hour light period
Data represents the average of 5 replicates in two repetitive experiments.
Numbers in parenthesis indicate ± SD

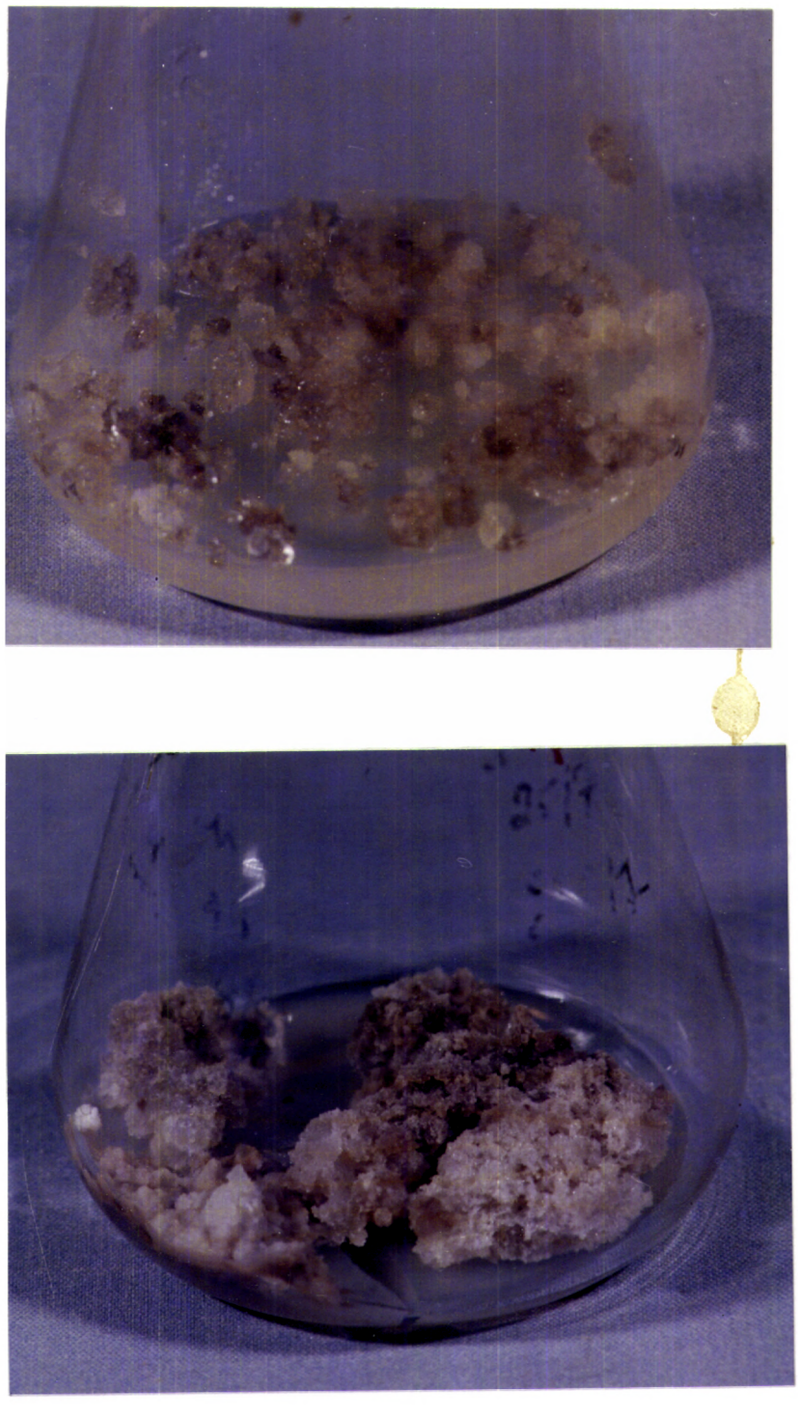


PLATE 2&3: CALLUS CULTURE OF *P. ROSEA*

(b) Optimization of media for callus growth

The proliferation of callus cultures were further optimized by varying the auxins and keeping BA in 0.3 mg/l level in all the trials. A total of 28 combinations were tried, wherein the individual auxins were varied between 0.5 to 1.5 mg/l and combination of two auxins were used in callus initiation. The fresh weight of the callus was noted on 20^{th} day of incubation and 5 replicates in two repetitive experiments were used for each set and mean values were given in Table 2.5 and Table 2.6. It can be observed that maximum weight of the callus was developed in the media containing 0.3 mg/l of BA, 0.5 mg/l of NAA and 1.0 mg/l of IAA. Slight increase in the concentration of NAA, 0.5 to 0.75 mg/l, has induced marginal reduction in the weight of the callus. From these experiments, it can be observed that both NAA and IAA are important to obtain maximum amount of callus. Plate 3 represents one-month-old callus of *P rosea* in optimized medium.

© Growth profile of *P. rosea* callus cultures

The growth profile of the callus culture of *P rosea* was studied in optimized MS medium (1 mg/l IAA, 0.5mg/l NAA and 0.3 mg/l BA). With the same concentrations of auxins and cytokinins, the growth of the callus was also observed in other two media viz. SH and B5. Fig. 2.1 and Table 2.8 represents the growth profile of callus cultures of *P rosea* in all the three media. It can be seen that among the three media tried, MS media was found to be the best to obtain maximum amount of callus. The growth parameters were calculated and found that the specific growth rate of the callus in MS, SH and B5 media was 0.0767 d^{-1} 0.0373 d^{-1} and 0.0493 d^{-1} respectively Accordingly, the doubling time of the callus was found to be 9, 18.5 and 14.0 days in MS, SH and B5 media, respectively. The specific

Table 2.7 Influence of cytokinins on growth and plumbagin production in *P rosea* callus cultures

Cytokinins	Concentration (mg/l)	Fresh Wt. (g/culture)	Plumbagin (mg/ g D. Wt.)
Basal (B)		2.135 (±0.23)	1.78 (±0.03)
B + Kinetin	0.1	1.377 (±0.13)	2.11 (±0.02)
	0.3	1.583 (±0.23)	2.38 (±0.07)
	0.5	1.572 (±0.18)	2.051(±0.01)
	0.75	1.565 (±0.24)	2.431 (±0.03)
	1.00	1.492 (±0.41)	1.983 (±0.021)
B + BAP	0.1	1.411 (±0.33)	1.893 (±0.11)
	0.3	1.571 (±0.43)	2.243 (±0.13)
	0.5	1.603 (±0.3)	2.347 (±0.08)
	0.75	1.566 (±0.17)	1.911 (±0.13)
	1.00	1.581 (±0.31)	2.113 (±0.31)

Basal (B): MS basal salts + sucrose 3 % + 0.5 g/l myoinositol
Inoculam 500 ± 30 mg fresh tissue in 40 ml medium
Incubation period: 20 days at 25 ± 2°C under 14 hour light period
Data represents the average of five replicates. Numbers in parenthesis indicate ± SD

Table 2.8 represents the growth of callus culture of *P rosea* in different standard media

No. of days	MS*	SH*	B_5*
		*(mg/culture)	
0	0.50 (±0.03)	0.500 (±0.043)	0.500 (±0.013)
4	0.513 (±1.12	0.501 (±0.01)	0.509 (±0.13)
8	0.725 (±0.09)	0.603 (±0.92)	0.573 (±0.13)
12	1.025 (±0.32)	0.625 (±0.071)	0.601 (±0.087)
16	1.825 (±0.11)	0.911 (±0.041)	0.755 (±0.093)
20	2.510 (±0.076)	1.325 (±0.011)	0.984 (±0.044)
24	2.623 (±0.23)	1.525 (±0.052)	1.202 (±0.056)
28	2.931 (±0.03)	1.779 (±0.066)	1.373 (±0.061)
32	3.193 (±0.009)	1.958 (±0.032)	1.610 (±0.012)
36	3.351 (±0.23)	2.213 (±0.037)	1.650 (±0.033)
40	3.433 (±0.067)	2.459 (±0.09)	1.706 (±0.082)
44	3.450 (±0.015)	2.587 (±0.018)	1.711 (±0.019)
48	3.440 (±0.02)	2.630 (±0.056)	1.713 (±0.04)
52	3.437 (±0.094)	2.631 (±0.046)	1.710 (±0.075)

Medium used: Basal medium + sucrose 3% + IAA 1.0 mg/l + NAA 0.5 mg/l ±BAP 0.3 mg/l

Data represents the average of 3 replicates and data in parenthesis indicate ± SD

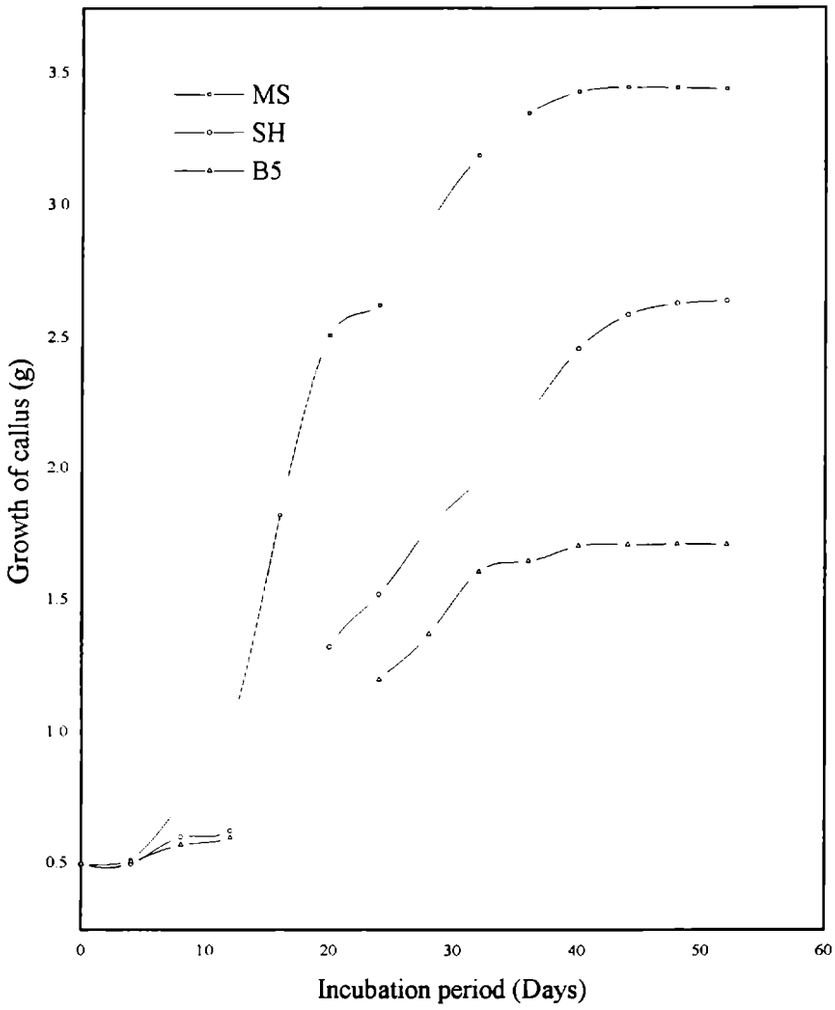


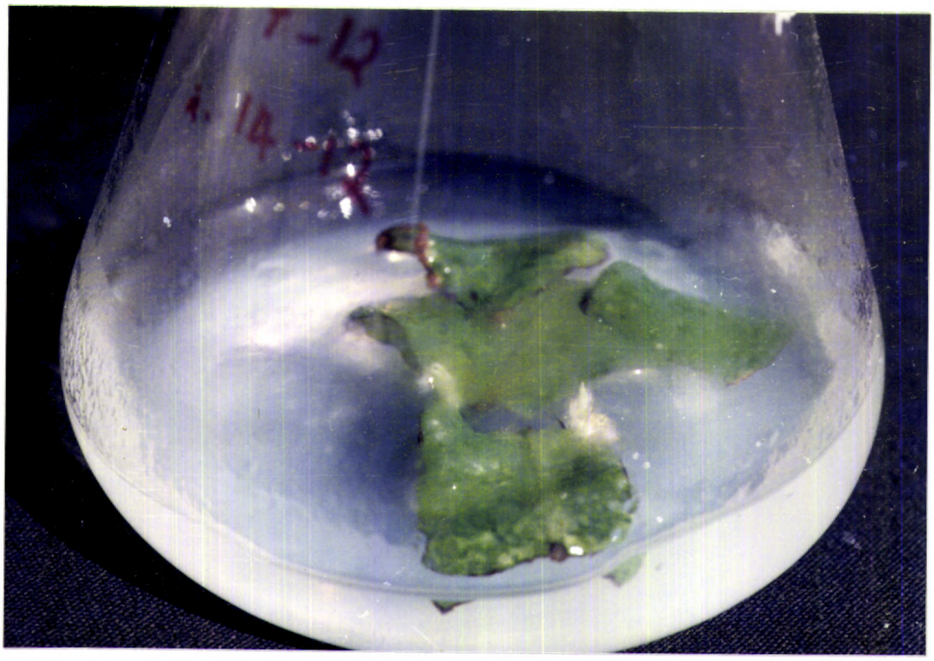
Fig. 2.1 Growth curve of *P. rosea* callus culture in various standard media

MS- Murashige and Skoog medium; SH- Schenk and Hildebrandt medium; B5-Gamborg'S medium

Initial inoculam: 500 mg / 40 ml medium

Medium used: MS basal medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + 0.5 g myoinositol

Data represents the average of 5 replicates in two repetitive experiments.



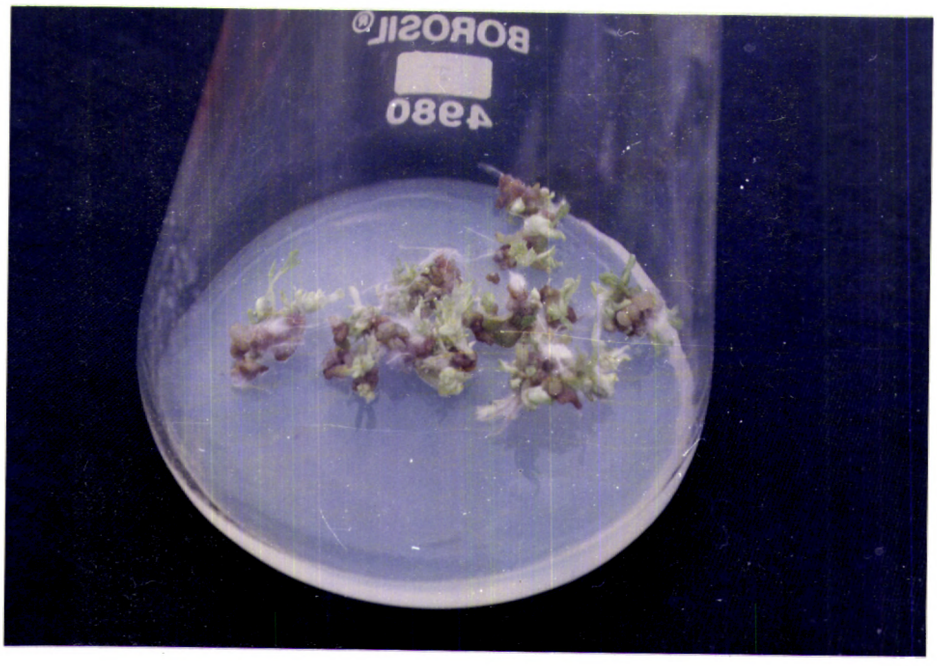


PLATE 4-7 : DIFFERENT STAGES OF SHOOT REGENERATION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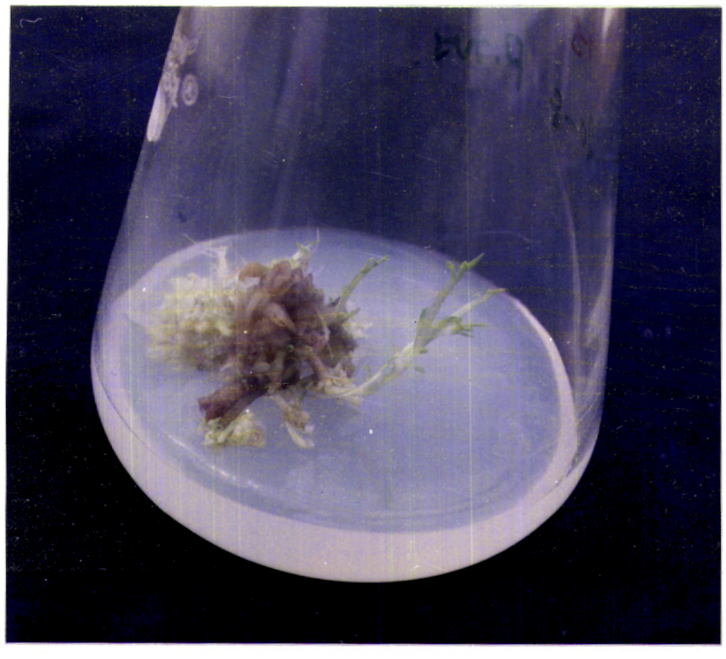

growth rate and doubling time were calculated in exponential phase only. Exponential phase was varying between 12-35 days. Whereas in the case of B5 medium, the callus did not multiply for a long time having lag phase up to 20^{th} day

(d) Shoot regeneration medium

The shoot regeneration of the callus cultures of *P rosea* was studied by varying auxin and cytokinin concentration and the percentage frequency of shoot formation was calculated. Table 2.9 represents the data obtained in MS medium after 35 days of incubation. A total of 16 combinations of auxins and cytokinins were used and at 1.0 mg/l of IAA and 1.0 mg/l of BA, 100% shoot formation was noticed. The addition of other auxins, NAA and 2,4-D has resulted in negligible shoot regeneration.

Occasionally, leaf explants grown in the above mentioned shoot regeneration medium form a narrow band of dividing cells on the vicinity of vascular bundles after 8-12 days in culture. Shoot primordia could be observed within 20 days; they have apical meristem with leaf primordia on their apical parts as well as tracheal elements connecting them to the mother plant on their basal parts. Regenerated shoots were able to create roots and complete regenerated plants could be obtained subsequently.

The shoot regeneration that observed during these trials are presented in Plate 4, 5, 6 and 7

(e) Embryogenic callus cultures

The embryogenic callus culture (Plate 8), was developed by selective screening and repeated sub-culturing in MS medium containing 1.0 mg/l of IAA, 0.5 mg/l of NAA and 0.3 mg/l BA. The growth of embryogenic callus and the plumbagin content (mg/g D. Wt.) were recorded for a period of 30 days and compared with normal callus culture (Table 2.10). It can be seen

Table 2.9 Effect of various auxins on shoot regeneration

Sl No.	NAA	IAA	2,4-D	BAP	1	2
1	0.5	0.5		0.3	Nil	
2	0.5		0.5	0.3	Nil	–
3		0.5	0.5	0.3	Nil	
4	0.5	0.5		0.5	Nil	
5		0.75	0.5	0.5	Nil	
6	0.5	1.0		0.5	5*	8.32 (±0.281)
7	0.5	0.75		1.0	19**	16.34 (±0.212)
8	0.75	1.0		1.0	19**	13.2 (±0.33)
9		0.75	0.75	1.0	Nil	
10	1.0	1.0		1.0	9**	6.78 (±0.463)
11		1.0	1.0	1.0	Nil	
12	1.0			1.0	Nil	
13		1.0		1.0	100	36.79 (±1.37)
14		1.5		1.0	30.0	16.21 (±2.1)
15				0.5	Nil	
16	--	1.0	--	0.5	3	2.33 (±0.5)

1 and 2 represent the frequency (%)of shoot formation and its mean value ± standard deviation.

Data were collected after 35 days of incubation in MS medium

- * indicating the formation of callus preceded by shoot formation
- ** indicates the formation of embryogenic callus

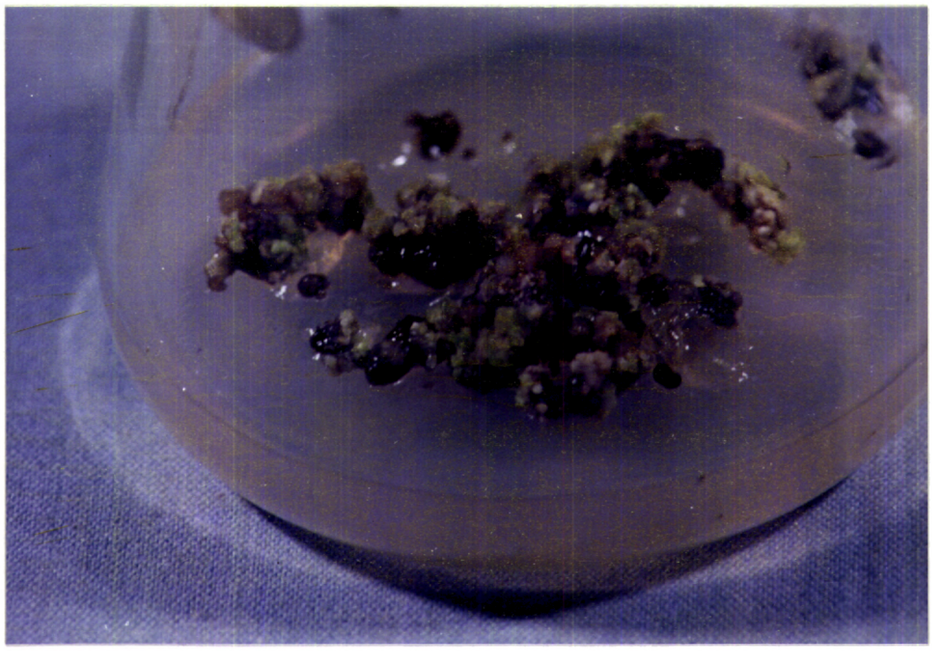

PLATE 8 : EMBRYOGENIC CALLUS CULTURE OF *P. ROSEA*

Table 2.10 Growth of *P rosea* callus culture in MS medium containing 1.0 mg/l IAA, 0.5 mg/l NAA and 0.3 mg/l BAP

No. of days	Fresh Wt. (g/culture)	Dry Wt. (g/culture)	Plumbagin (g/culture)
0	0.5 (±0.022)	0.043	1.823 (±0.34)
8	0.563(±0.11)	0.052	1.843 (±0.061)
10	0.647(±0.03)	0.059	1.981 (±0.11)
12	0.739 (±011)	0.064	2.342 (±0.77)
14	0.836 (±0.33)	0.078	2.648 (±0.055)
16	0.911 (±0.052)	0.084	2.909 (±0.092)
18	1.021 (±0.007)	0.093	3.223 (±0.54)
20	1.203 (±0.31)	0.112	3.331 (±0.043)
22	1.289 (±0.41)	0.116	3.401 (±0.212)
25	1.325 (±0.06)	0.124	3,403 (±0.55)
28	1.306 (±0.08)	0.116	3.401 (±0.32)
30	1.283 (±0.92)	0.116	3.400 (±0.613)

Data represents the average of 4 replicates.

that the growth of the embryogenic callus was rather slow in comparison to normal callus cultures. It was observed that the embryogenic callus was green and compact whereas normal callus was cream in colour and fragile. The texture of the callus was also found to be different. The embryogenic callus was compact whereas the undifferentiated callus was puffy. The plumbagin content was found to be almost double in embryogenic culture in comparison to undifferentiated callus. Rarely, small amount of pink coloured callus was observed among the embryogenic callus.

(e)Hairy root culture

Hairy root culture of *P.rosea* was developed by infecting the shoots with *Agrobacterium rhizogenes.* The hairy roots thus formed after 3 weeks (Plate 9) were sub-cultured repeatedly in hormone free media (Basal medium). Table 2.11 and Fig 2. 2 presents the fresh weight of hairy roots in MS, SH and B5 media and compared with normal roots in MS basal medium. The hairy roots were incubated for a period of 75 days on an orbital shaker (80 rpm) at 25 (± 2°C) at 10/14 photo period. Among the three media tried MS media was found to be the best. The specific growth rate was 0.024 d^{-1} in case of MS medium which was further decreased to 0.014 d^{-1} in both SH and B5 media. In comparison, the normal roots have very low specific growth rate of 0.005 d^{-1} The doubling time of hairy roots were found to be 29 days in MS medium, which is 3 times higher than the callus culture. The plumbagin content during the growth phase was estimated at a regular interval of 5 days and presented in Table 2.12. It can be seen that the plumbagin content was 2 to 2.1 mg/g D. Wt. from the 20^{th} day onwards up to the 75 days in MS medium. In comparison, SH and B5 media resulted in lower yield of plumbagin. The plumbagin content was found to be slightly (1.913 mg/g D. Wt. in callus culture and 2.33 mg/g D.

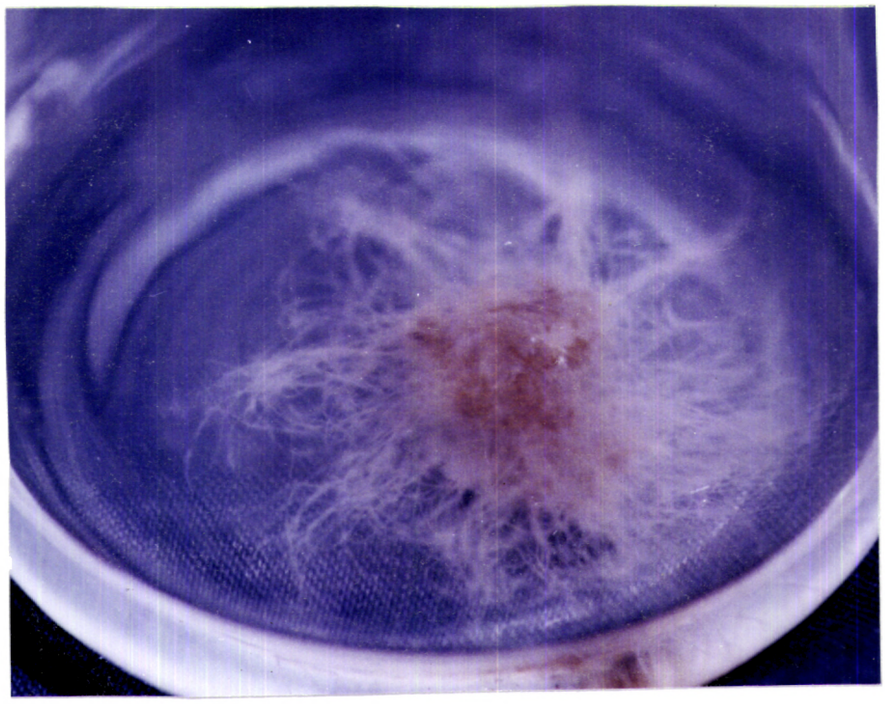

PLATE 9 : HAIRY ROOT CULTURE OF *P. ROSEA*

Table 2.11 Influence of various media on biomass accumulation in *P rosea* hairy root culture

	Hairy roots		Normal roots	
No. of days	MS (B)	SH (C)	B_5 (D)	MS (E)
0	I.5	1.5	1.5	I.5
5	2.02	1.680	1.721	1.532
10	2.231	1.723	1.728	1.623
15	2.367	1.834	1.733	1.783
20	2.568	2.111	1.911	1.911
25	2.839	2.316	2.30	2.088
30	3.313	2.347	2.327	2.119
35	3.69	2.857	2.381	2.143
40	3.873	2.791	2.566	2.207
45	4.031	2.988	2.804	2.317
50	4.391	3.232	3.117	2.374
55	4.453	3.263	3.123	2.408
60	4.251	3.263	3.169	2.469
65	4.231	3.241	3.162	2.503
70	4.238	3.298	3.183	2.561
75	4.239	3.298	3.188	2.598

Biomass is expressed in g/culture

Medium used: MS basal medium supplemented with 3% sucrose

Data represents the average of 3 replicates in two repetitive experiments.

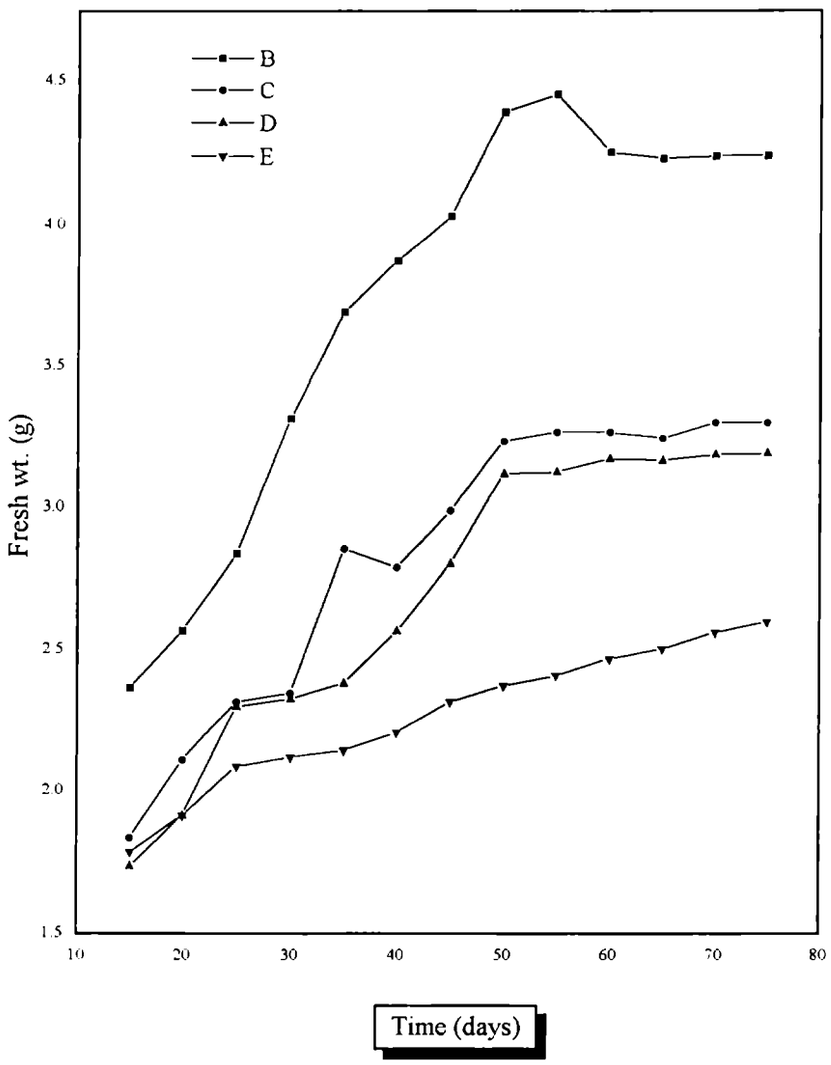

Fig.2.2 Effect of various media on biomass accumulation in hairy root culture of *P rosea*

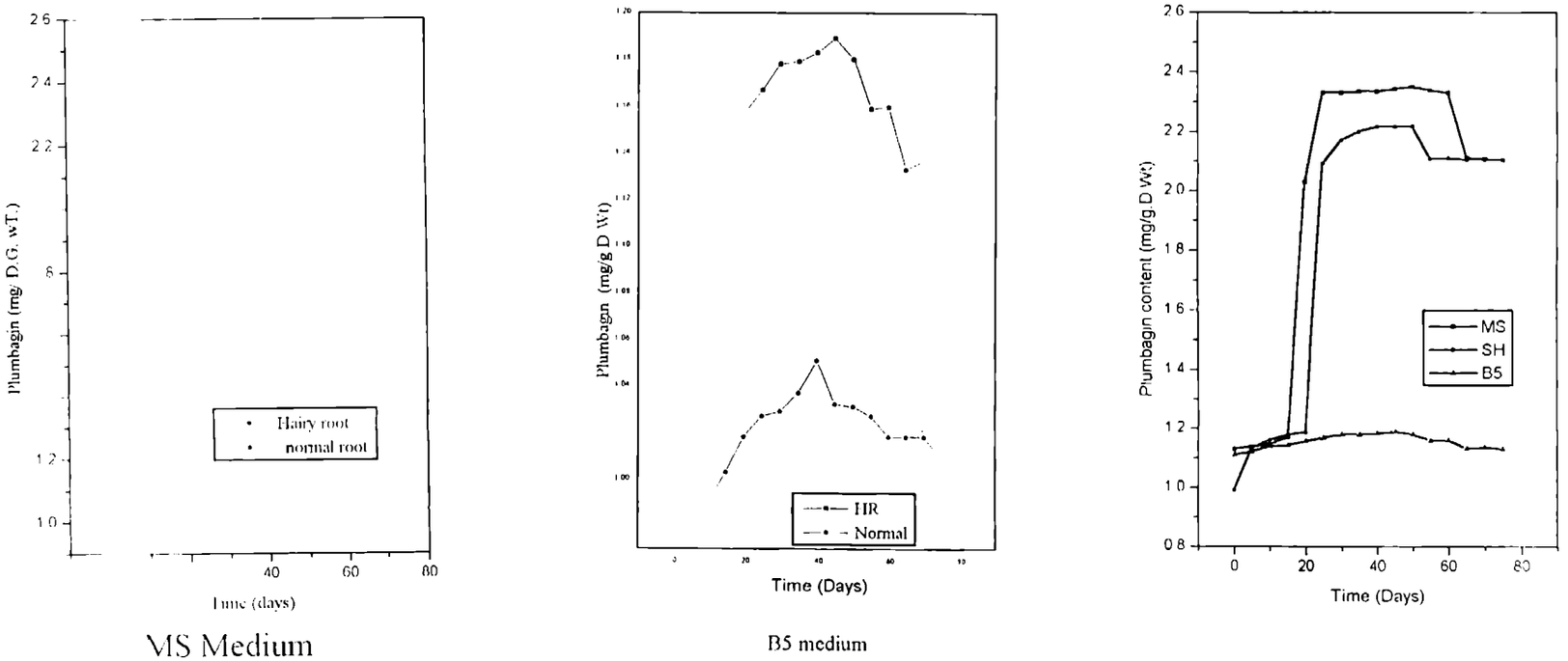

Fig.2.3. Effect of various media on Plumbagin production in hairy root Culture of *P. rosea*

Table 2.12 Effect of various basal media on plumbagin production in *P rosea* hairy root culture

No. of days	Normal roots			Hairy roots		
	MS	SH	B5	MS	SH	B5
0	0.987	0.987	0.984	1.13	1.13	1.13
5	0.999	9.989	0.989	1.139	1.133	1.126
10	1.009	0.993	0.991	1.145	1.161	1.138
15	1.091	1.038	1.003	1.17	1.178	1.143
20	1.113	1.043	1.018	2.03	1.186	1.157
25	1.129	1.053	1.027	2.11	2.093	1.169
30	1.137	1.061	1.029	2.331	2.17	1.178
35	1.148	1.067	1.037	2.333	2.2	1.179
40	1.159	1.077	1.051	2.333	2.217	1.183
45	1.172	1.091	1.032	2.341	2.216	1.189
50	1.166	1.110	1.031	2.349	2.217	1.18
55	1.163	1.09	1.027	2.338	2.108	1.159
60	1.158	1.109	1.018	2.117	2.11	1.16
65	1.158	1.107	1.018	2.109	2.103	1.133
70	1.156	1.105	1.017	2.103	2.103	1.14
75	1.151	1.105	1.009	2.103	2.103	1.137

Production of plumbagin expressed on mg/g D. Wt.

Medium used: MS basal medium supplemented with 3 % sucrose.

Data represents the average of 3 replicates in two repetitive experiments.

Wt. in hairy roots) higher in hairy roots when compared to callus culture. However, it was found significantly lower than that of embryogenic callus cultures (3.4 mg/g D. Wt. in embryogenic callus culture and 2.331 in hairy root cultures).

There are a very few reports on the effect of different media on growth and micropropagation of *P rosea.* Harikrishnan and Hariharan have used MS medium supplemented with various dosages of BA, IAA and 2,4-D.[12] The addition of 2.0 mg/l of 2,4-D in MS medium has resulted in massive callus formation. However, no shoots or roots were observed. Supplementation of BA 1.0 mg/l and IAA 0.5 mg/l showed 20-25 shoots and 4-6 roots per flask. Increase of BA slightly to 2.0 mg/l has lowered the number of shoots and roots. Use of White's medium for callus development has showed poor response. Satheesh and Bhavanandan (1989)[11] have also studied the influence of auxins and cytokinins on callus formation in *P rosea.* They have observed that 1.5 mg/l of 2,4-D and 0.5 mg/l of kinetin in MS medium has resulted in good callus formation. IAA 1.5 mg/l has induced root formation from the callus. In the present investigations, we have noticed that NAA at 1.0 mg/l in MS medium has given the best result (100% callus induction). The influence of 2,4-D was lower than that of NAA at all the concentrations tried (Table 2.4). The shoot generation could be achieved by varying the composition of IAA to 1.0 mg/l and BA to 1.0 mg/l (100% frequency of shoot formation as shown in Table 2.6) without any structure or developmental abnormalities which are commonly observed when higher hormone concentrations are used. It has been reported earlier that the same combination of growth hormone caused varied response in callus induction in each plant. This might be due to the variation in the internal hormonal content and the specific metabolic and the physiological

state prevalent in the explant tissue. These results are in variation to earlier reported values with optimized media composition. In the present investigations, the influence of 2,4-D either for callus formation or shoot regeneration was poor. None of the earlier reports indicate the growth profile and productivity. In the present study, the growth of the callus used was related to the plumbagin content. One of the significant observations of the present study is the higher productivity of embryogenic callus over undifferentiated callus. The metabolite content was almost double in embryogenic callus to that of undifferentiated callus. It was reported earlier that the undifferentiated callus of *Digitalis lanata* did not accumulate digitoxin unless it differentiated into embryogenic callus (Alfermann et al. 1985).[20]

The present investigations revealed that embryogenic callus of *P. rosea* accumulate higher quantity of naphthoquinones than the normal undifferentiated cultures. Among all the standard media tried MS medium was found to be the best. All further experiments were carried out using this optimized media composition.

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CHAPTER III

PERMEABILIZATION

INTRODUCTION

Plant cell cultures offer an important source of commercially valuable secondary metabolites particularly pharmaceuticals.[1-3] The application of plant cell cultures for the production of these compounds on a commercial scale is still in a very early phase. There are a few generally accepted reasons for this delay. The most important one is the low productivity of the plant cell in culture. Usually the productivity of cells drop dramatically when callus is formed from plant parts, and again when a suspension culture is started from callus culture. Another draw back is the slow growth of *in vitro* grown cultures, combined with the fact that most plant cell products, with few exceptions such as capsaicin [4] and berberine [5], are stored with in the cells.

During the past years, a series of permeabilization methods (Table 3.1) have been developed, especially for the microorganisms to solubilize the membrane partially or totally to release the products.[6-8] Cells can be permeabilized without lysis or destruction of the whole inner organization. The choice of a special permeabilization procedure depends on the composition of the cell wall and of cell membrane. Permeabilization can only be effected if an agent can penetrate the cell wall and reach the cell membrane. In most cases, the plasma membrane is damaged in such a way that the morphology of the cells remains intact, yet low molecular weight molecules can freely enter and leave the cell. For example, Schnaitman [9] found that when *E. coli* cells were treated with non-ionic detergent Triton X-

100, removed native lipids and lipopolysaccharide but did not affect the normal morphology of the cell wall. Studies conducted with Transmission Electron Microscopy also revealed that the permeabilization with 2 M Guanidine and 2 % Triton X-100 resulted in the complete solubilization of the inner membrane. However, in the outer wall, alteration occurs at the molecular level as there was no evidence of extensive disruption of the peptidoglycan layer.[10] Part of this resistance to detergent action must be due to a stabilizing effect of divalent cations.[11]

In plants, vacuole is the main storage site of inorganic salts such as phosphate[12] neutral sugars,[13] aminoacids[14] and secondary metabolites.[15,16] It has been suggested that more acidic nature of vacuole than cytosol play an important role in trapping of hydrophilic and polar secondary plant products.[17] The mechanism of extracellular excretion have been described by Renaudin and Guern (1982) [18] and Renaudin *et. al.* (1989) [19] with the interesting conclusion that the mechanism of secondary product translocation across the tonoplast and the plasmalemma membranes show a high degree of similarity and both are involved in the regulation of metabolic activities of the cells. These factors make it inevitable to destroy the biomass that has been obtained so laboriously. If it were possible to force the cells to excrete their products, while preserving the cell viability and production capacity, the chances for plant cell culture based products would become much better.

Permeabilized cell systems have also been employed for the studies on complex metabolic processes such as replication of nucleic acids and synthesis of nucleic acids and proteins.[20-23] As the treated cell system constitute a more physiological state as in intact cell, it is well suited for investigations on enzyme kinetics.[24-26] Certain substances are released from

the cell wall during the permeabilization procedure. Some proteins and other molecules can even leave the cell without any effect on the cell wall integrity.[27]

Organic solvents as permeabilizer:

Toluene is one of the most frequently used permeabilizing agents for microorganisms. Investigations were carried out on both prokaryotic and eukaryotic systems to study the effect of toluene on cells.[28-30] Jackson and De Moss[28] found that 25% of the total *E. coli* proteins were secreted into the surrounding medium after permeabilization. Freeze-fracture electron microscopy shows that toluene causes considerable damage to the cytoplasmic membrane while membranes of other sub-cellular particles remain relatively intact.

Toluene has been known to attack the lipid layer of the cytoplasmic membrane of bacteria,[22] and fungi.[31] It has also been reported that this treatment increases permeability to exogenous molecules like nucleotide triphosphate and thereby lead to enhanced nucleic acid and protein synthesis.[32-34]

Howell and Walker[35] studied the effect of toluene on plant system. *Chlamydomonas reinhardii* cells treated with 0.5 % toluene carried out DNA synthesis, however, the organelles except chloroplast lose its integrity

Hoffmann-Berling[36] was the first one to use ether for the direct measurement of DNA synthesis in *E. coli* cells. In the case of ether, permeabilization of cells is effected through pore formation (due to solubilization of cell wall proteins) in membrane structure rather than disruption of the membrane integrity

Table 3.1 Permeabilization methods

Methods	References
1. Organic solvents	
Benzene	[71,72]
n-Butanol	[73]
Chloroform	[8,74,75]
Dimethyl sulfoxide	[8, 41,50]
Ether	[6,36,76,77]
Methanol	[78]
Phenethyl alcohol	[8, 37,38, 40]
Toluene	[22, 31,34]
n-propanol	[30,34]
2. Antibiotics	
N-acetyl candidin	[79]
Amphotericin B	[80]
Nigericin	[81,82]
Nystatin	[6]
Polymixin	[83,84,85]
3. Thionins	[86]
4. Detergents	
Brij 58	[87,88]
CTAB	[57,60]
Sodium dodecyl sulfate	[94]
Triton X-100	[8,9,56,88,89]
Tween 80	[90]

Dextran sulfate 500	[91]
Nonidet P-40	[87]
Sodium deoxycholate	[92]
DEAE dextran	[93]
Sodium docyl glycol	[55]
5. Protamine	[9,95,97]
6. Chitosan	[65,70]
7 Lysolecithin	[98]
8. Digitonin	[58,96,99]
9. Physical methods	
Cold shock	[22,32]
Ultrasonic treatments	[102]
Osmotic shock	[100, 101]
Temeperature shock	[100]
10. chelating agents (e.g. EDTA)	[9,103,104]

Phenethyl alcohol, structurally close to toluene, has been used for the permeabilization experiments in microorganisms.[37-40] The effect of phenethyl alcohol is reversible as it does not disturb the synthesis of macromolecules and the membrane structures.

Dimethyl sulfoxide (DMSO) is the widely used permeabilizing agent in eukaryotic system.[41,42] De Bruijne and Van Steveninck[43] analysed the kinetics of DMSO influx and efflux through yeast cell membrane. DMSO treated yeast cells were employed successfully for the extraction of protein and water soluble sterol components without cell wall disruption.[44] It has also been postulated that treatment of cells with DMSO makes the membranes less rigid due to the dissolution of cholesterol and phospholipids.

This allows certain membrane proteins to aggregate, which in turn cause channel formation through that compounds can enter the cells.[42]

DMSO has also been found suitable for the release of intracellular plant secondary metabolites. For example, *Chenopodium rubrum* cells treated with 10 % (v/v) DMSO accumulated higher amount of amaranthine within the cells as well as in the medium.[45] Brodelius and Nilsson [42] reported that the treatment of *Catharanthus roseus* cells with 5% DMSO for 10 minutes completely released the alkaloids in the medium. Parr et al.[46] suggested that high concentration of DMSO was required for the release of intracellular quinoline alkalids from *Cinchona ledgeriana* cells. Similar observation was reported from *Berberis stolonifera*[47] cells also, and the cells were irreversibly damaged. The treatments of *Digitalis lanata* cell cultures with DMSO concentration higher than 11% caused the release of only negligible amount of cardenolides with considerable amount of damage to the cells.[48]

Park and Martinez[49] have reported that plant cell's adaptability to higher concentration of DMSO could be improved by exposing the cells to lower concentration of DMSO initially. The preconditioned cells of *Coleus blumei* could maintain the viability at 0.5-1.5 % of DMSO where as the unconditioned cells lost their viability at that level. However, a second conditioning step with 0.5 % DMSO has resulted in the decrease of biomass.

Plant cells treated with higher concentration of DMSO could be employed for 'biotransformation' experiments too. Presence of 10% DMSO in the culture medium enhanced the conversion of naringenin into eriodactyol and dihydrokaempferol in the suspension culture of *Haplopappus gracilis*.[50] The untreated cultures showed much lower level of production of the same compounds.

Detergents as permeabilizing agents (Table 3.1)

Detergents such as Triton X-100, Brij 58, Polyethylene glycol, Nonidet P-40, sodium dodecyl sulfate and Tween 80 are another important category of chemicals frequently used for altering the permeability of living cells.

Structure of biological membrane

The biological membrane consists of supramolecular aggregates of lipids and proteins to which carbohydrates are found covalently bounded. Lipids form a very heterogeneous group of molecules including hydrocarbons, pigments, cholesterol phospholipids, glycolipids and detergents. They all contain apolar groups of aliphatic or aromatic nature and most lipids have polar groups such as phosphate, amino, sulphate and carboxyl groups or neutral groups such as hydroxyl, carbonyl or protonated carboxyl groups. The aliphatic or aromatic groups are hydrophobic; they are readily soluble in non-polar solvents but sparingly soluble in water. The hydrophilic groups in lipids are called 'heads' and hydrophobic groups 'tails' particularly when they are alkyl groups. Molecules, which are partly hydrophilic and partly hydrophobic, are called amphiphiles which constitutes the major lipid groups in biological membranes.[51,52]

Mechanism of action of detergents:

According to Helenius and Simons, (1975), the detergent molecules bound to the hydrophobic domain and not to the hydrophilic part of the amphipathic protein.[53] A part of the bound molecules interact directly with the protein and the rest bind co-operatively to form a micelle-like region on the surface of the proteins.[54] The important feature of this scheme is that the milieu around the hydrophobic domain of the protein remains apolar, and the milieu around the hydrophilic parts, aqueous throughout the delipidation. Therefore, the orientation of the protein in two different detergents mimics the lipid environment in the membrane sufficiently well

to support the continued activity of the protein. It is attractive to speculate that the detergent molecules (for example, Triton X-100 and sodium dodecyl glycol ether sulphate) in ordered structure at or above the critical micelle concentrations, interact through their hydrophobic moieties with lipid A portion of the lipo-polysacharide. Thereby, substituting the phospholipid molecules which are the normal partners of lipo-polysaccharides with divalent cations and proteins in the construction of the outer membrane. The balance of hydrophilic and hydrophobic properties of the lipo-polysaccharide molecule appears to be the decisive factor in the formation membrane-like vesicles.[55]

Triton X-100:

Several studies have indicated that the non-ionic detergent Triton X-100 has a specific solubilizing effect on the cytoplasmic membrane of *E. coli*.[56] Triton X-100 appears to attack predominantly on proteins, which are bound to the membrane lipids by hydrophobic interactions. Usually, binding of the detergent do not lead to major conformational change of the protein and hence no loss of activity. [9] (Fig. 3.1)

Cetyl trimethyl aminobromide (CTAB):

CTAB, a cationic detergent, have been used for the treatment of cells of *E. coli* [57] *Saccharomyces cervisiae* [59,60] and *Kluyveromyces fragilis*.[58] Gowda *et al.*[58] have shown the increased activities of a number of intracellular enzyme after treatment with CTAB. This was suggested to be due to the enhanced transport of small molecular weight compounds such as substrates, products and co-factors across the cell membranes as a result of increased permeability.

Chitosan (β-1,4 glycosamine) is widely distributed in nature. It has been used for elicitation[61,62] and permeabilization[63,64] purposes in plant cell

cultures. This polycationic polymer appears to be involved as a regulatory molecule in a number of different biological system.[65-67] Hahl *et al.* suggested that the effect of chitosan as permeabilizing agent is due to the electrostatic interaction with the cell membrane.[68,69]

Studies conducted in plant cells showed that the complete release of products stored in the intracellular compartments require a very high concentration of permeabilizing agent. Brodelius *et al.* (1988) found that 90 percentage release of intracellular products from *Chenopodium rubrum*, *Thalictrum rugosum* and *Catharanthus roseus* require 35% DMSO, 230 % Triton X-100 and 84 % hexadecyltrimethyl ammonium bromide, respectively. [8] The cell viability was lost upon permeabilization except in *Catharanthus roseus* with Triton X-100 and DMSO. Despite the claims of Lundberg *et al.* (1986)[70] that DMSO permeabilized/immobilized cells of *Catharanthus roseus* may release their products without showing severe growth inhibition, there seem to be no experimental data available indicating that chemical induced release of metabolites in significant levels is tolerable for cells. Chemical permeabilization for the release of intracellular secondary metabolites appears to be suitable for plant systems, unless the cell viability is not required.

The cell cultures of *P. rosea* release only about 0.1 percentage of plumbagin per milligram dry weight of callus tissues. Hence, attempts were made to enhance the secretion of intracellular plumbagin into the medium by permeabilizing agents.

3.2 **Materials and methods**

Suspension culture of *Plumbago rosea:*

The suspension culture of *Plumbago rosea* was maintained on MS medium supplemented with 1.00mg/l of IAA, 0.5mg/l of NAA and 0.3mg/l of BA at pH 5.8 (± 0.2) in 250 ml. Erlenmeyer flasks. The cultures were transferred to fresh medium to an inoculam density of 1.0g fresh weight per 100 ml. of the medium, which was used as the stock culture. From the above stock culture, 0.250 g of cells were transferred to pre-weighed Erlenmeyer flasks containing 40ml. Sterilized MS medium and these cultures were treated with various permeabilizing agents for fifteen and thirty minutes. After seven days, cells were harvested and plumbagin content was estimated. All the cultures were maintained under 14 hour light period at room temperature of 28 (±1°C) on a rotary shaker at 80 rpm. For each set of experiments five replicates of samples were used.

Permeabilizing agents:

Cetyltrimethylammonium bromide (CTAB) was obtained from Sd Fine Chemicals, India. Dimethylsulfoxide (DMSO) and Triton X-100 were purchased from Merck. Crude Chitosan was procured from Microbiology Unit, Central Food Technological Research Institute, Mysore.

Stock solutions of permeabilizing agents were prepared as follows. Milligram per litre (ppm) of CTAB and Triton X-100 were prepared in MS basal medium and sterilized in an autoclave. Percentage, (v/v) solution of DMSO was mixed with sterilized MS basal medium and used without further sterilization. Acetylated chitosan was dissolved in MS basal medium and sterilized. All sterile operations were done in a sterilized Laminar flow chamber

Preparation of acetylated Chitosan:

Acetylation of chitosan was done according to the modified procedure of Mitchell et al (1994).[105] Crude chitosan (100mg) obtained from crab shell wall was dissolved in 4ml, of 10% (v/v) acetic acid at 80°C for 2 hours and cooled to room temperature. Methanol (9ml) containing 100µl of acetic anhydride, was added drop-wise with vigorous shaking. After 3 hours the partially acetylated chitosan was first dialyzed with excess 10mM acetic acid for 5 hours, followed by distilled water. In the distilled water, dialysis was carried out for 24 hours. Then the sample was centrifuged for 10 minutes at 5,000 rpm to remove insoluble materials. Samples were lyophilized (Hetosic lyophilizor) at ⁼40°C and dried it. Flakes of this acetylated chitosan were used for permeabilization experiments.

Permeabilization procedure:

For the permeabilization experiments, cells in logarithmic phase were used. In *P rosea* culture logarithmic phase was from 10-20 days. The standard procedure used for the permeabilization of cells was as follows:

Seven days old culture of *P rosea* was collected by filtration and the culture (0.250g) was transferred to fresh MS medium. Required amount of MS medium containing permeabilizing agents was added to cell suspension cultures (40 ml) and shaken for 15 and 30 minutes on a gyratory shaker (80 rpm) in 150 ml. Erlenmeyer flasks. After the treatment, cells were washed thoroughly with sterile fresh culture medium for 3 times to remove the permeabilizing agent completely. The treated cells were transferred to fresh culture media and allowed to grow for seven more days and harvested. The cells were collected by centrifugation at 5,000 rpm for 10 minutes at 4°C and washed with distilled water three times. The plumbagin content of the

biomass as well as the supernatant were estimated. For each set of experiments 5 replicates were taken.

Determination of cell viability:

Viability of the cells was determined after 24 hours by staining with Evan's Blue reagent.[106] To 1 ml of the cell culture equal volume of 0.025% Evan's blue solution was added and kept for 1 minute. Cells were washed thoroughly with fresh MS medium to remove the excess stain from the medium. Cells were separated from the staining solution by centrifugation at 4°C (5,000 rpm) for 10 minutes using Hitachi Refrigerated centrifuge. Cells were again suspended in 1 ml. fresh MS medium. A drop of the stained culture was placed on a clean slide and viable cells were counted under a light microscope (Nikon (Type 129), Japan). For each sample, cells from 10 different fields were counted and percentage viability was calculated. Dead cells adsorb the dyes and become blue in colour.

Extraction and analysis of plumbagin:

After the permeabilization experiments, the cells were incubated for 7 days in MS fresh medium. The cells were separated from the culture medium by filtering under vacuum with Millipore filter unit. Cells were washed thoroughly with distilled water for three times. Fresh weight of the biomass was determined. The cells were lyophilized for 3 hours to remove the moisture content. After lyophilization, dry weight of the biomass was also determined.

Metabolites from the dried biomass was extracted with methanol (100%) in soxhlet apparatus for 3 hours (Crouch et al., 1990).[107] The methanol fraction was separated from the plant material by filtration through Whatman No.1 filter paper. The filtrate was reduced to dryness under

vacuum at temperature below 40°C, resuspended in distilled water and washed twice with petroleum ether and diethyl ether.. Then, extracted with chloroform thrice and evaporated to dryness under vacuum, resuspended in 3ml. of 80% HPLC methanol for analysis.

Alkaloid from the medium was isolated as described above. Chloroform phase was collected and evaporated under vacuum. The dried sample was dissolved in 3ml. HPLC grade methanol and used for analysis.

HPLC analysis of plumbagin:

The analysis of plumbagin was done according to the procedure of Lee *et al.* (1985).[108] Following solvent extraction, the crude extract dissolved in methanol was loaded onto a reversed phase column (μ-Bondapack C18, 300mm X150 mm I.D., Shimadzu LC-A 10 AD) connected with an UV detector (SPD 10A) The detection wavelength was 254 nm. The solvent mixture of methanol and water (80:20) at pH 3.5 adjusted with triethyl amine was eluted at a flow rate of 0.8 ml/min. The quantitative analysis was carried out by comparing the peak area of the sample with authentic plumbagin (Sigma).

3.3 Results

The cells of *Plumbago rosea* were harvested at logarithmic phase (14 days old) and suspended in medium containing various permeabilizing agents such as CTAB, DMSO, Triton X-100 and chitosan at different concentrations. After required treatment time, the cells were removed from the medium containing permeabilizing agents, washed thoroughly with fresh MS medium to remove the traces of adhering permeabilizing agents, and suspended in fresh MS medium. The cells were incubated for 7 days at 25

Table 3.2 Influence of CTAB on plumbagin production in *P rosea* cultures

Concn. of CTAB (ppm)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% release of plumbagin	Viability (%)
0.00	0.077 [± 0.008]	3.181 [± 0.013]	3.258	2.36	93.13 [±1.81]
0.001	0.632 [± 0.05]	5.443 [± 0.323]	6.075	10.40	93.1 [± 1.57]
0.005	0.892 [± 0.06]	5.981 [± 0 .433]	6.873	12.978	92.01 [± 1.83]
0.01	1.627 [± 0.19]	3.271 [± 0.01]	4.898	33.21	47 13 [± 1.02]
0.05	2.895 [± 0.24]	2.394 [± 0.413]	5.289	54.74	45.04 [± 2.06]
0.1	3.038 [± 0.25]	1.977 [± 0.014]	5.015	60.57	39.88 [± 1.87]
0.5	3.672 [± 0.23]	1.315 [± 0.11]	4.987	73.63	18.12 [± 2.41]

Treatment period: 15 minutes

Medium Used: MS medium + IAA 1.0 mg/l+ NAA 0.5 mg/l + BAP 0.3 mg/l + with or without (control) CTAB

Age of the culture: 14 days; Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml. Medium

Incubation period: 7 days at 25 ± 2°C under 14 hours light period

Data represent the average of 5 replicates in two repetitive experiments.

Numbers in parenthesis indicate ± SD

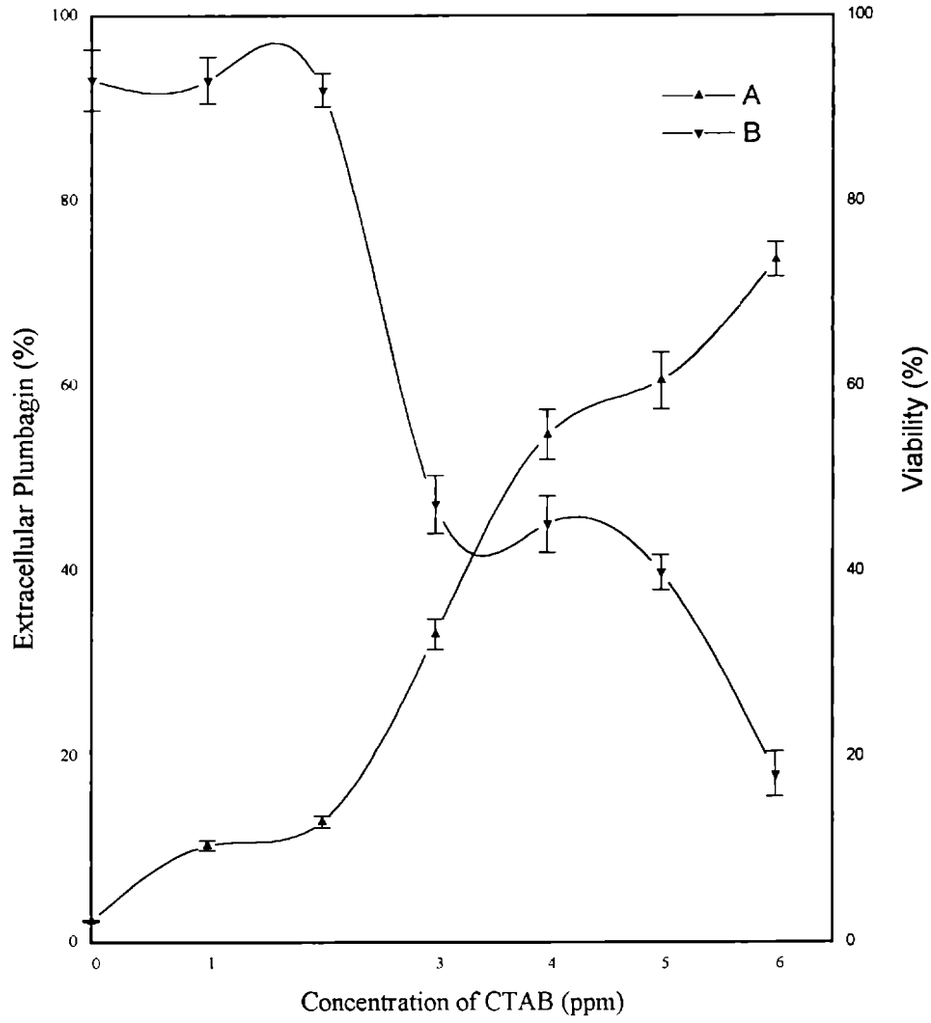

Fig. 3 1 Effect of CTAB on permeabilization of *P rosea* cell cultures

Period of treatment with CTAB: 15 minutes

A -- Extracellular plumbagin; B --- Viability of cells

Medium used: MS basal medium supplimented with IAA 1.0mg/l, NAA 0.5mg/l and BAP 0.3mg/l

Age of the culture: 14 days;

Initial inoculam:500mg/40ml medium

Table 3.3 Influence of CTAB on plumbagin production in *P rosea* cultures

Concn. of CTAB (ppm)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% release of plumbagin	Viability (%)
0.00	0.077 [± 0.008]	3.181 [± 0.22]	3.258	2.36	93.13[±1.81]
0.001	1.066 [±0.114]	5.354 [± 0.02]	6.420	16.6	81.24[±2.73]
0.005	1.515 [±0.164]	3.98 [± 0.02]	5.495	27.57	76.22[±1.11]
0.01	2.031 [± 0.091]	3.556 [± 0.237]	5.587	36.35	59.34[±1.88]
0.05	2.709 [± 0.011]	2.989 [± 0.02]	5.698	47.54	43.60[±2.31]
0.1	4.013 [± 0.266]	2.131 [± 0.024]	6.144	65.32	36.91[±1.95]
0.5	4.038 [± 0.37]	1.641 [± 0.04]	5.679	71.1	11.13[±1.81]

Treatment period with CTAB: 30 minutes

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l with or without (control) CTAB

Age of the culture: 14 days; Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml medium

Incubation period: 7 days at 25 ± 2°C under 14 hours light period

Data represent the average of 5 replicates in two repetitive experiments. Data in parenthesis indicate ± SD

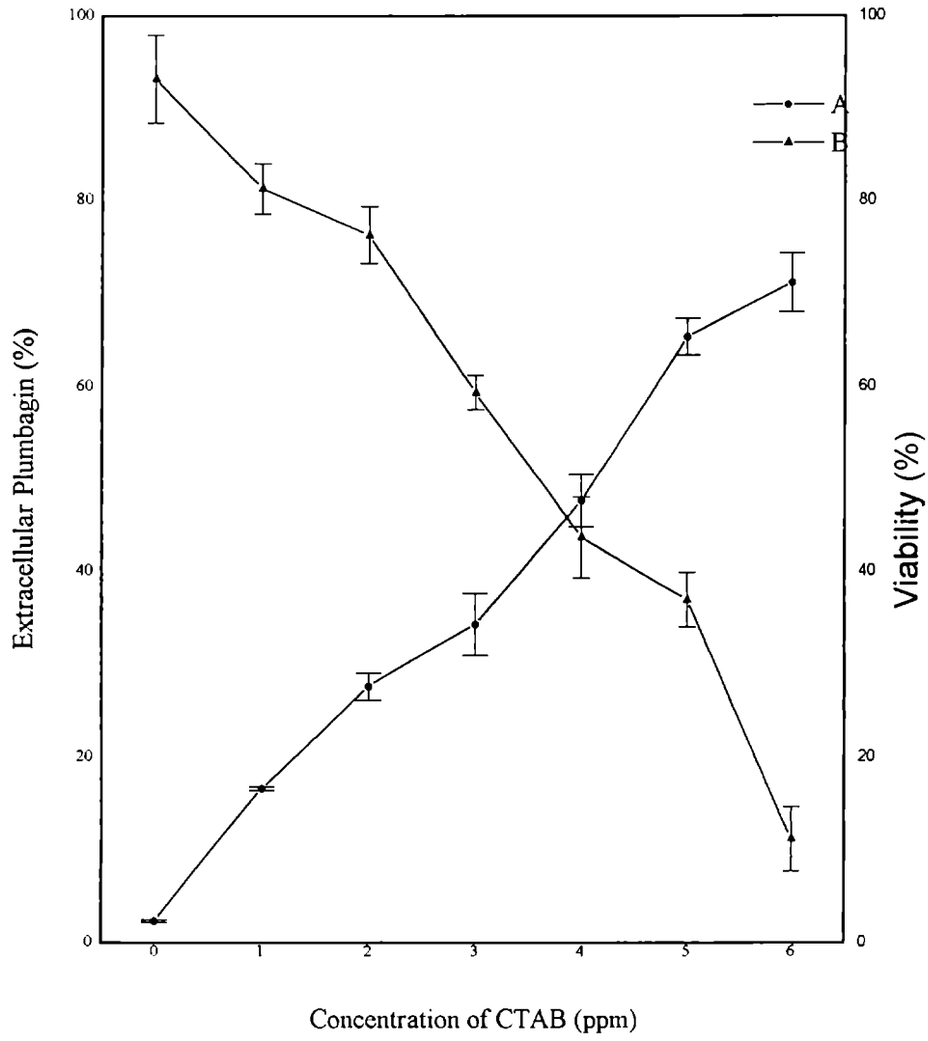

Fig. 3.2 Effect of CTAB on permeabilization of *P rosea* cell cultures

Period of treatment with CTAB 30 minutes
Symbols: A -- Extracellular Plumbagin; B -- Viability of cells
Medium used: MS basal medium with IAA 1 0mg/l,
NAA 0.5 mg/l and BAP 0.3 mg/l
Age of the culture: 14 days;
Initial inoculam: 500mg/40ml medium

(±2°C) at a photoperiod of 14 hours. The viability of the cells was determined after 12 hours using Evan's blue reagent. The cells were separated from the medium and plumbagin was estimated both in the cells as well as in the medium.

Influence of CTAB on plumbagin production:

At lower concentration of CTAB and shorter treatment time (15min.), the cell viability was found 93.1% and is similar to cells, which were not treated (Control). Even at lower concentration of CTAB, higher contact time 30 min. was found detrimental for cell viability. At the lowest concentration tried (0.001 ppm), the viability was only 81.24 per cent after 30 minutes of treatment. Fig. 3.1 & 3.2 represent the percentage of plumbagin released into the medium and percentage viability at various concentration of CTAB. In the case of untreated cells, negligible amount of plumbagin (0.077 mg/g. D. Wt.) was found in the culture medium whereas the major portion of metabolite remains inside the cells. The fraction of the plumbagin released was 2.36 per cent only. It can be observed from the graphs (3.1& 3.2) that the permeabilization of tissue has shown a drastic effect on cell viability as well as on metabolite production. It can be seen that the percentage of metabolite released enhanced with the increase in the concentration of permeabilizing agent. For a contact time of 15 minutes, 73.63 per cent of total plumbagin was found in the medium when the concentration of the permeabilizing agent was adjusted to 0.5 ppm. Similarly at higher contact time of 30 minutes, 71 1 percent of total product was leaked out of the modified membrane after the treatment with 0.5 ppm of CTAB. On the contrary, higher contact time and higher concentration of permeabilizing agent has detrimental effect on cell viability The cell viability was drastically reduced when the cells were treated with 0.5 ppm of CTAB and

Table 3.4 Influence of DMSO on plumbagin production in *P rosea* cultures

Concn. Of DMSO (%,v/v)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% release of plumbagin	Viability (%)
0.00	0.075 [±0.043]	3.366 [±0.31]	3.391	2.21	93.22[± 1.34]
0.001	0.219 [±0.01]	8.813 [±0.15]	8.062	2.72	93.00[±0.23]
0.005	0.232 [±0.041]	7.93 [+0.093]	8.162	2.84	91.06 [±1.79]
0.01	0.252 [+0.09]	8.566 [±0.214]	8.819	2.86	86.77[±1.21]
0.05	0.278 [±0.145]	8.52 [±0.156]	8.798	3.16	83.70 [±1.22]
0.1	0.299 [±0.213]	8.556 [±0.104]	8.855	3.38	83.8 [±0.983]
0.5	0.36 [±0.099]	8.06 [±0.02]	8.420	4.28	80.99[±1.97]
1.0	0.561 [±0.013]	7.335 [±0.31]	7.896	7.84	78.43 [±3.22]

Treatment period: 15 minutes

Medium used: MS basal medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + with or without (control) DMSO

Age of the culture: 14 days; Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml medium

Incubation period: 7 days at 25 ± 2°C under 14 hours light period

Data represents the average of 5 replicates in two repetitive experiments.

Data in parenthesis indicate ± SD

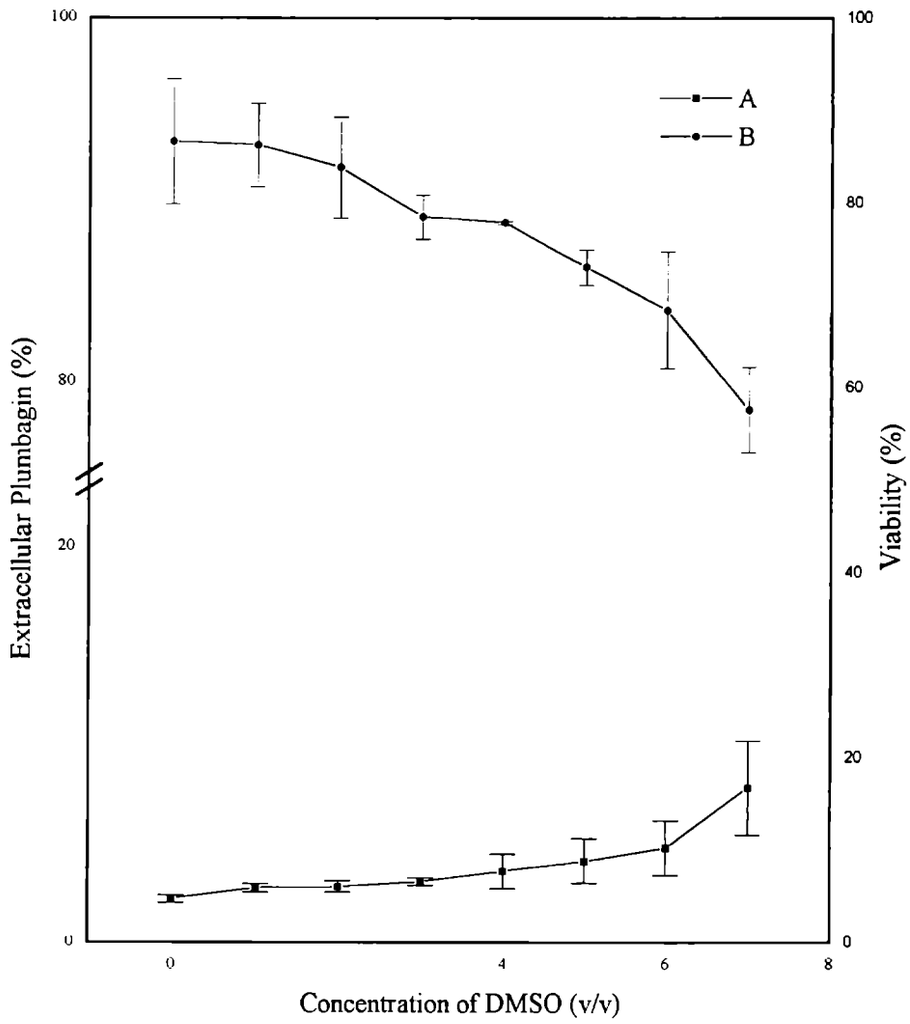

Fig: 3.3 Effect of DMSO on permeabilization of *P rosea* cell cultures

Period of treatment with DMSO: 15 minutes
Concentration of DMSO used:0-Nil; 1-0.001, 2-0.005; 3-0.01, 4-0.5; 5-0.1, 6-1.0
Symbols used: A -- Extracellular Plumbagin; B -- Viability of cells
Medium used: MS basal medium with IAA 1.0mg/l, NAA 0.5mg/l and BAP 0.3mg/l
Age of the culture: 14 days; Initial inoculam: 500mg/40ml medium

Table 3.5 Influence of DMSO on plumbagin production in *P rosea* cultures

Concn. Of DMSO [%, (v/v)]	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% release of plumbagin	Viability (%)
0.00	0.075 [±0.043]	3.366 [± 0.31]	3.391	2.21	93.22 [± 1.34]
0.001	0.238 [± 0.039]	8.015 [±0.093]	8.253	2.88	91.22 [± 0.33]
0.005	0.255 [± 0.011]	7 76 [± 0.041]	8.015	3.7	82.11 [± 1.65]
0.01	0.450 [± 0.083]	7.593 [± 0.076]	8.045	5.59	78.372 [± 1.82]
.05	0.464 [± 0.017]	7.517 [± 0.043]	7.981	5.814	53.23 [± 0.99]
0.1	0.507 [± 0.21]	6.014 [± 0.009]	6.521	7.8	48.73 [± 2.47]
0.5	0.754 [± 0.17]	5.697 [± 0.011]	6.451	11.69	35.66 [± 2.01]
1.0	0.987 [± 0.091]	5.221 [± 0.023]	6.208	15.898	32.13 [± 1.38]

Treatment periods: 30 minutes

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + with or without (control) DMSO

Age of the culture: 14 days; Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml medium

Incubation period: 7 days at 25 ± 2°C under 14 hours light period

Data represents the average of 5 replicates in two repetitive experiments.

Numbers in parenthesis indicate ± SD

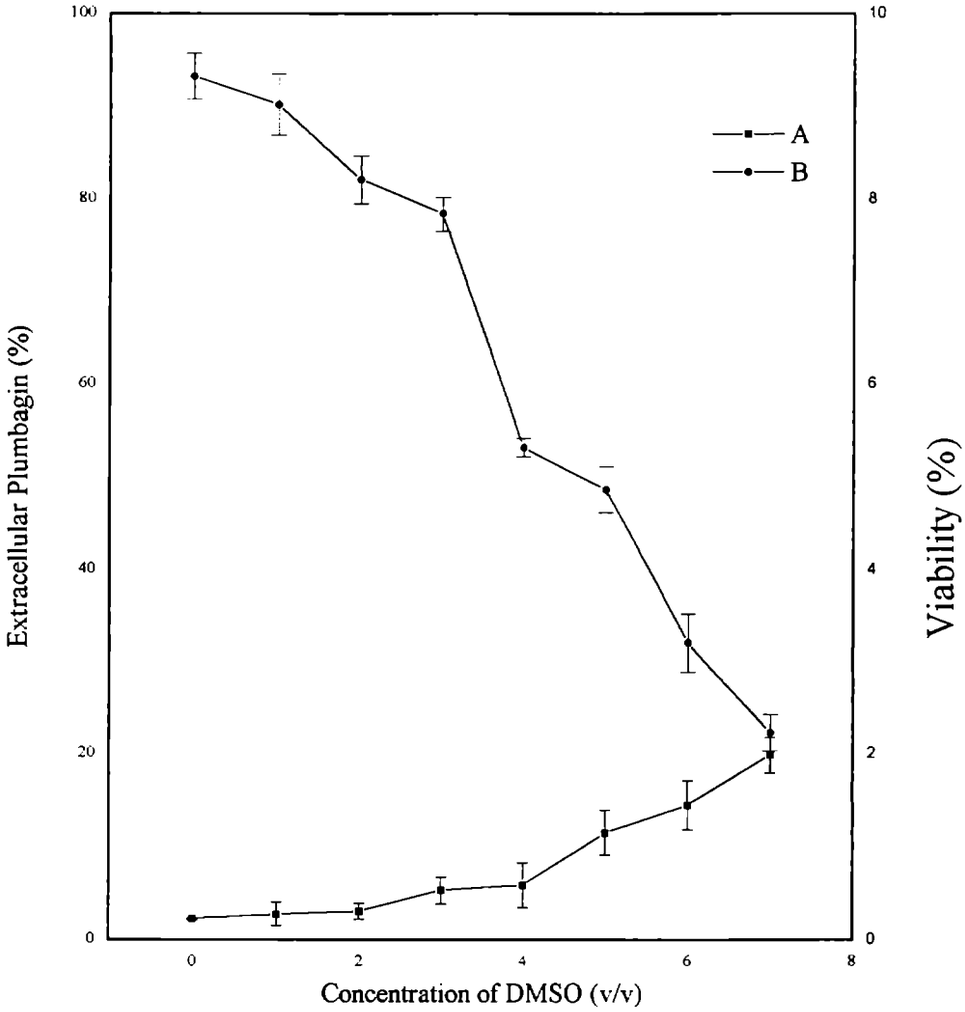

Fig. 3.4 Effect of DMSO on permeabilization of *P rosea* cell cultures

Period of treatment with DMSO: 30 minutes
Concentration of DMSO used: 0-Nil; 1-0.001, 2-0.005; 3-0.01, 4-0.05; 5-0.1, 6-0.5; 7- 1.0
Symbols used: A - Extracellular Plumbagin; B - Viability of cells
Medium used: MS basal medium with IAA 1.0mg/l, NAA 0.5mg/l and BAP 0.3 mg/l
Age of the culture: 14 days;
Initial inoculam: 500mg/40ml medium

incubated for 30 minutes (11.13 per cent). The reduction in the contact time has slightly improved the viability, however, at higher concentration of CTAB even at 15 minutes of contact time (shortest contact time attempted) appears to be highly detrimental to the cells. From the above results, it can be inferred that the treatment of the cells with CTAB is not suitable for repeated use of the biomass However, the procedure can be adopted for *in situ* extraction of metabolites from the cells to the bulk medium.

Pretreatment with Dimethyl sulfoxide (DMSO):

The experiments carried out with DMSO at various concentrations and with treatment duration of 15 minutes and 30 minutes have indicated the positive influence of pretreatment. It can be seen from the figures(3.3 & 3.4) and tables (3.4 & 3.5) that at shorter contact time of 15 minutes, the cell viability was 78.43 percent even at highest concentration of permeabilizing agent used (1.0 %, v/v). The percentage of total plumbagin released into the medium was found to be lower and only 3.5 times more plumbagin was released in comparison to the control when 1.0 per cent DMSO (v/v) was used and treatment time was adjusted to 15 minutes.

When the cells were subjected to permeabilization with DMSO for longer duration (30 min.), the cell viability decreased gradually with increase in concentration of permeabilizing agent. At the same time, percentage of metabolite released into the medium was improved. At 1.0 %, (v/v) of the permeabilizing agent, the cell viability was found to be 32.13 per cent, but the percentage of plumbagin released was 15.898 % which is nearly 7.2 times more than the control (2.21%). From the data, it can be seen that the effect of DMSO as permeabilizing agent is moderate at shorter contact time, but its effect on cell viability and plumbagin release was drastic when the contact time was increased. The effect of contact time is more pronounced

Table 3.6 Influence of Triton X-100 on plumbagin production in *P rosea* cultures

Concn. of TX-100	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% release of plumbagin	Viability (%)
0.00	0.063 [±0.004]	3.321[±0.021]	3.344	1.883	93.07 [±1.35]
0.001	0.166 [±0.011]	8.694 [±0.071]	8.814	1.883	92.08[±1.683]
0.005	0.242 [±0.031]	7.894 [±0.01]	8.136	2.97	91.13[±0.972]
0.01	0.344 [±0.019]	6.677 [±0.003]	7.021	4.9	88.43 [±1.49]
0.1	0.379 [±0.012]	5.17 [±0.014]	5.549	6.83	84.37[±1.54]

Treatment period with Triton X-100: 15 minutes

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + with or without (control) Triton X-100.

Age of the culture:14 days; Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml medium

Incubation period: 7 days at 25 ± 2°C under 14 hour light period

Data represents the average of 5 replicates in two repetitive experiments.

Numbers in parenthesis indicate ± SD

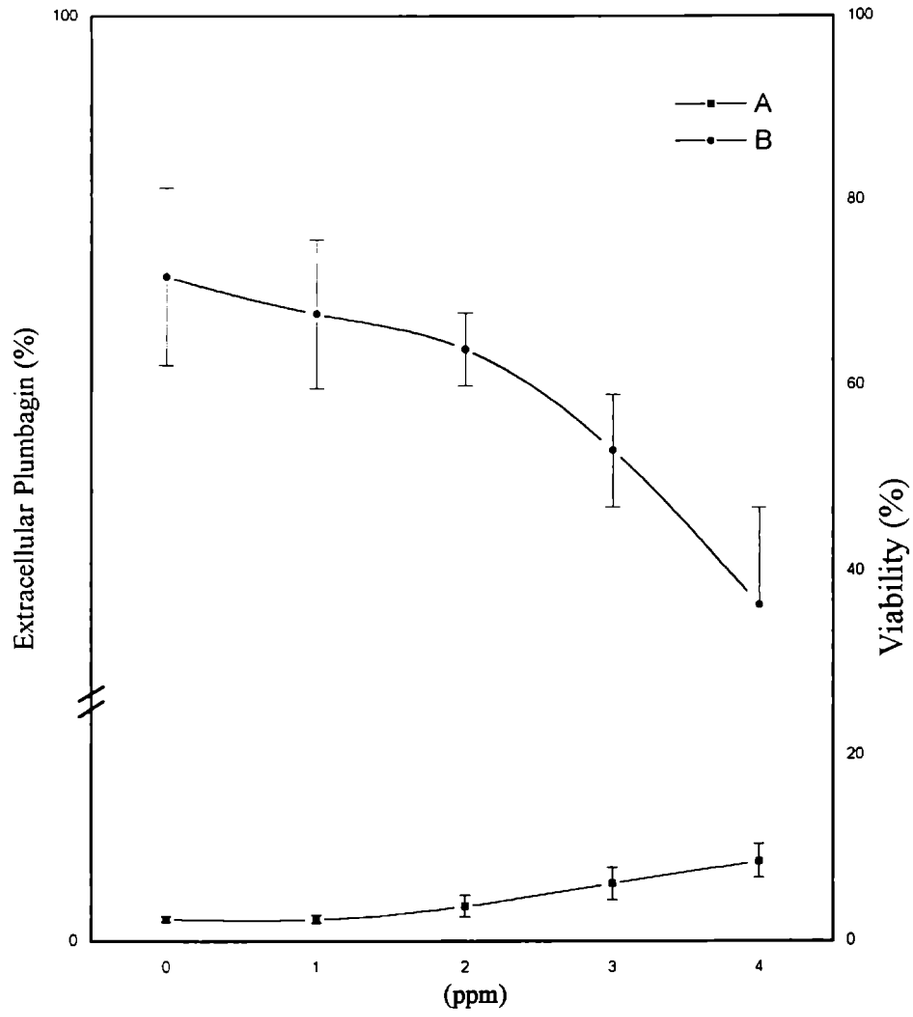

Fig. 3.5 Effect of Triton X-100 on permerabilization of *P rosea* cell cultures

Period of treatment with Triton X-100: 15 minutes
Symbols used. A -- Extracellular Plumbagin; B -- Viability of the cells
Concentration of Triton X-100 used: 0 - Nil; 1.0.001ppm; 2-0.005ppm; 3-0 01ppm,4-0.1ppm
Medium used: MS basal medium with IAA 1.0mg/l, NAA 0.5mg/l and BAP 0.3mg/l
Age of the culture: 14 days; Initial Inoculam: 500mg/40ml medium

Table 3.7 Influence of Triton X-100 on plumbagin production in *P rosea* cultures

Concn. of T X-100 (ppm)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% release of plumbagin	Viability (%)
0.00	0.063 [±0.004]	3.322 [±0.021]	3.345	1.883	93.07 [±1.39]
0.001	0.32 [±0.018]	7 751 [+±.01]	8.071	3.96	92.01 [±1.48]
0.005	0.369 [±0.023]	7.461[±0.012]	7.83	4.95	90.17 [±1.76]
0.01	1.016 [±0.026]	6.469 [±0.009]	7.485	13.573	85.47[±1.114]
0.1	1.09 [±0.63]	4.845 [±0.007]	5.935	18.37	80.1 [±1.423]

Treatment period 30 minutes

Medium used: MS medium + IAA 1.0mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + with or without (control) Triton X-100

Age of the culture: 14 days; Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml. Medium

Incubation period: 7 days at 25 ± 2°C under 14 hours light period

Data represents the average of 5 replicates in two repetitive experiments.

Numbers in parenthesis indicate ± SD

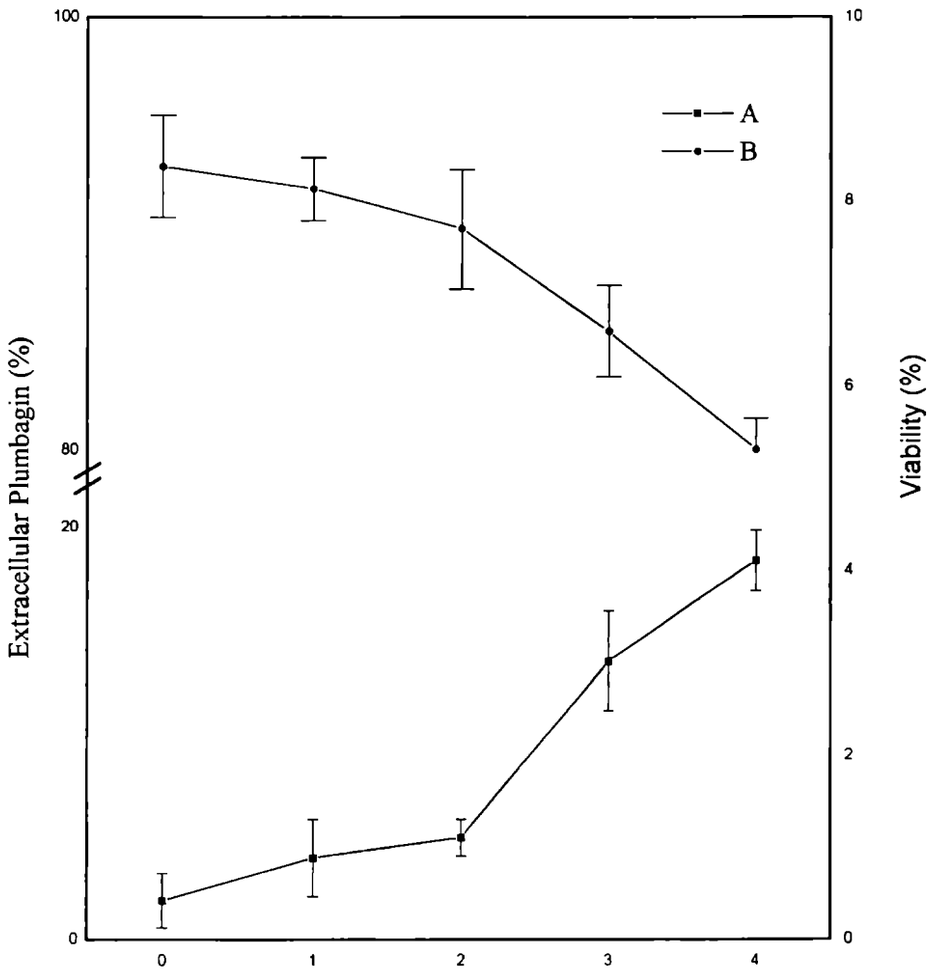

Fig. 3.6 Effect of Triton X-100 on permeabilization of *P rosea* cell cultures

Period of treatment with Triton X-100: 30minutes
Symbols used: A -- Extracellular plumbagin; B -- Viability of the cells
Concentration of Triton X-100 used: 0 -Nil;1-0.001ppm;2-0.005; 3-0.01ppm;4-0 1ppm
Medium used:MS basal medium with IAA 1.0 mg/l, NAA 0.5mg/l and BAP 0.3mg/l
Age of the culture:14 days; Initial inoculam: 500mg/40ml medium

than the concentration of permeabilizing agent on the cells. For example, 0.005 percent, (v/v) of DMSO, the cell viability was 91.06 % and the plumbagin released was 2.84 %. When the contact time extended to 30 minutes, the cell viability decreased to 82.11 per cent, which is about 8.95% less whereas the percentage of plumbagin released improved only to 2.84 to 3.7, an increase of about 1.0 %. Therefore, the effect of DMSO is more drastic at higher contact time and at higher concentration.

The influence of Triton X-100 on cell Viability and product release:

When the cells were treated with Triton X-100 at various concentrations for 15 minutes and 30 minutes, it was found that the cell viability marginally affected at all the concentrations tried, at both treatment periods. The percent of plumbagin released gradually increased with increase in concentration of permeabilizing agent and the cell viability reduced. However, even at highest concentration of permeabilizing agent tried (0.1 ppm), 80.1 to 84.37 of cell viability could be maintained in both cases. It was observed that longer contact time facilitated higher amount of product release into the media. For example, when the cells were treated with 0.1 % of Triton X-100 for 15 minutes, the percent release of plumbagin was 6.83, whereas at the same concentration, longer contact time of 30 minutes has resulted in 2.7 times higher yield of plumbagin in the medium (18.37 % of total plumbagin released). The treatment of the cells with 0.1 % of Triton X-100 for half an hour has enhanced the metabolite release into the media nearly 10 times and the cell viability was only marginally affected (80.1 percent of the cells were viable). Unlike, in the case of CTAB and DMSO, the cells treated with Triton X-100 were only marginally effected to the increase in contact time as evidenced by higher cell viability (viability percent was reduced only from 84.37 to 80.1).

Table 3.8 Influence of chitosan on plumbagin production in *P rosea* cultures

Concn. of Chitosan (mg/L)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% release of plumbagin	Viability (%)
0.00	0.075 [± 0.03]	3.366 [± 0.11]	3.391	2.21	93.7[± 1.63]
0.01	0.634 [± 0.026]	3.731 [± 0.019]	4.365	14.52	62.84 [± 0.05]
0.05	0.805 [± 0.008]	3.683 [± 0.015]	4.488	17.93	38.39 [± 1.91]
0.1	1.023 [± 0.01]	2.896 [± 0.06]	3.919	26.10	13.62 [± 2.25]

Treatment period: 30 minutes

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + with or without (control) chitosan

Age of the culture: 14 days; Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml medium

Incubation period: 7 days at 25 ± 2°C under 14 hours light period

Data represents the average of 5 replicates in two repetitive experiments.

Date in parenthesis indicate ± SD

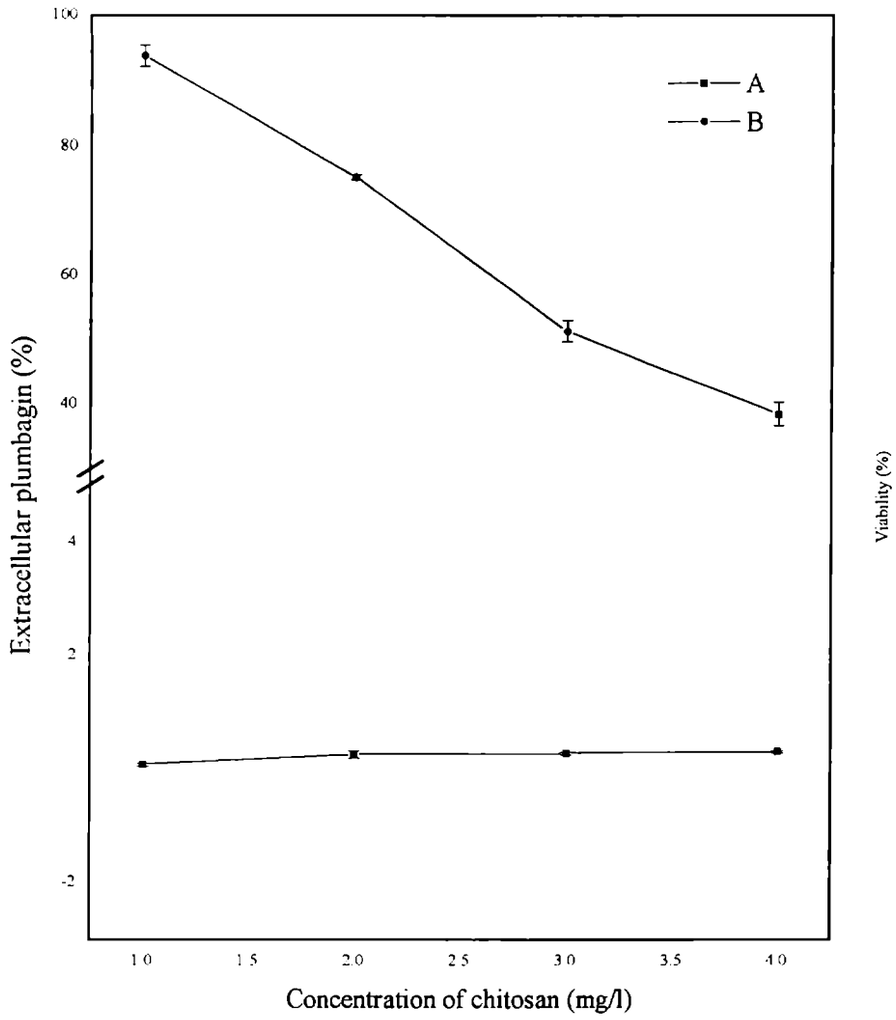

Fig. 3.7 Effect of chitosan on Plumbagin production in *P rosea* cultures

Period treatment with chitosan: 15 minutes

Symbols used: A-Extracellular plumbagin; B- Viability

Concentration of chitosan used: 0-Nil; 1-0.01, 2-0.05; 3-0.1

Ageof the culture: 14 days; Initial inoculam: 500 mg/40 ml medium

Table 3.9 Influence of chitosan on plumbagin production in *P rosea* cultures

Concn. of Chitosan (mg/L)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% release of plumbagin	Viability (%)
0.00	0.075 [± 0.03]	3.366 [± 0.11]	3.391	2.21	93.7 [± 1.63]
0.01	0.268 [± 0.07]	8.404 [± 0.123]	8.672	3.09	75.30 [± 0.341]
0.05	0.283 [± 0.023]	8.329 [± 0.16]	8.612	3.287	51.41 [± 1.66]
0.1	0.313 [± 0.017]	7.172 [± 0.02]	7.485	4.182	38.45 [± 1.78]

Treatment period 15 minutes

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + with or without (control) chitosan

Age of the culture: 14 days; Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml medium

Incubation period: 7 days at 25 ± 2°C under 14 hours light period

Data represents the average of 5 replicates in two repetitive experiments.

Numbers in parenthesis indicate ± SD

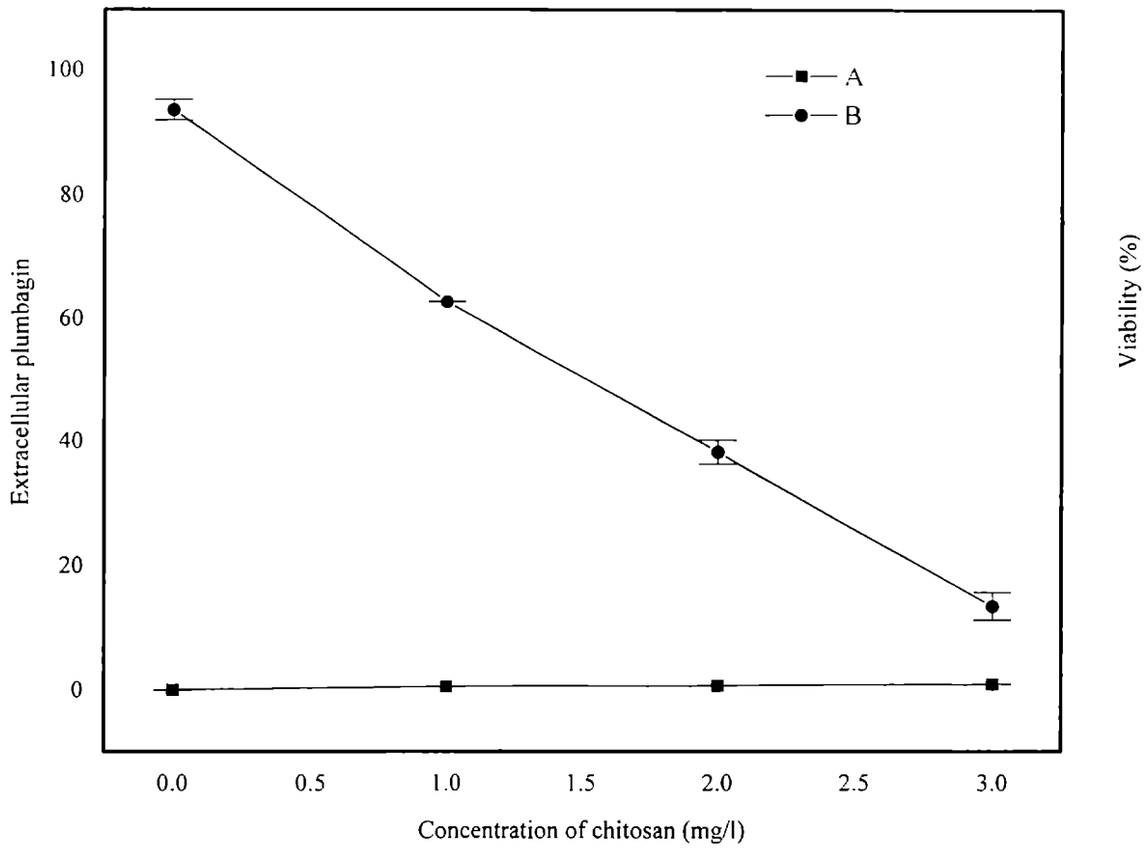

Fig. 3.8 Effect of chitosan on plumbagin production in *P rosea* cultures

Period of treatment with chitosan:30 minutes
A --Extracellular plumbagin; B - Viability
Concn. of chitosan used:0-Nil; 1-0.01; 2-0.05; 3-0.1

Chitosan as permeabilizing agent:

Experiments were also carried out using chitosan as permeabilizing agent and its effect on cell viability and plumbagin release into the medium was studied. Different concentration of chitosan (0.01, 0.05 and 0.1 mg/l) was used as permeabilizing agent and the cells were treated for 15 min. and 30 min. Concentration of chitosan higher than 0.1 mg/l affected the cells very drastically, hence concentration higher than 0.1 mg/l were not attempted further. It can be seen from figures (3.7 and 3.8) and graphs (3.7 and 3.8) that the cell viability was drastically affected at higher concentrations of chitosan as well as at higher contact time. The release of plumbagin was slightly (4.182 percent against 2.21% untreated cells) improved when the cells were treated with 0.1 ppm of chitosan for 15 minutes. But, the cell viability was almost reduced by 62 percent. At the same concentration of permeabilizing agent, when the incubation period was doubled, the release of metabolite was enhanced to 12 folds (26.1% against 2.21 %). However, the cell viability drastically reduced and 13.62% of cells were viable. The increase in contact time was found to have a drastic effect on cell viability, even at lowest concentration of permeabilizing agent tried (0.01 ppm) only 62.84 % of cells were viable when the cells were treated for 30 minutes.

Among the four permeabilizing agents tried, chitosan even at lower concentration and shorter contact time, severely affected the cell viability. Whereas, the influence of Triton X-100 was very marginal on the cell. The viability reduced to 80 percent at highest concentration and longer treatment time. In the case of DMSO and CTAB, the cell viability was drastically affected depending upon the concentration of the permeabilizing agent and the treatment period. The effect was more intensive in case of CTAB in comparison to DMSO. It can be seen from all the experiments that the total

plumbagin present after 7 days of incubation was in the range of 3.9 to 8.8 mg per g. dry biomass. Whereas in the case of untreated cells, the total plumbagin content was in the range of 2.3 to 3.39 mg per g dry weight. It can be observed from these results that the permeabilization has stimulated the secondary metabolism considerably as evidenced by increased yield of plumbagin. This can be attributed to the stress inducing nature of the permeabilizing agent. Since plumbagin (a naphthoquinone) being a stress induced metabolite (Nahalka *et al.* 1996),[109] naturally its production might be influenced by the stress causing agents such as permeabilizing agents. The treatment has increased the yield to 2-2.5 folds more than the control. However, the presence of plumbagin in the medium is influenced by the magnitude of the treatment procedure. It can be seen from the tables that the percentage release of plumbagin into the medium is depending upon the nature of the permeabilizing agents, contact time and concentration. A higher concentration of CTAB (0.5ppm) has resulted in 71.1–73.63% of plumbagin into the medium. Whereas 0.1%, (v/v) of DMSO and Triton X-100 (ppm) has accounted 7.84 and 6.83%, respectively, of plumbagin released into the medium when treated for 15 minutes. When the treatment time was increased to 30 minutes, the product release improved to 15.9 % and 18.37 %, respectively. Though, the cell viability was drastically affected by chitosan treatment, the release of plumbagin was only moderate and 26.1% of plumbagin released when cells were treated with 0.1 mg/l of chitosan for 30 minutes. From these experiments, the following conclusions can be drawn: -

- Among the four permeabilizing agents, CTAB was the best (73% of the plumbagin released), however, the cell damage was quite severe (82% of the cells died)
- Cells treated with chitosan affected significantly and cell viability reduced considerably by higher treatment duration. The percent release of plumbagin is not proportional to the percent cell damage.
- Among Triton X-100 and DMSO, Triton X-100 was found to be mild and 80% of the cell viability can be maintained even after treating the cells with 0.1 ppm of the chemicals for a duration of 30 minutes. At the same concentration of DMSO and contact time, the cell viability was only 48.73 %. The percentage of alkaloid released with DMSO and Triton X-100 was moderate and only 7.8 and 18.37% at the highest concentration and longer duration of treatment, respectively.
- CTAB, though very effective to release the alkaloid into the medium cannot be used as a permeabilizing agent, because of severe damage to cells. From the above studies, it can be inferred that for repeated use of the cells, Triton X-100 at 0.1ppm and contact time of 30 minutes was the best. Whereas the treatment with CTAB, resembles *in situ* extraction of metabolite and cells can not be reused.

3.4 Discussion

With a view for using plant cells in biotechnological process, it is desirable to harvest secondary compounds without destroying the cells. Since the majority of the plumbagin, is stored within the cells, we investigated the influence of various permeabilizing agents on metabolite release and cell viability Reports are available on the influence of DMSO

and chitosan as permeabilizing agent for *Chenopodium rubrum* for the release of amaranthine.[64] The authors have reported that at higher concentrations of the permeabilizing agents the cell's damage was severe and DMSO at a concentration of 0.42 mg/g fresh weight, chitosan 0.77 mg/g fresh weight have found to be optimum for amaranthine release without loosing the cell activity. The treatment period was 4-8 days. However, in the present investigations we have kept the treatment period, 15-30 minutes only. Despite the shorter contact time, chitosan treatment appears to be very detrimental to cell viability. Whereas DMSO has found to be, moderate both in cell viability as well as in product release. Knorr and Teutonico [110] also suggested that chitosan gel can serve concurrently as an effective immobilizing and permeabilizing agent for cultured plant cells as in *Amaranthus tricolor.* DMSO is one of the widely used permeabilizing agents in plant cells and the treatment periods vary from few hours to 12-25 days. Parr, et al. (1984)[46] has reported severe damage to the cells at above 0.5percentage of DMSO concentrations. However, the alkaloid release was negligible at that level. Literature so far available indicated that either lower concentration of DMSO for longer duration or higher concentration for shorter contact time was sufficient for release of the metabolites. However, in the present investigations we have fixed the contact time for only 15-30 minutes and the concentration of DMSO 0.001% to 0.5% (v/v). However, the cell viability and alkaloid release varied significantly From these studies, it can be inferred that the influence of DMSO on plant cells cannot be generalized but specific to each cell line. Brodelius (1988) has studied the effects of DMSO and Triton X-100 on the permeability of *Catharanthus roseus, Chenopodium rubrum and Thalictrum rugosum.*[8] This study indicated that only *C. roseus* cells could withstand the treatment with DMSO

and Triton X-100 without loosing viability Parr et al. (1984) have observed that the suitability of DMSO for permeabilization varies with the culture and demonstrated that *Cinchona ledgeriana* cells were permanently damaged by the levels of DMSO necessary to release products.[46] However, Brodelius has reported the successful use of DMSO at the same concentrations with *C. roseus*.[8]

Accoring to Young *et al.* (1982),[111] chitosan binds to polygalacturonate, a plant cell wall component, and induces the leakage of low molecular weight compounds as well as some proteins (> 5,000 D). The increase in cell permeability of chitosan may be due to disruption of the intermolecular bonding responsible for maintaining an intact membrane, change in membrane fluidity, or effects on the components associated with membrane transport (Young and Kauss, 1983).[112]

Chemical permeabilization for the release of intracellularly stored product appears to be appropriate only in limited number of cell lines if sustained cell viability is desired. The low viability of the treatments most likely not due to the toxic nature of permeabilizing agent but due to destruction of cell compartmentation and to the release of toxic compounds and degradive enzymes (e.g. proteases). However, this technique may find use in future in downstream processing of cultured plant cells. Further permeabilized plant cells may be also used for bio-conversion of substrates that are not taken up by intact cells (Fuller and Bartlett, 1985).[113]

The present study employed four different permeabilizing agents at various concentrations and resulted in varied degree of cell damage and product release depending upon the contact times (15 min. and 30 min.). Unlike the earlier reports, we have used very low concentrations of permeabilizing agent and very short treatment duration. But, even these

mild conditions appear to be very severe to the cells of *Plumbago rosea.* Our results are in agreement with the earlier studies conducted by Brodelius (1988)[8] and Parr, *et al.* (1984)[46] where in authors were reported that the cell's response to the permeabilization treatment vary with cell lines. Only *C. roseus* cells were suitable for permeabilization at a level where product release can be accepted. A number of speculative suggestions relating to different behavior of *P rosea* compared to that of *C. rosea* can be put forward. The product exchange may be very rapid in *C. roseus* whereas in other cell lines, this transport may be slow. The involvement of cytoplasmic membrane protein in product transport has to be investigated. Our results clearly demonstrate that enhanced plumbagin release can be accomplished from the cells of *P rosea* by treating the cells with Triton X-100 for shorter duration without cell damage. Significant release can be achieved by the use of CTAB, but the cells loose viability.

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CHAPTER IV

ELICITATION

INTRODUCTION

Cell cultures of higher plants have long been recognized as a potential source of commercially valuable biochemicals such as pharmaceuticals and other biologically active compounds.[1-5] However, it is a characteristic feature of cultured plant cells that they fail to produce the secondary metabolites over extended periods in amounts comparable to those found in the whole plant. For instance, *Catharanthus roseus* cell culture do not accumulate the dimeric indole alkaloids, as occur in leaves and as had been expected.[6] So far, the manipulation of culture media, culture conditions and phytohormone levels have, in general fail to produce the phytochemicals to a level economically feasible for commercial scale production. In number of cases, it has also been suggested that the biosynthesis of compounds have to be often related to differentiation of cells. For example, in *Papaver somniferum*, absence of morphinan alkaloids was due to the lack of laticifers in the unorganized culture.[7] In some cases, production of favoured compound requires nutritional components entirely different from the medium which supports the optimum growth of biomass.[8,9] Therefore, the usage of two stage of culture practice is essential for the successful production of compounds in the cell culture.

Usually, plant cells under culture initially proceed through a dormant phase of growth, the lag phase, which is characterized by no apparent life activities. During that period, the cells gather all essential components required for primary metabolism through catabolic pathways

(e.g. Glycolysis, Pentose Phosphate Shunt and Tricarboxylic acid Cycle). The succeeding exponential phase is the most important and lengthy period in a cell cycle, which is favored by the accumulation of primary metabolites such as carbohydrates, amino acids, proteins, nucleic acids and its precursors. The last, but the most important phase as far as the synthesis of secondary products is concerned, is the stationary phase during which period all the key intermediary products of primary metabolism (e.g. sugars, amino acids, nucleotides, acetyl CoA etc.) are utilized for the synthesis of secondary metabolites. For example, cyanogenic glycosides and glucosinolates are derived from sugars. Acetyl CoA is the key precursor of terpenes and steroids. The nucleotide bases are the precursors to purine and pyrimidine alkaloids (e.g. nicotine and caffeine). Tryptophan is the starting material of many important secondary metabolic pathways such as indole alkaloids (ajmalicine and catharanthine) and quinoline alkaloids (e.g. quinine and quinidine). For the purpose of increasing the yield of secondary metabolites in cell culture, it would be beneficial to be able to "switch on" secondary metabolism early in the growth cycle. For example, the removal of regulatory repressors, genetic manipulation of enzyme pathways, or the addition of specific metabolic inducers could dramatically increase secondary metabolism.[10]

Plant cell cultures have often been shown to increase the production of secondary metabolites under specific environmental or physiological conditions such as stress or exposure of plant tissue to cell wall fragments of microbes.[11-13] The microbe-derived molecules which stimulate secondary metabolism have been called 'elicitors' Elicitors can be derived from microbial culture fluids or plant and fungal cell walls and include oligosaccharides, chitin oligomers, peptides, proteins, glycopeptides and

lipids.[14-17] The abiotic elicitor comprises UV light,[18] pH changes[19] and heavy metal ions.[20]

Generally, the tissues accumulate low molecular weight compounds showing antibacterial properties called 'phytoalexins' at the site of infection.[21-23] Numerous phytoalexins of varying chemical structures have been isolated from a great number of plants.[24,25] The structure of phytoalexins seems to be a characteristic for the genus or family of plant taxonomy Plants from the *Leguminosae, solanaceae, Compositae* and *Convolvulaceae* produce predominantly isoflavanoids, carbocyclic sesquiterpenoids, polyacetylenes and furano-sesquiterpenoids, respectively.[22,26,27] Though, very different in chemical structure, phytoalexins by definition formed only under stress or infectious conditions which documents the great potential for the formation of new secondary compounds.

Elicitor-induced accumulation of secondary metabolites have received wide acceptance because it can improve the efficiency of metabolite production in systems where the product formation appears near or after the exponential phase. In addition, the products accumulate within a short exposure period of 24-72 hours after elicitation as compared to 2-3 weeks or more in non-elicited cultures. The technique also serves to eliminate media exchange as a means of promoting secondary product formation. Metabolites, typically accumulate not only within the cells, but, in several instances large amounts are found excreted into the medium.[28] In *Capscicum frutescens* cell cultures, elicitor treatment leads to the initiation of a secondary pathway which is only present in one developmental stage (fruit ripening) in the whole plant. Furthermore, elicited cells can be used for the biotransformation reactions due to the induction of key enzymes.[29,30]

Biotic elicitors

Although molecules from various sources have shown the property of inducing secondary metabolite production, the most extensively studied elicitors are obtained from fungal sources. One of the most extensively used elicitors for the induction of plant secondary metabolism is from the fungus *Phytophthora megasperma*.[14,44] The yeast (*Saccharomyces cerevisiae*) or yeast extract is another more commonly used elicitor. With yeast extract, the production of berberine from *Thalictrum rogosum* cells was increased upto 4 times and the production of alkaloids by *Eschscholtzia californica* upto 30 times.[31] Elicitors from *Botrytis cinerea, Cercospora lingustr* and *Pythium ultimum* have been tested in *Tagetus patula* and have shown an increase in the production of thiophene about 100 times.[32,33] For *Bidens sulphureus*, the fungi *Pythium aphanidermatum* and *Phytophthora dreschleri* increased the alkaloid nproduction 20 times as high as without elicitor.[34] Eilert, *et. al.* (1984) reported that the co-culture of *Rhodotorula rubra* with cells of *Ruta graveolens* resulted in 100-fold increase in acridone epoxide alkaloid synthesis.[35] Other elicitors of microbial origin that are commonly used for increasing the production of plant secondary metabolites are *Aspergillus niger, Rhodotorula rubra, Colletotrichum lindemuthianum, Botrytis Sps., Verticillium dahliae, Pythium aphanidermatum, Bacillus subtilis* and *Pseudomonas Sps.*

Abiotic elicitors

Abiotic elicitation has been used to investigate the metabolic pathway involved in the biosynthesis of phytoalexins and to aid in the dissection of complex plant-microbial infections.[36,37] Threlfall and Whitehead (1988)[38] reported that metal ions are effective in inducing the formation of phytoalexins of sesquiterpenoids type in cell suspension cultures of *Datura*

stramonicum. It was observed that metals even at low concentration cause drop in medium pH as well bring about cell aggregation. Studies also confirmed that metals, in many cases, form complexes with the cell wall proteins whose structure is altered consequently, and this conformational change then acts as a trigger to a chain of events, which culminates in the *de novo* synthesis of phytoalexins. In certain cases, metal ions may act by causing the formation of hydroxyl radical OH, which may damage cell membrane, due to oxidative burst.[39]

There are some encouraging reports on the use of metal ions to induce or enhance the production of pharmaceuticals in cell suspension cultures. Fujita *et al.* (1981)[8] reported that the addition of copper at a level of 30 times higher than normal medium stimulated a dramatic increase in shikonin production in the cell suspension cultures of *Lithospermum erythrorrhizon*. At elevated levels of Cu^{2+} induce sharp increase in plasma membrane permeability and hence ionic imbalance, thereby, sometimes may lead to osmotic stress within the system.[40] Smith *et al.* (1987)[41] reported that vanadium sulphate (10-500mg/l) when added to *Catharanthus roseus* cell culture increased the intracellular accumulation of catharanthine and ajmalicine.

Elicitation through metal ions seems to depend on cell line, type of culture, stage of culture, choice of metal ions as well as toxicity of metal ions. However, being a cheaper source, ready availability and defined chemical structure, it offers many advantages over biotic elicitors.

DiCosmo and Misawa (1985)[9] reviewed the use of elicitors and other inducers as modulators of secondary metabolism in cultured plant cells. The technique showed great promise for allowing the induction and accumulation of commercially valuable phytochemicals. Table 4.1

illustrates a range of compounds that are synthesized rapidly, in increased amount by cultured plant cells in response to elicitation using various elicitor molecules.

Table 4.I Elicitor stimulated accumulation of secondary metabolites in cells cultured *in vitro*

Compound	Plant cell culture	Elicitor	References
Capsaicin Capsidiol	*Capsicum frutescens*	*Gliocladium* deliquescens	Holden, *et al*,1986 [45]
solavetivone	*Solanum eleagnifolium*	*Aspergillus niger*	Paniego *et al.* 1995 [46]
Ajmalicine Catharanthine	*Catharanthus roseus*	*Chrysosporium palmorum*	DiCosmo *et al* 1987 [47]
Indole alkaloids	*C. roseus*	Vanadium sulfate	Smith *et al* 1987 [41]
Callose Coumarins	*Petroselinum crispum*	Chitosan	Conrath *et al* 1989 [48]
Capsaicin	*C. frutescens*	Chitosan	Johnson *et al* 1991[49]
Tagetus patula	*A. niger*		Buitelaar *et al*,1992[50]
Medicarpin Maackiain	*Cicer arietinum*	Yeast	Mackenbrock *et al* 1993[51]
Tropane alkaloids	*Datura innoxia*	Calcium chloride	Gontier *et al.* 1994 [52]
Catharanthine Ajmalicine	*C. roseus*	*Pythium*	Nef-campa *et al* 1994[53]

Catharanthine Ajmalicine	*C. roseus*	*Penicillium* sps.	Sim *et al.* 1994 [54]
Acridone Epoxide	*Ruta graveolens*	*Rhodotorula rubra*	Bohlmann, 1994 [55] Park *et. al* 1995 [56]
Coumaryl Aminoacids	*Ephedra distachya*	Yeast	Song *et al.* 1995 [57]
Xanthotoxin Bergapten	*Petroselinum hortense*	*Phytophthora megasperma*	Hauffe, *et al.* 1986 [58]
Codeine Morphine	*Papaver somniferum*	*Verticillum dahliae*	Heinstein *et al.* 1985 [59]
Shikonin	*Lithospermum erythrorrhizo*	Agaropectin	Fukui, *et al.* 1983 [43]
Diosgenin *deltoidea*	*Dioscorea arrhizus*	*Rhizopus*	Rokem *et al* 1984 [60]
Sanguinarine	*P somniferum*	*Botrytis sps.*	Eilert *et al.* 1985 [61]
Isoflavonoids Daidzein	*Vigna angularis*	*P. megasperma* glucan	Hattori & Ohta, 1985 [62]
Glyceollin	*Glycine max*	*P. megasperma* Spores	Keen & Horsch, 1972 [63]
Glyceollin	*Glycine max*	*P. megasperma* Cell wall	Ebel *et al* 1976 [60]
Ajmalicine Catharanthine	*C roseus*	*Pythium aphanidermatum*	Tallevi *et al.* 1986 [64]
Anthra-quinones	*Cinchona ledgeriana* *Rubia tinctorum*	*Aspergillus niger*	Wijnsma *et al.* 1986 [65]
Cephalotaxus Alkaloids	*Cephalotaxus harringtonia*	*Verticillum dahliae* spore	Heinstein, 1985 [59]

Debneyol	*Nicotiana tabacum*	*V dahliae*	Heinstein, 1982 [67]
Gossypol	*Gossypium arboreum*	*V dahliae*	Heinstein 1982 [67]
Phytuberol	*N. tabaccum*	*Pseudomonas solanacearum*	Fujimori *et al.* 1983 [68]
Sanguinarine	*P. somniferum*	*Rhodotorula rubra*	Eilert *et al.* 1985 [69]
Furano-Coumarins	*Glehnia littoralis*	Yeast extract	Kitamura *et al.* 1998 [70]
Podophyllotoxin	*Juniperus*	Chito-oligosaccharide	Murannaka, 1998[71]
Solasodine	*Solanum surattense*	Aspergillus & Fusarium	Malpathak & David, 1992[72]
Capsidiol	*N. tabccum*	*Phytophthora cryptogea*	Milat,et.al. 1991[73]
Acridone	*Ruta graveolens*	Yeast extract	Baumert *et al.* 1991[74]
Phytuberin	*Solanum tuberosum*	*Phytophthora infestans*	Ersek & Sziraki, 1980[75]

P rosea cell cultures produce plumbagin and many other compounds belonging to the category of naphthoquinones. Durand and Zenk[42] suggested that the biosynthesis of plumbagin be through the polyketide-mevalonate pathway, which is concerned with the synthesis of phytoalexins in plants. More over, the effectiveness of elicitation on naphthoquinone production has been mentioned in the literature. For example, in *Lithospermum erythrorrhizon* cell culture, addition of agaropectin increases shikonin, a naphthoquinone, production 4 times higher than non-elicited cells.[43] In this

study, we were attempted to study the influence of various biotic elicitors from fungi, bacteria and yeast and abiotic elicitors on the production of plumbagin.

4.2 Materials and methods

Plant cell culture:

The cell suspension cultures of *P rosea* were maintained in MS medium supplemented with 1.0 mg/l of IAA, 0.5 mg/l NAA and 0.3 mg/l of BA at pH 5.8 (± 0.2) in 250 ml. Erlenmeyer flasks. The cultures were transferred to fresh medium to an inoculam density of 1.0 g fresh weight per 100 ml. of medium which was used as the stock culture. From the above stock culture, 0.250 g. of cells were transferred to pre-weighed Erlenmeyer flasks containing 40 ml. of sterilized MS medium and these cultures were treated with different concentrations of elicitors prepared from various sources such as fungi, bacteria, yeast and metal ions. Incubation period of 48-72 hours was given for biotic elicitors. Cultures were grown in medium containing 10, 20 and 30 times the concentration of inorganic salts in standard MS medium for a period of 16 days to study the effect of metal ions.. After the required treatment period, cultures were harvested and both biomass and plumbagin content was estimated. All the cultures were maintained under 14 hour light period at a room temperature of 28 (± 1 °C) on a rotary shaker at 80 rpm. For each treatment, five replicates of samples were used.

Preparation of elicitor of fungal origin:

The fungi used for elicitation experiments were strains of *Aspergillus niger* and *Rhizopus nigricans*. An isolate of *A. niger* (RRL 103), the culture collections at the Regional Research Laboratory, Trivandrum maintained on agar-solidified (1.5%) potato dextrose broth containing dextrose 20g/l and

potato 200g/l at pH 5.6 (± 0.2) were used as inoculam. *R. nigricans* culture was purchased from culture collections at National Chemical Laboratory, Pune which was also maintained at the same culture conditions.

Pieces of fungal mycelial mat (approximately $1cm^2$) from a seven-day old fungal culture were used to inoculate 40 ml. liquid MS basal medium containing 3 % sucrose in 250 ml. Erlenmeyer flasks. The cultures were kept at 28° C for 7 days as static culture at room temperature. After sporulation (at stationary phase) the culture medium was filtered through Whatman No.1 filter paper placed on a Buchner funnel. The spent medium was filter sterilized with sterile 0.22μ filter paper (Millipore) using a sterilized Millipore filter unit. Volume of the sterilized spent medium was made up to 50 ml. with sterilized MS basal medium. The mycelial mass was washed thoroughly with double distilled water and was then homogenized with acid treated sand in 15-20 ml of MS basal medium for 15 minutes, and subsequently autoclaved for 20 minutes at 121°C. The autoclaved homogenate was filtered through sterilized Whatman No.1 filter paper using Buchner funnel and the volume of the mycelial hydrolysate was made up to 50 ml. with MS basal medium. Both sterilized spent medium and mycelial hydrolysate were used as elicitors without further purification.

Preparation of elicitor from *Saccharomyces cerevisiae*:

Stock cultures of *S. cerevisiae* was maintained on MGYP medium containing malt extract 3 g/l, glucose 30 g/l, yeast extract 3 g/l and peptone 5g/l at pH 6.4 (± 0.4). the pH was adjusted with 1-3 drops of 0.1 N sterilized tartaricacid. All the cultures were maintained in culture tubes as slant culture.

One to three loopful of bacterial stock culture was transferred to 40 ml. MGYP broth in 250 ml. Erlenmeyer flasks and kept on a gyratory shaker (120 rpm) at room temperature (32 °C) for 4 days. The cells were separated from the medium by centrifugation at 8,000 rpm for 10 minutes. The supernatant was decanted and discarded. The pellet was suspended in double distilled water and again centrifuged. The same process was repeated for 3 times with 25 ml. distilled water. The pellet was suspended in 50 ml. double distilled water and autoclaved for 20 minutes (121° C). The sterilized crude extract was used as elicitor source.

Elicitor from bacterial source:

The bacteria used for elicitation was *Bacillus cereus*, (provided by PCBT unit, Central Food Technological Research Institute, Mysore). The bacterial stock culture was maintained on Nutrient Agar medium (Bacto-beef extract 3 g/l, Bacto-peptone 5g/l and agar 15 g/l) at room temperature.

From the seven-day old *B. cereus* cultures two to three loopful of bacterial culture was inoculated in Nutrient broth and kept at room temperature on a gyratory shaker at 120 rpm for 3 days. The culture was centrifuged at 8,000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed three times with 25 ml. of double distilled water and centrifuged. Finally, the pellet was suspended in 50ml. double distilled water and autoclaved for 20 minutes at 121°C. This preparation was used as crude elicitor for all the experiments.

Method of elicitation:

Elicitors prepared from various microbial sources were added at a concentration of 0. 5%, (v/v) to 2.5 %, (v/v) to 40 ml. of 14-day old cell suspension culture containing approximately 0.5g of cells. Control cultures received the same amount of sterilized distilled water. Cultures were

harvested after 48 hours and 72 hours, respectively The effect of day of elicitation on plumbagin production was performed by adding the best elicitor concentration on 5^{th} 8^{th} 12^{th} and 16^{th} day of culture period and treated the cultures for 72 hours. The inorganic salts used in MS medium (e.g. calcium chloride, sodium molybdnate, Manganese sulfate, zinc sulfate and copper sulfate) was added at 10, 20 and 30 times the concentration of standard medium for studying the influence of metal ions. Cells were incubated in medium containing required amount of metals ions and harvested at 5^{th}, 8^{th}, 12^{th} and 16^{th} day of incubation. After the required period of elicitor treatments, cells were harvested from the culture medium and total plumbagin content was determined. Effect of medium pH on metabolite production was also studied by growing the cultures at pH varying between 3.5 to 7.0.

Alkaloid extraction and analysis was done as described in Chapter III.

4.3 Results

Even though many reports are available on the possibility of using biotic and abiotic elicitors for the enhancement of secondary metabolite in culture systems, no elicitor have been found to have a general effect on many systems or no system has been found to respond to all the elicitors. Hence, the screening of elicitors have been done based on the available reports and on the availability of the materials. Moreover, since the concentration of elicitor, and incubation period required for maximum elicitation differs, with the kind of elicitor and cell system, an empirical screening programme has been adopted to find out the suitable elicitors for maximum plumbagin production. The elicitors screened in this study

included the media filtrate and mycelia/cell extracts of various fungi, yeast and bacteria and abiotic elicitors such as metal ions.

Our preliminary studies showed that callus of *P rosea* in the exponential phase was suitable for elicitation process and hence for all experiments, 14 days old cultures were used. It was also noticed that the *P rosea* cultures showed maximum response to elicitation within 72 hours of incubation with various biotic elicitors. Hence, all further studies were limited to 48 hours and 72 hours. The elicitors from microbial sources were used at five different levels (0.5, 1.0, 1.5, 2.0 and 2.5%, v/v) and compared to a control without elicitor. On the other hand, the metal ions were used at a concentration of 10, 20, and 30 times the concentration of corresponding salts in the standard MS basal medium and the response was monitored at every 5 days interval. Since, the microbial growth itself affects the secondary metabolism of plant cultures, all the fungi except yeast and *Bacillus cereus* were grown in MS basal medium. The elicitor activity of PDA and MGYP medium alone was tested in the preliminary experiments and found that 2.5% level of the growth medium had no virtual influence on the production of secondary metabolites in *P rosea* cultures.

Influence of fungal elicitors

In the present study, elicitors derived from *Aspergillus niger, Rhizopus nigricans, R. arrhizus, Bacillus cereus, Pseudomonas Sps.* and *Saccharomyces cerevisiae* were screened and used for active elicitor source. Elicitor inducing plumbagin concentration >110% (control=100%) were considered as an active elicitor and used for further studies. Hence, *Pseudomonas Sps.* and *R. arrhizus* were not used for further studies.

Table 4.2 Influence of *Aspergillus niger* spent medium as elicitor on plumbagin production in *P rosea* cultures

Concn. of elicitor (%, v/v)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt	% increase over control
0.00	0.156 [±0.03]	2.943 [±0.111]	3.099	00.00
0.5	0.220 [±0.009]	6.365 [±0.364]	6.585	I12.49
1.0	0.171 [±0.016]	8.91 [±0.237]	9.081	193.03
1.5	0.240 [±0.033]	9.530 [±0.293]	10.977	215.263
2.0	0.124 [±0.27]	9.812 [±0.148]	9.936	220.62
2.5	0.167 [±0.048]	10.01 [±0.194]	10.177	228.396

Period of treatment with elicitor: 48 hours

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + sucrose 4 % + with or without (control) elicitor

Age of the culture:I4 days

Inoculam:500 mg ±30 mg fresh tissue in 40 ml. medium

Data represents the average of 5 replicates in two repetitive experiments.

Data in parenthesis indicates ± SD

Table 4.3 Influence of *Aspergillus niger* spent medium as elicitor on plumbagin production in *P rosea* cultures

Concn. of elicitor (%, v/v)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% increase over control
0.00	0.123 [±0.009]	2.983 [±0.364]	3.106	00.00
0.5	0.220 [±0.019]	4.875 [±0.279]	5.095	64.04
1.0	0.190 [±0.110]	7.748 [±0.166]	7.938	155.57
1.5	0.213 [±0.092]	9.60 [±0.301]	9.813	215.94
2.0	0.234 [±0.026]	8.392 [±0.118]	8.626	177.72
2.5	0.205 [±0.211]	8.072 [±0.906]	8.277	166.48

Period of treatment with elicitor: 72 hours

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + sucrose 4 % + with or without (control) elicitor

Age of the culture: 14 days

Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml. medium

Data represents the average of 5 replicates in two repetitive experiments.

Data in parenthesis indicates ± SD

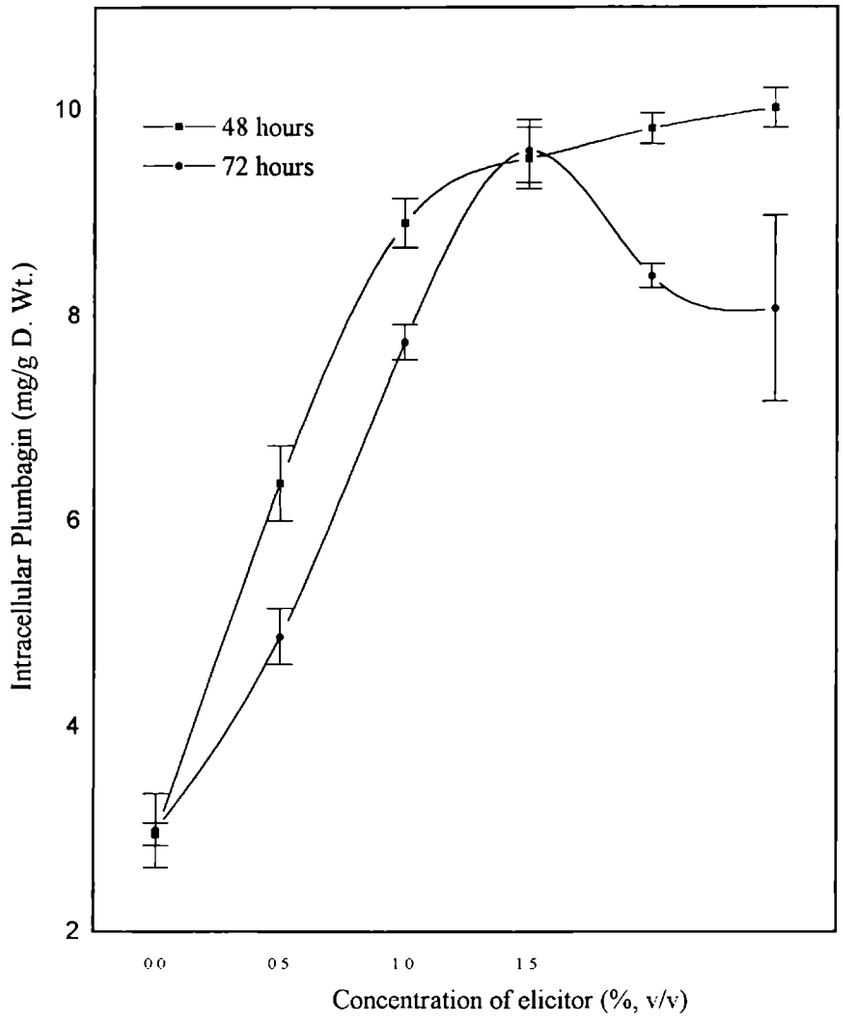

Fig. 4.1 Effect of *A. niger* spent medium as elicitor on plumbagin production in *P rosea* cultures

Incubation period: 48 hours and 72 hours.
Age of the culture:14 days.
Initial cell bimass: 500 mg/40 ml medium.
Medium used: MS basal medium + IAA 1.0mg/l
+ NAA 0.5 mg/l + BAP 0.3 mg/l

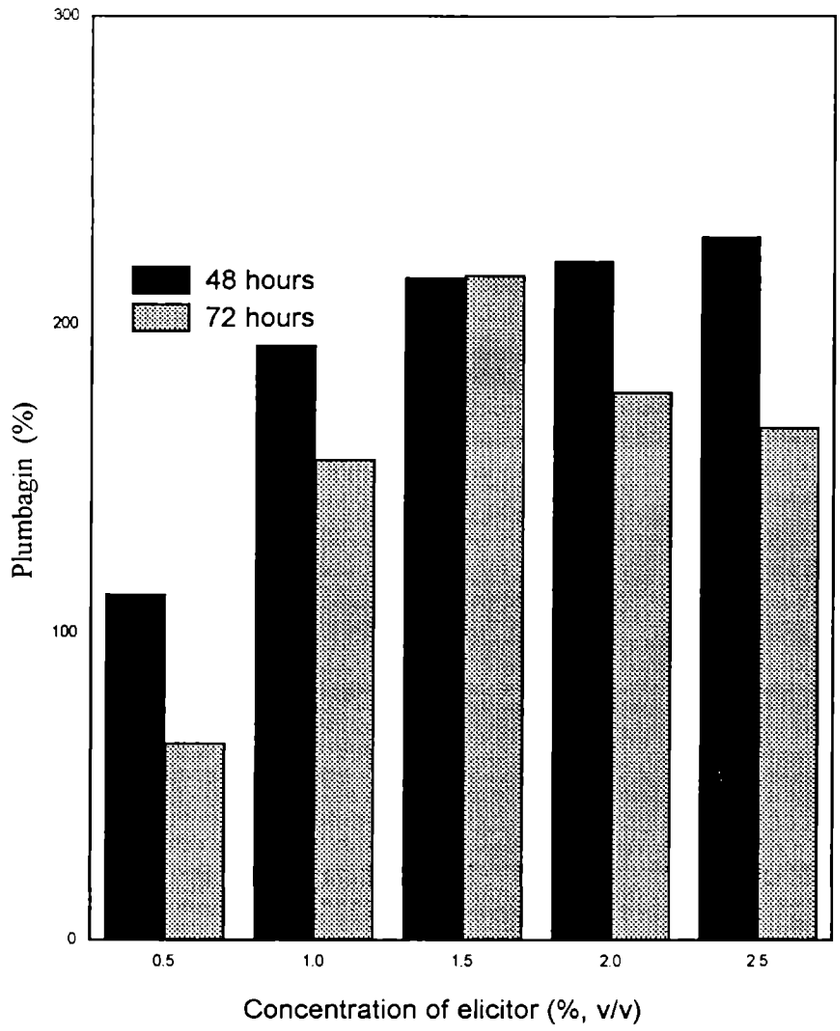

Fig. 4.2 showing the percentage increase of plumbagin production over untreated cultures at various concentration of elicitor and incubation periods

Source of elicitor: ***A. niger* spent medium**
Incubation periods: 48 hours and 72 hours; Age of the culture: 14 days
Initial biomass of the culture: 500mg/ 40 ml medium
Medium used: MS basal medium + IAA 1.0 mg/l
+ NAA 0.5 mg/l + BAP 0.3 mg/l

Table 4.4 Influence of *Aspergillus niger* mycelial hydrolysate as elictor on plumbagin production in *P rosea* cultures

Concn. of Elicitor (%, v/v)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% increase over control
0.0	0.118 [±0.03]	3.198 [±0.237]	3.316	00.000.
0.5	0.145 [±0.064]	3.700 [±0.149]	3.845	15.953
1.0	0.230 [±0.033]	4.586 [±0.208]	4.816	45.240
1.5	0.209 [±0.17]	4.770 [±0.169]	4.979	50.151
2.0	0.167 [±0.045]	4.821 [±0.11]	4.988	50.422
2.5	0.187 [±0.038]	4.895 [±0.276]	5.082	53.260

Treatment period: 48 hours

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + sucrose 4 % + with or without (control) elicitor

Age of the culture: 14 days

Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml. medium

Data represents the average of 5 replicates in two repetitive experiments.

Data parenthesis indicates ± SD

Table 4.5 Influence of *Aspergillus niger* mycelial hydrolysate as elicitor on plumbagin production in *P rosea* cultures

Concn. of eliciotr (%, v/v)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% increase over control
0.0	0.154 [±0.022]	3.157 [±0.451]	3.311	00.00
0.5	0.177 [±0.07]	3.78 [±0.337]	3.957	19.511
1.0	0.231 [±0.041]	7.940 [±0.349]	8.171	146.78
1.5	0.209 [±0.12]	8.120 [±0.181]	8.329	151.56
2.0	0.178 [±0.063]	8.450 [±0.237]	8.628	160.59
2.5	0.167 [±0.084]	8.312 [±0.119]	8.479	156.09

Incubation period: 72 hours

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + Sucrose 4 % + with or without (control) elicitor

Age of the culture: 14 days

Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml. medium

Data represents the average of 5 replicates in two repetitive experiments.

Data in parenthesis indicates ± SD

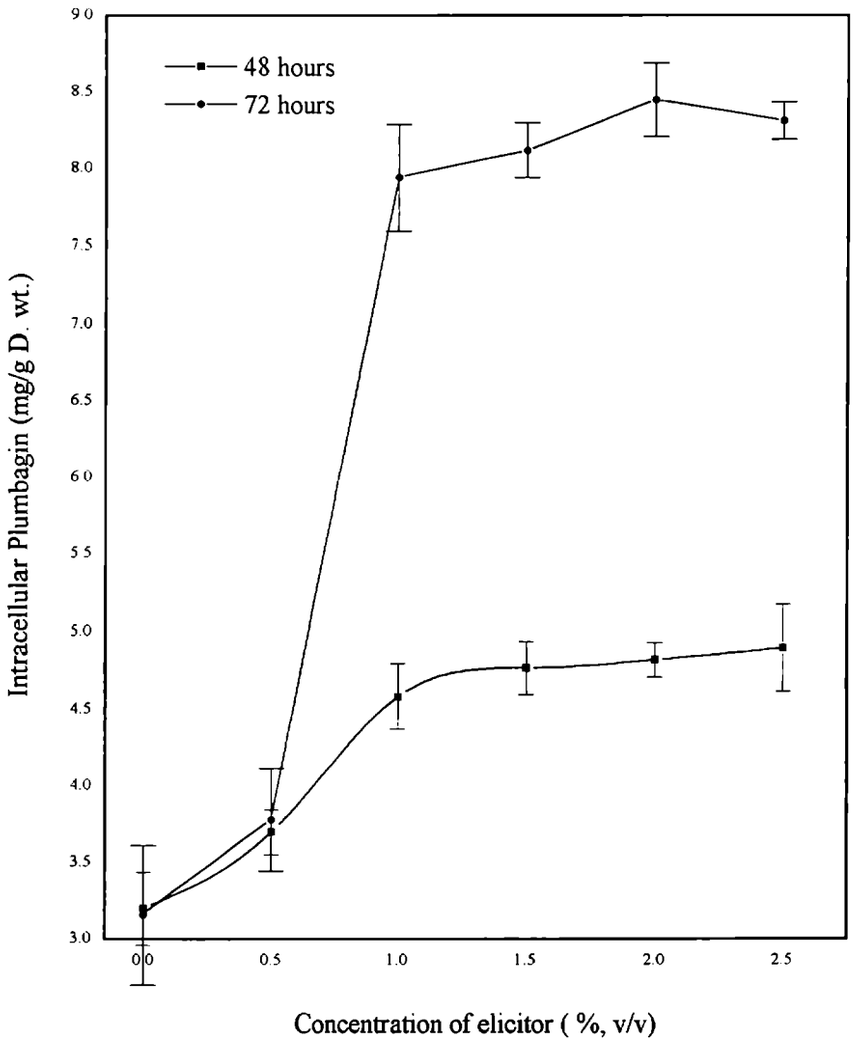

Fig. 4.3 Effect of *A. niger* mycelial hydrolysate as elicitor on plumbagin production in *P. rosea* cultures
Incubation period: 48 hours and 72 hours
Age of the culture: 14 days
Initial cell biomass: 500 mg/40 ml medium
Medium used: MS basal medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l

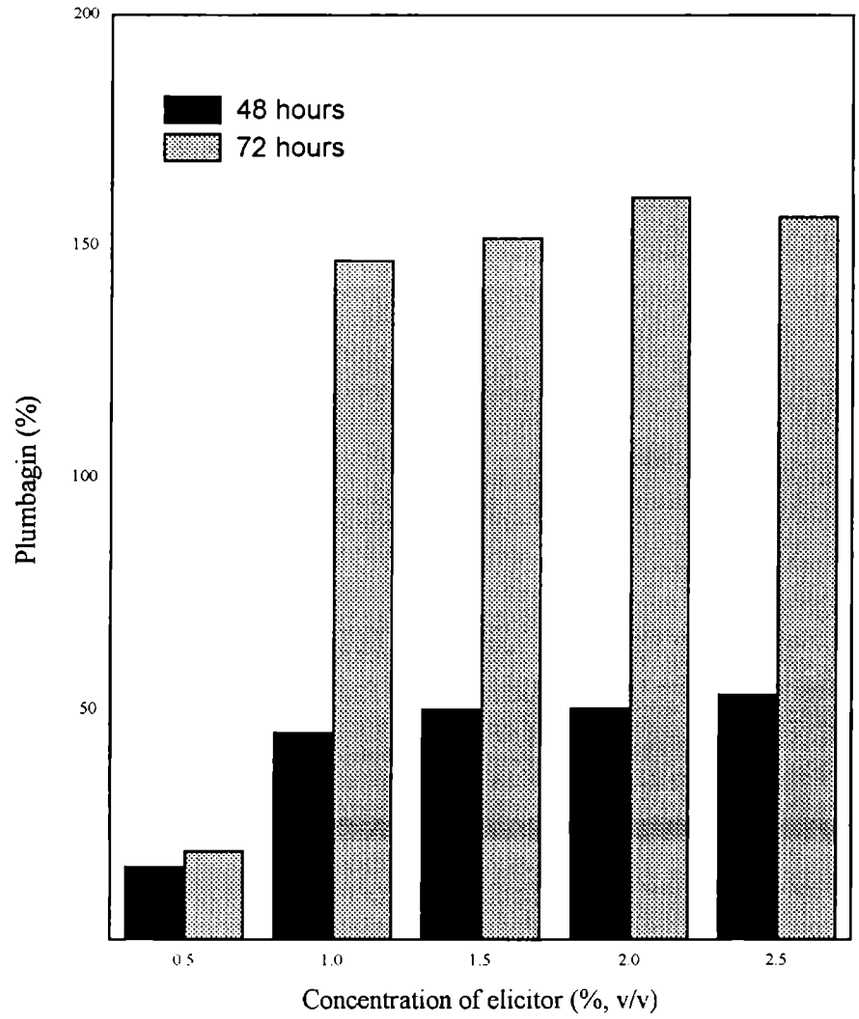

Fig. 4.4 showing the percentage increase of plumbagin production over control at various levels of elicitor and treatment periods

Source of elicitor: A. niger mycelical hydrolysate;
Treatment periods: 48 hours and 72 hours; Age of the culture: 14 days
Initial biomass of the culture: 500mg/40 ml medium

Elicitor from *Aspergillus niger*

A comparison of efficacy of medium filtrate (MF) and mycelial hydrolysate (MH) filtrate of *A. niger* revealed that medium filtrate was superior to mycelial filtrate when plumbagin production was measured. When the cultures were incubated with medium filtrate, the maximum accumulation of product was noticed at 2.5% level in 48 hours (10.177 mg/g D. Wt.) which was 3.3 times higher than the untreated cells. Moreover, at 72 hours of incubation, media filtrate above 1.5% level inhibited the synthesis of plumbagin (Table 4.3). At 72 hours of incubation with media filtrate, maximum plumbagin produced was 9.813 mg/g D. Wt. at 1.5% level. However, the maximum response of cells to media filtrate from *A. niger* was not much different at 48 hours and 72 hours (228.396 % and 215.94 % increase over control, respectively).

The response of cultures to mycelial hydrolysate of *A. niger* was not very significant in comparison to media filtrate. Elicitors from mycelial filtrate induced the production of plumbagin over control, 160.59 % only Incubation with mycelial filtrate (2.5%) for 48 hours enhanced 1.5 times product accumulation than the untreated cells. But, it was, 2.61 times in cultures incubated for 72 hours at 2.0% level. It can be seen from the Table. 4. that higher concentration of elicitor (MH) had virtually no effect on product synthesis at 72 hours. Whereas medium filtrate adversely affected the culture in 72 hours (Table 4.3) as evident from the amount of plumbagin produced.

Elicitor from *R. nigricans*

Table 4 6 and 4.7 represent the accumulation of plumbagin when *P rosea* culture were incubated with various dosage of elicitor from *R. nigricans* media filtrates for 48 hours and 72 hours. The response of the

Table 4.6 Influence of *Rhizopus nigricans* spent medium as elicitor on plumbagin production in *P rosea* cultures

Concn. of elicitor (%, v/v)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% increase over control
0.0	0.114 [±0.033]	3.302 [±0.343]	3.416	00.00
0.5	0.230[±0.072]	4.962 [±0.277]	5.192	51.99
1.0	0.187 [±0.077]	5.341 [±0.209]	5.528	61.83
1.5	0.143 [±0.021]	6.421 [±0.337]	6.564	92.155
2.0	0.156 [±0.03]	8.824 [±0.196]	8.98	162.88
2.5	0.212 [±0.043]	8.790 [±0.217]	9.002	163.524

Period of incubation with elicitor: 48 hours

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + Sucrose 4 % + with or without (control) elicitor

Age of the culture: 14 days

Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml. medium

Data represents the average of 5 replicates in two repetitive experiments.

Data in parenthesis indicates ± SD

Table 4.7 Influence of *Rhizopus nigricans* spent medium as elicitor on plumbagin production in *P rosea* cultures

Concn. of Elicitor (%, v/v)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/ g D. Wt.]	% increase over control
0.0	0.170 [±0.025]	3.228 [±0.179]	3.398	00.00
0.5	0.230 [±0.117]	5.744 [±0.266]	5.974	75.81
1.0	0.213 [±0.007]	6.783 [±0.374]	6.996	105.89
1.5	0.243 [±0.09]	6.941 [±0.173]	7.184	111.42
2.0	0.229 [±0.056]	7.633 [±0.223]	7.862	131.37
2.5	0.283 [±0.099]	8.150 [±0.371]	8.433	148.18

Period of incubation with elicitor: 72 hours

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + sucrose 4 % ++ with or without (control) elicitor

Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml. medium

Age of the culture: 14 days

Data represents an average of 5 replicates in two repetitive experiments.

Data in parenthesis indicates ± SD

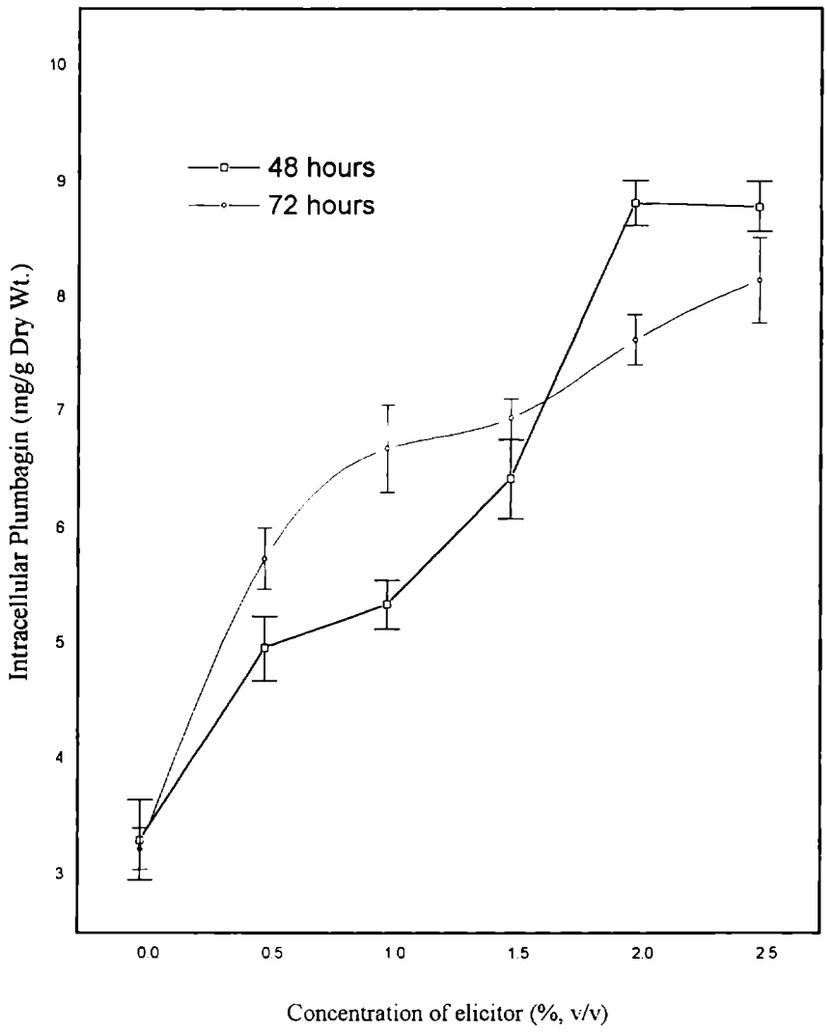

Fig. 4.5 Effect of *Rhizopus nigricans* spent medium as elicitor on plumbagin production in *P rosea* cell cultures

Incubation periods: 48 hours and 72 hours
Age of the culture 14 days; Initial cell biomass: 500 mg/40 ml medium
Medium used: MS basal medium with IAA 1.0 mg/l, NAA 0.5 mg/l and BAP 0.3 mg/l

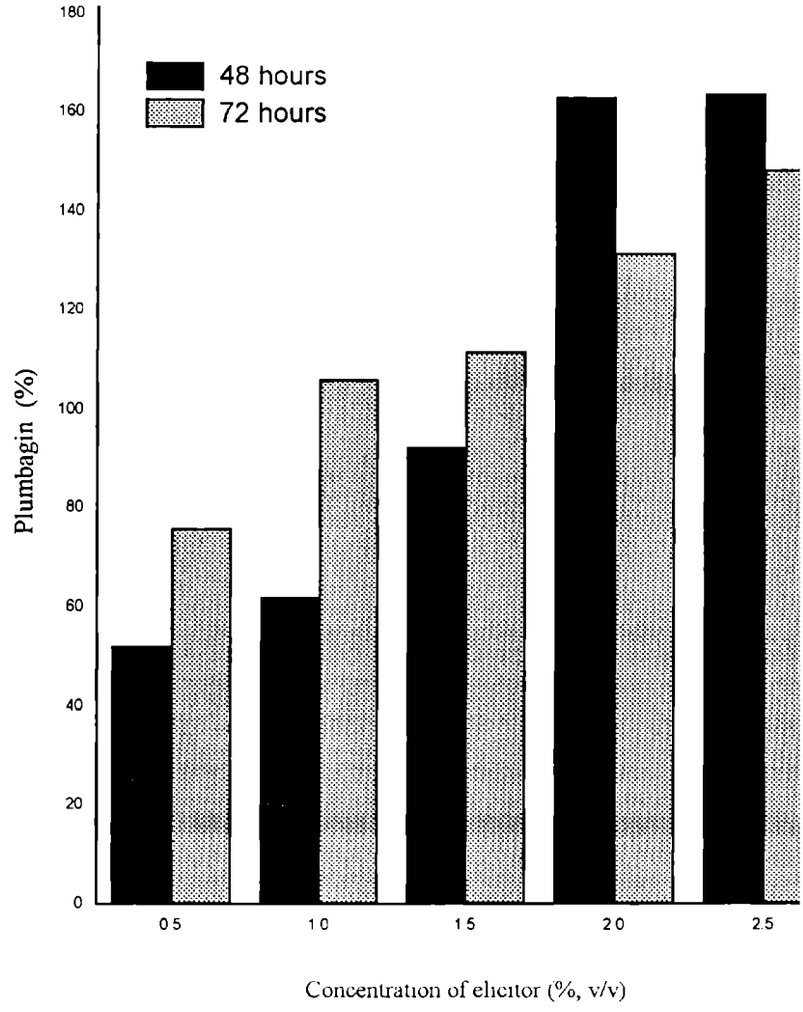

Fig. 4.6 showing the percentage increase of plumbagin over untreated cells at various levels of elicitor and treatment periods

Source of the elicitor: *Rhizopus nigricans* spent medium
Treatment periods: 48 hours and 72 hours; Age of the culture: 14 days
Initial biomass of the culture: 500 mg/ 40 ml medium
Medium used: MS basal medium with IAA 1.0 mg/l, NAA 0.5 mg/l and BAP 0.3 mg/l

Table 4.8 Influence of *Rhizopus nigricans* mycelial hydrolysate as elicitor on plumbagin production in *P rosea* cultures

Concn. of Elicitor (%, v/v)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% increase over control
0.0	0.113 [±0.038]	2.841 [±0.113]	2.954	00.00
0.5	0.185 [±0.009]	3.126 [±0.230]	3.311	12.09
1.0	0.193 [±0.071]	4.447 [±0.193]	4.640	57.075
1.5	0.263 [±0.034]	6.178 [±0.249]	6.441	118.04
2.0	0.287 [±0.15]	7.223 [±0.321]	7.510	154.232
2.5	0.334 [±0.109]	8.54 [±0.317]	8.874	200.041

Period of incubation with elicitor: 48 hours

Medium used: MS medium + IAA 1.0mg/l + NAA 1.0 mg/l + BAP 0.3 mg/l + sucrose 4 % + with or without (control) elicitor

Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml. medium

Age of the culture: 14 days

Data represents an average of 5 replicates in two repetitive experiments.

Data in parenthesis indicates ± SD

Table 4.9 Influence of *Rhizopus nigricans* mycelial hydrolysate as elicitor on plumbagin production in *P. rosea* cultures

Concn. of elicitor (%, v/v)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% increase over control
0.0	0.109 [±0.01]	2.934 [±0.149]	3.043	00.000
0.5	0.133 [±0.027]	3.652 [±0.233]	3.785	24.383
1.0	0.147 [±0.033]	5.631 [±0.34]	5.778	89.880
1.5	0.187 [±0.11]	6.778 [±0.248]	6.965	128.89
2.0	0.214 [±0.201]	9.482 [±0.374]	9.696	218.63
2.5	0.203 [±0.31]	9.833 [±0.203]	10.036	229.81

Period of incubation with elicitor: 72 hours

Medium used: MS basal medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + Sucrose 4% + with or without (control) elicitor

Inoculam: 500mg ± 30 mg fresh tissue in 40 ml. medium

Data represents the average of 5 replicates in two repetitive experiments.

Date in parenthesis indicates ± SD

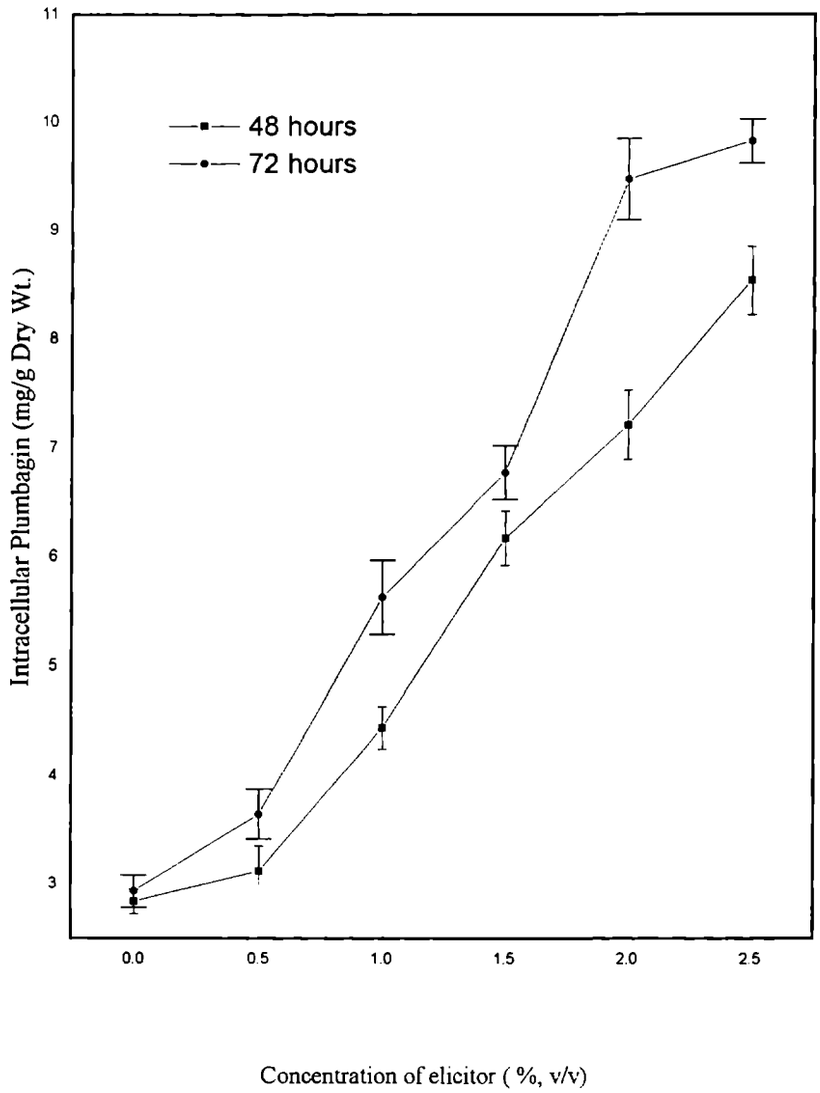

Fig. 4.7 Effect of *Rhizopus nigricans* mycelial hydrolysate as elicitor on plumbagin production in *P. rosea* cell cultures

Incubation periods: 48 hours and 72 hours;
Age of the culture: 14 days Initial cell biomass: 500 mg/40ml medium
Medium used: MS basal medium with IAA 1.0 mg/l, NAA 0.5 mg/l and BAP 0.3 mg/l

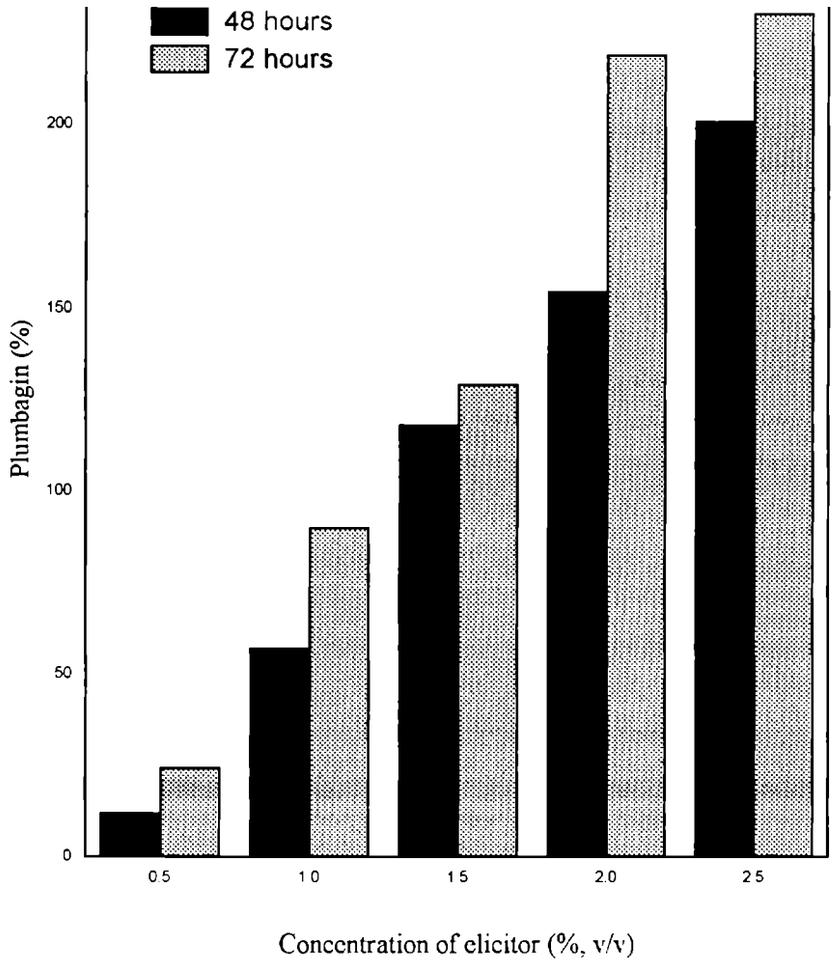

Fig. 4.8 showing the percentage increase of plumbagin production over untreated cells at various levels of elicitor and treatment periods

Source of the elicitor: *Rhizopus nigricans* mycelial hydrolysate
Treatment periods: 48 hours and 72 hours; Age of the culture: 14 days
Initial biomass of the culture: 500mg/40ml medium
Medium used: MS basal medium with IAA 1.0 mg/l, NAA 0.5 mg/l and BAP 0.3 mg/L

cultures in both incubation periods was found to be dependent on the dosage level of elicitors. The plumbagin accumulation was enhanced to 163.524 % over control in 48 hours at 2.5% dosage level (Fig. 4.6). However, the same dosage gave 148.18% over control in 72 hours. It is interesting to note that the percentage excretion of plumbagin was almost constant when treated with media filtrate for 72 hours and highly unstable for 48 hours, although the total production was increased (Fig. 4.7).

In the case of mycelial filtrates of *R. nigricans* as shown in Table 487 and 4.9, the total plumbagin production was increased with dosage of elicitor and gave a maximum response of 10.036 mg/g D. Wt. at 2.5 % level when incubated with the elicitor for 72 hours (Fig. 4.9). The plumbagin accumulation was enhanced to 229.81 % higher than the control in 72 hours in cultures treated with elicitor from mycelial filtrate (Fig. 4.9), whereas, with medium filtrates 163.524 % over control (Fig. 4.6) was obtained indicating the more effectiveness of mycelial filtrates as elicitor in the induction of plumbagin production.

***Saccharomyces cerevisiae* as elicitor**

The elicitor obtained from *S. cerevisiae* showed that accumulation of metabolites increased with the concentration of elicitors and incubation period (Table 4.10 and Table 4. 11). The cells responded more effectively at 72 hours and accumulated 8.326 mg/g D. Wt. plumbagin at 2.5% level in 48 hours (6.154 mg/g D Wt. at 2.0% level). In this case, it is interesting to note that the percentage excretion of plumbagin is not constant in 48 hours whereas it is almost stable in 72 hours of incubation. In comparison to other fungal elicitors, elicitor from yeast had shown least effect at all the level tried.

Table 4. 10 Influence of *Saccharomyces cerevisiae* as elicitor on plumbagin production in *P rosea* cultures

concn. of elicitor (mg/l)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% increase over control
0.00	0.194 [±0.03]	3.105 [+0.194]	3.209	00.00
0.5	0.204 [±0.033]	4.123 [+0.037]	4.327	34.83
1.0	0.311 [+0.351]	4.272 [+0.109]	4.583	42.82
1.5	0.221 [+0.073]	4.663 [+0.076]	4.884	52.196
2.0	0.179 [+0.043]	5.975 [+0.097]	6.154	91.77
2.5	0.213 [+0.045]	5.768 [+0.088]	5.981	86.382

Period of treatment with elicitor: 48 hours

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + Sucrose 4 % + with or without (control) elicitor

Inoculam: 500 mg + 30 mg fresh tissue in 40 ml. medium

Data represents the average of 5 replicates in two replicative experiments.

Data in parenthesis indicate + SD

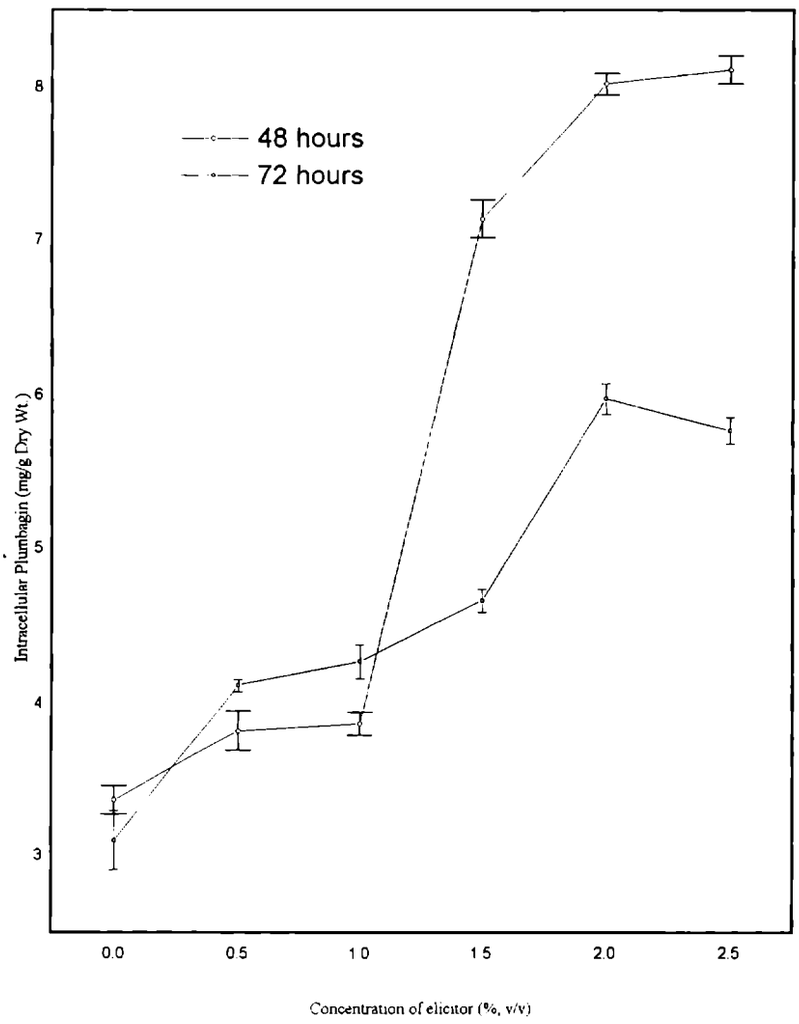

Fig. 4.9 Effect of elicitor obtained from S. cerevisiae on plumbagin production in *P rosea* cell cultures

Incubation period:48 hours and 72 hours;
Age of the culture:14 days
Medium used: MS basal medium with IAA 1.0 mg/l, NAA 0.5 mg/l and BAP 0.3 mg/l

Table 4.11 Influence of *Saccharomyces cerevisiae* as elicitor on plumbagin production in *P rosea* cultures

Concn. of Elicitor (mg/l)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% increase over control
0.0	0.104 (±0.093)	3.367 (±0.092)	3.471	00.00
0.5	0.104 (±0.007)	3.823 (±0.132)	3.927	13.14
1.0	0.111 (±0.114)	3.871 (±0.076)	3.982	14.722
1.5	0.221 (±0.035)	7.141 (±0.121)	7.362	112.1
2.0	0.179 (±0.007)	8.018 (±0.071)	8.197	136.16
2.5	0.213 (±0.11)	8.113 (±0.093)	8.326	139.87

Period of incubation with elicitor: 72 hours

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l+ with or without (control) elicitor

Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml. medium

Data represents the average of 5 replicates in two repetitive experiments.

Date in parenthesis indicates ± SD

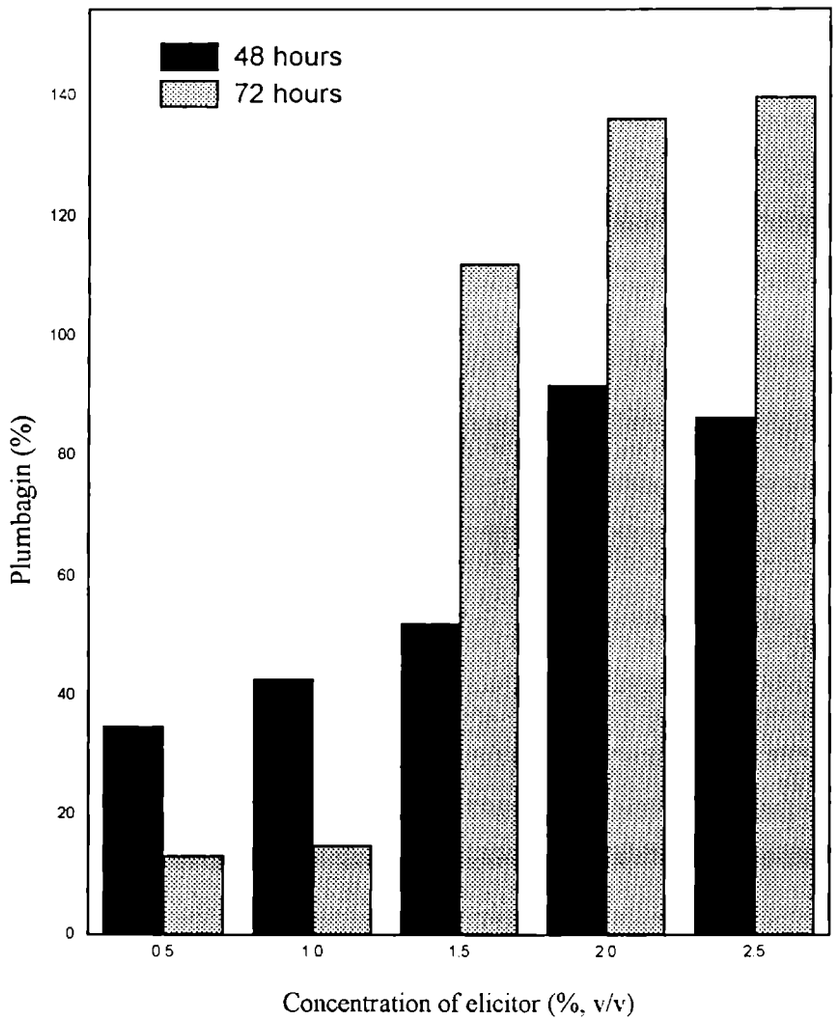

Fig. 4.10 Showing the percentage increase of plumbagin production over untreated cells at various levels of elicitor and treatment periods

Source of the elicitor: *Saccharomyces cerevisiae* culture
Treatment periods: 48 hours and 72 hours; Age of the culture: 14 days
Initial biomass of the culture: 500 mg/40ml medium
Medium used: MS basal medium with IAA 1.0 mg/l, NAA 0.5 mg/l and BAP 0.3 mg/l

Table 4.12 Influence of *Bacillus cereus* as elicitor on plumbagin production in *P rosea* cultures

Concn. of elicitor (mg/l)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% increase over control
0.00	0.163 [+0.031]	2.983 [+0.311]	3.146	00.00
0.5	0.172 [+0.019]	3.328 [+0.093]	3.500	11.252
1.0	0.173 [+0.034]	3.929 [+0.174]	4.102	30.388
1.5	0.202 [+0.11]	3.95 [+0.241]	4.152	31.98
2.0	0.178 [+0.073]	4.137 [+0.089]	4.315	37.158
2.5	0.166 [+0.01]	4.220 [+0.279]	4.386	39.415

Period of incubation with elicitor: 48 hours

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + Sucrose 4% + with or without (control) elicitor

Inoculam: 500 mg ± 30 mg fresh tissue in 40 ml. medium

Data represents the average of 5 replicates in two repetitive experiments.

Data in parenthesis indicates ± SD

Table 4.13 Influence of *Bacillus cereus* as elicitor on plumbagin production in *P rosea* cultures

Concn. of elicitor (mg/l)	Extracellular plumbagin [mg/g D. Wt.]	Intracellular plumbagin [mg/g D. Wt.]	Total plumbagin [mg/g D. Wt.]	% increase over control
0.0	0.071 (±0.078)	2.943 (±0.117)	3.114	00.00
0.5	0.165 (±0.017)	3.954 (±0.083)	4.119	32.37
1.0	0.170 (±0.031)	3.974 (±0.07)	4.144	33.076
1.5	0.217 (±0.101)	4.521 (±0.093)	4.738	52.152
2.0	0.234 (±0.029)	4.772 (±0.113)	5.006	60.76
2.5	0.178 (±009)	4.866 (±0.191)	5.044	61.98

Period of incubation with elicitor:72 hours

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + Sucrose 3 % + with or without (control) elicitor

Inoculam: 500 mg +± 30 mg fresh tissue in 40 ml. medium

Data represents the average of 5 replicates in two repetitive experiments.

Data in parenthesis indicates ± SD

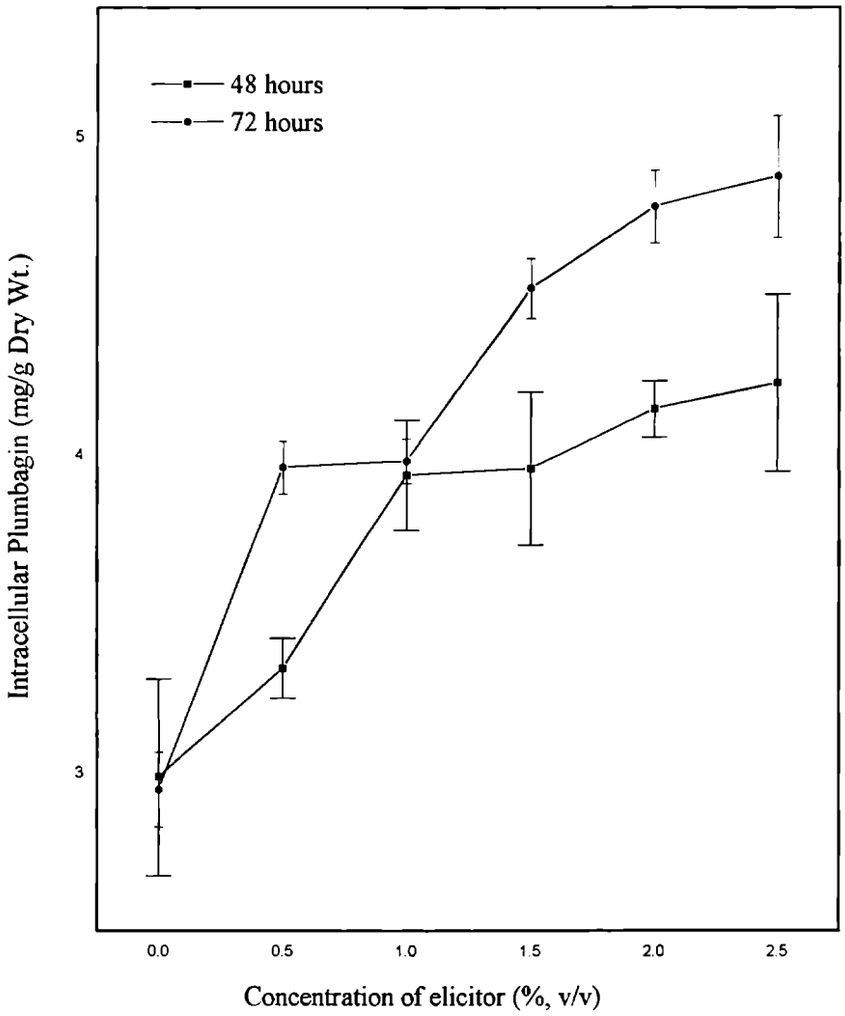

Fig. 4.11 Effect of elicitor obtained from *Bacillus cereus* on plumbagin production in *P. rosea* cell cultures

Incubation periods: 48 hours and 72 hours; Age of the culture: 14 days
Initial biomass of the culture: 500mg/40ml medium
Medium used:MS basal medium with IAA 1.0mg/l, NAA 0.5mg/l and BAP 0.3mg/l

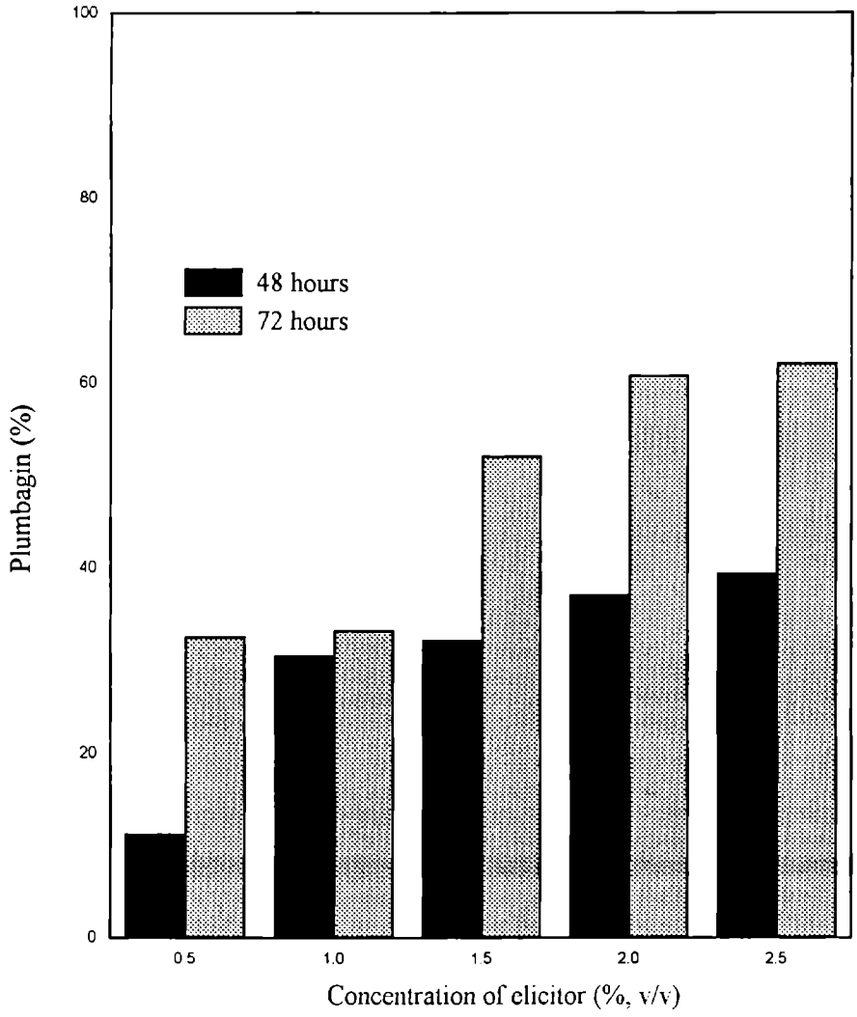

Fig. 4.12 showing the percentage increase of plumbagin production over untreated cells at various levels of elicitor and treatment periods

Source of the elicitor: *Bacillus cereus* culture
Treatment periods: 48 hours and 72 hours; Age of the culture: 14 days
Initial biomass of the culture: 500mg/40ml medium
Medium used: MS basal medium with IAA 1.0 mg/l, NAA 0.5 mg/l and BAP 0.3 mg/l

Table 4.14 Time course studies on plumbagin production in *P rosea* cultures when elicited with *R. nigricans* mycelial hydrolysate

Time of addition of elicitor (days)	Intracellular plumbagin (mg/g D. Wt.)	Extracellular plumbagin (mg/g D. Wt.)	Total plumbagin (mg/g D. Wt.)
5	2.955 (±1.03)	0.138 (±0.098)	3.093
8	3.475 (±0.078)	0.139 (±0.11)	3.606
12	6.472 (±1.37)	0.273(±0.022)	6.745
16	6.447 (±0.19)	0.268 (±0.049)	6.625

Amount of elicitor added: 1.0 % (v/v)

Incubation period with elicitor: 72 hours

Medium used: MS basal medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.5 mg/l + 0.5 g/l myoinositol

Data represents the average of 4 replicates in two repetitive experiments. Data in parenthesis indicates ± SD

Table 4.15 Time course studies on plumbagin production in *P rosea* cultures when treated with *A. niger* mycelial hydrolysate

Time of addition of elicitor (days)	Intracellular plumbagin (mg/g.D.Wt.)	Extracellular plumbagin (mg/g D. Wt.)	Total plumbagin (mg/gD.Wt.)
5	3.078 (±0.107)	0.099 (±0.003)	3.177
8	4.561 (±1.21)	0.117 (±0.017)	4.678
12	5.811 (±0.762)	0.142 (±0.037)	5.953
16	5.121 (±0.063)	0.178 (±0.045)	5.299

Amount of elicitor added: 1.0 % (v/v)

Incubation period with elicitor: 72 hours

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + 0.5 g/l myoinositol

Data represents the average of 4 replicates in two repetitive experiments. Data in parenthesis indicates ± SD

Table 4. 16 Time course studies on the porduction of plumbagin by *P rosea* cultures when treated with elicitor from *Saccharomyces cerevisiae*

Time of addition of elicitor (days)	Intracellular plumbagin (mg/g D. Wt.)	Extracellular plumbagin (mg/g D. Wt.)	Total plumbagin (mg/g D. Wt.)
5	2.602 (±1.2)	0.114 (±0.018)	2.715
8	4.13 (±0.073)	0.144 (±0.051)	4.277
12	5.018 (±0.009)	0.246 (±0.061)	5.264
16	4.392 (±0.038)	0.218 (±0.053)	4.610

Amount of elicitor added: 1.0 % (v/v)

Incubation period with elicitor: 72 hours

Medium used: MS medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + 0.5 g/l myoinositol

Data represents the average of 4 replicates in two repetitive experiments. Data in parenthesis indicates ± SD

Table 4. 17 Time course studies on the production of plumbagin by *P. rosea* cultures when treated with elicitor from *Bacillus cereus*

Time of addition of elicitor (days)	Intracellular plumbagin (mg/g D. Wt.)	Extracellular plumbagin (mg/g D. Wt.)	Total plumbagin (mg/g D.Wt.)
5	3.286 (±0.087)	0.136 (±0.01)	3.422
8	3.320 (±0.121)	0.173 (±0.073)	3.493
12	3.524 (±0.099)	0.182 (±0.01)	3.705
16	4.207 (±0.913)	0.174 (±0.061)	4.381

Amount of elicitor added: 1.0 % (v/v)

Incubation period with elicitor: 72 hours

Medium used: MS basal medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + 0.5 g/l myoinositol

Data represents the average of 4 replicates in two repetitive experiments. Data in parenthesis indicates ± SD

Elicitor from *B. cereus*

Tables 4.12 and 4.13 represent the response of *P rosea* cultures to the elicitor obtained from *B. cereus* cultures. It can be seen that cells responded positively to these elicitors at 48 hours and 72 hours. However, in comparison to elicitor from other sources, it gave a least response. Cultures incubated with 2.5% elicitor for 72 hours accumulated 5.044 mg/g D. Wt. of plumbagin, which was the highest figure, obtained in this case (Fig. 4.12). Only forty to sixty percentage increases in plumbagin over control was noticed when cells were challenged with maximum dose of elicitor treated for 48 hours and 72 hours, respectively.

Metal ions as abiotic elicitor

Inorganic constituents of incubation medium have reported to have an influence on the secondary metabolite production in cells grown *in vitro*. We have selected five important salts viz. sodium molybnate, potassium iodide, copper sulphate, zinc silphate and calcium chloride as abiotic stress and added at a concentration of 10, 20 and 30 times the normal MS medium. However, except, in the calcium chloride all other salts shows a negative response at all the levels tried.(Table 4.19 Calcium chloride at a concentration of 0.44 mg/l (10n) and 0.88 mg/l (20n) have significantly affected the production of plumbagin during exponential phase of growth.

Effect of pH on plumbagin production

The pH of the medium was kept in the range of 3.5 to 7.5 to study the effect of pH on the metabolite production. When the medium pH was at 3.5 and 4.5, after 10 days, it became 4.88 and 4.89 In all other cases, pH of the medium was lowered than at initial conditions. Except, at pH 4.5, the growth of the cells was affected severely and at that level an increase of 20.14 % in the fresh weight was observed. The production of plumbagin

Fig. 4.18 Effect of pH on growth and plumbagin production in *P rosea* callus cultures

PH (0)	pH (exp.)	Delta pH*	Increase in FW (%)	Plumbagin (mg/g D. Wt.)
3.5	4.88	-1.38	-61.79	3.8
4.5	4.89	-0.39	20.14	4.776
5.0	4.92	0.08	-8.51	4.934
5.5	5.02	0.48	-18.23	3.584
5.7	4.94	0.76	-37.57	3.352
5.9	4.85	1.05	-37.00	2.981
6.0	4.90	1.10	-27.39`	2.720
6.1	5.00	1.10	-53.36	3.638
6.3	5.02	1.28	-20.81	3.381
6.5	5.35	1.15	-40.44	3.813
7.0	6.23	0.77	-33.47	3.216
7.5	6.56	0.94	-44.43	3.705

pH (0) – pH of the cultivation medium before sterilization; pH (exp) – pH of the medium in exponential phase of growth;

- delta pH = pH(0) – pH (exp.)

Incubation Period: 12 days; -ve indicates decrease in growth.

Medium used: MS basal medium + IAA 1.0 mg/l + NAA 0.5 mg/l + BAP 0.3 mg/l + 0.5 g/l myoinositol

Data represents the average of 3 replicates.

Table 4.19 Effect of inorganic salts on the plumbagin production in *P rosea* cultures

(a) Sodium molybnate

No. of days	control	10 n	20 n	30 n
(a) Sodium molybdnate				
5	1.766	0.58	0.19	0.34
8	1.843	0.4	0.20	0.12
12	2.342	0.13	0.57	0.10
16	2.809	0.04	0.07	0.20
(b) Copper sulphate				
5	1.766	0.38	0.17	0.25
8	1.843	0.52	0.082	0.074
12	2.342	0.61	0.013	0.058
16	2.809	0.73	0.03	0.054
(c) Zinc sulphate				
5	1.766	0.462	0.616	0.624
8	1.843	1.2	1.54	1.26
12	2.342	1.202	1.386	1.196
16	2.809	0.98	1.201	1.11
(d) Potassium iodide				
5	1.766	0.08	0.04	0.05
8	1.843	0.045	0.073	0.02
12	2.342	0.045	0.073	0.02
16	2.809	0.017	0.27	0.05

(e) Calcium chloride

5	1.766	1.027	0.724	0.822
8	1.843	2.314	1.784	1.832
12	2.342	2.513	2.561	2.013
16	2.809	2.423	2.937	1.964

All the chemicals were used at a concentration of 10, 20 and 30 times the normal (control) MS basal medium.

Data indicates the average of 3 replicates.

also was influenced by the increase of medium pH and at pH 5.0 maximum production of plumbagin has seen (4.776 mg/ g D Wt.) which was nearly 0 5 times more than the control at pH 5.9 It has also been noticed that pH in the acidic range influenced the production of plumbagin more significantly than at pH at usual level.

4.4 Discussion

Effect of biotic factors on the production of plumbagin

In recent years, plant-pathogen interactions were mainly studied using various plant cell cultures and fragments of fungal cell wall constituents called elicitors. It has been noticed that plants challenged by pathogenic microorganisms respond with a rapid activation of various defense mechanisms. The specific compounds excreted extracellularly in the medium have been demonstrated widely Though initially plant pathogens were used for elicitation, several non-pathogenic microbial species were also been proven as an effective inducer of secondary metabolites. In our studies, we have used both bacterial culture (Bacillus cereus) and fungal cultures (Aspergillus niger and Rhizopus nigricans). Apart from trying mycelial hydrolysate as elicitor compounds, we have also tried fungal spent culture medium as elicitor source. Our work revealed that the spent culture medium was found to be an effective source of elicitor to accelerate plumbagin accumulation in the cells of *P rosea*. There are very few reports on the use of spent culture medium as an elicitating compound. Ramakrishna et al [82] have reported the use of spent culture medium of plant pathogen *Rhizoctonia solani*. The authors have reported a moderate

pathogen *Rhizoctonia solani.* The authors have reported a moderate response of the cells of Hyoscyamus muticus. Though the phytoalexin yield was not as high as the mycelial biomass hydrolysate, the use of spent culture medium found to be cost effective. In the present investigations, the use of spent culture medium of Aspergillus niger and Rhizopus nigricans have been found to be very effective and the yield of plumbagin was 148-229 % higher than nonelicited cells. When compared with fungal mycelial hydrolysate of *R. nigricans*, the yield of naphthoquinone was slightly lower in medium filtrate. However, in case of *A. niger* the spent culture medium has resulted in higher yield of metabolites. Since MS medium was used for fungal culture, the elicitor activity of medium can be ruled out. Thus whatever component responsible for elicitation could be derived from the fungus.

Different research workers have followed different protocol of the preparation of elicitor compounds. The widely used method was to autoclave the fungal biomass for prolonged period of 3 hours.[82,83] Whereas some authors recommended that autoclaving the biomass for 20 minutes was sufficient (Tyler *et al.* 1988)[28] In many of these preparations, the heat labile constituents of the hydrolysates were destroyed. The elicitor used in the present study derived from the spent medium was sterilized through membrane filtration and hence the preparations include the heat labile compounds also. To the best of our knowledge, there are no reports on the use of spent culture medium of *A. niger* and *R. nigricans* as elicitors.

The preparation of elicitors from Saccharomyces cerevisiae has been attempted by several workers (Grosskopf, 1991).[84] Buitelaar *et al* [50] have reported negligible response of *Tagetus patula* hairy roots to the yeast elicitor whereas *A. niger* was found to be highly effective in thiophene

Baumert *et al.*[74] have reported that the dramatic response of the cells to the yeast elicitor The production of rutacridone enhanced from 36 μg/g.D.Wt. to 235.24 μg/g.D.Wt. in 18 days culture. Similarly, rutacridone epoxide has also increased to 135.5 μg/g.D Wt. from 53 6 μg/g. D.wt. However, in the present study we have observed that the cell response to the elicitor preparation of yeast was not compared to fungal elicitors. But, at 2.5 % dosage 144.9% higher yield of plumbagin was obtained in 48 hours culture.

Few workers have reported the use of Bacillus cultures for mimicking the pathogenic signaling in plant cells. Buitelaar *et al.* (1992)[50] have used elicitor from *Bacillus subtilis*. The authors reported that at 2% (v/v) dosage, 50% higher yield of thiophene was obtained when compared to control. The response of *P rosea* cells was found to be similar (40-60% higher yield) with *B. cereus* used in the present study However, the cell response was minimum with Bacillus cultures in comparison to all other elicitor preparations used. The dose response curve profile was also found to follow the similar trend. The dosage saturation occurs at 1% level itself and beyond that concentration the yield of plumbagin was nearly the same (4.1 mg/g. D. Wt. – 4.3 mg/g. D.Wt.) in 48 hours culture. Similar trend was observed in 72 hours culture and dose saturation attained at 1.5 % itself (the highest dosage tried). However, at 5 times higher concentration of elicitor, the yield of metabolites increased by 61 98%.

Sim. *et al.*[54] reported that in *Catharanthus roseus* hairy root culture, elicitors from *A. niger* and *Penicillium Sps* shows different response. *A. niger* homogenate increases the production of catharanthine 2.5 times. But, the amount of ajmalicine was not greatly affected. However, at the same time, elicitor from *Penicillium* cultures enhanced the production of both

catharanthine and ajmalicine. In *P rosea* cell cultures, elicitor from both spent medium as well as mycelial hydrolysate of *A. niger* enhanced the production of plumbagin. *P rosea* cultures gave a maximum response (10.177 mg/g D. Wt.) at 48 hours of incubation with *A. niger* spent medium. At the same time, mycelial hydrolysate elicited 8.628 mg/g D. Wt. of plumbagin production in 72 hours.

Since there is no way to predict which organism would result in elicitation of *P rosea* the selection of elicitors was mainly based on reported literature. Among the four elicitors, *R. nigricans* has been found to be highly effective for elicitation purposes. Both fungal hydrolysate and spent medium have influenced the cells for higher accumulation of metabolites.

The elicitation experiments were carried out with crude preparations of mycelial hydrolysate and spent culture medium. Usage of purified components from the crude preparations for elicitation purposes may increase the cell response to elicitor molecules. At this stage, it is difficult to speculate which portion of the fragment or the excreted products have effected the higher metabolite accumulation. For the further understanding of the detailed mechanism of alkaloid stimulation of elicitation, further studies of the secondary metabolic enzymes need to be carried out.

Effect of abiotic factors on plumbagin production

The production of phytoalexins by plant and cell cultures could be stimulated by exposure to abiotic elicitors such as salts of metals. Parry *et al.* (1994)[86] reported a 6 to 7 fold increase in the production of medicarpin alalfa cell cultures when treated with cupric chloride. However, Yoshikawa (1978) reported that copper sulphate induced a 65% inhibition of glyceollin in soybean cultures.[87] Stafford (1990) also reported the negative effect of copper and zinc on isiflavone synthase activity[88] Ino et al. (1993)[89]

suggested that divalent metal ions including copper and zinc affect the enzymes involved in malonylation and glucosulation. Alternatively, a disruption in primary metabolism may affect the supply of co-factors and substrates necessary for isoflavonoid synthesis.

Lidon and Henriques (1993)[90] have shown that Cu^{2+} induced damage to cell membranes results in disturbances of respiration metabolism, photosynthesis and other essential physiological processes. Demidchik *et al.* 1997) [91] also suggested that Cu^{2+} induced dramatic alterations in plant cell plasmalemma permeability are caused mainly by non-selective conductance increase and electrogenic pump inhibition.

Various reports are showing the influence of calcium ions on the secondary production of metabolites. It is suggested that calcium ion act as a second messenger in the regulation of secondary metablite production by plant cells (Kurosaki *et al.* (1987)[91] Buitelaar *et al.*[50] also had shown the positive effect of calcium and high pH on the production of thiophene by hairy roots. Nahalka *et al.*[92] also has reported that plumbagin production could be enhanced by increase in medium pH in Drosera cell culture.

Since the production of Plumbagin (a naphthoquinone) by the cells is under stress conditions, its accumulation by mild elicitation could be explained as stress related response. The cells also responded to permeabilization which is also a stress causing protocol, it can be speculated that the plumbagin accumulation and release can be accomplished by creating stress conditions to the cells. One has to try other methods such as temperature, nutrient limitations, addition of toxins, etc, which were generally considered as stress causing event for the cells.

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CHAPTER V

SYNERGISTIC EFFECT OF IMMOBILIZATION, PERMEABILIZATION, ELICITATION AND *IN SITU* PRODUCT RECOVERY

INTRODUCTION

Plant cell cultures have been drawing increasing attention because of their potentiality to synthesize a broad spectrum of useful products comparable to the original plants. Though few attempts have been made to produce these molecules synthetically, by and large, plants remain main source for these compounds. Since the cultivation of plants is usually limited to specific areas and seasons, plant tissue culture offers advantage of obtaining desired product constantly regardless of place and climate. In case of slow growing species such as trees, the plant tissue culture offers higher productivity. However, often the synthesis of many compounds are associated with specific organs such as leaves, roots, flowers, fruits etc. and hence the differentiation of cells is required for the synthesis of products *in vitro*.. When compared to microbial cultures, the plants have several disadvantages. The growth of the plant cells is generally low and stabilization of potential cell lines under optimal conditions is rather difficult. More over, the low concentration of the biomolecules in fully grown plant or in cultured plant tissue poses serious limitations for their commercial exploitation. In this context, immobilization of plant tissue found to be an attractive alternative protocol [1-4] Immobilization of plant cells, similar to microbial cells has been attempted to overcome some of the limitations of free cells. It has been demonstrated that immobilized cell system offer several advantages over free cells such as high mechanical

stability, cell to cell contact (which is beneficial for structural and biochemical differentiation) and facility to use repeatedly. Immobilization of plant cells will render down stream processing simple. It is possible to remove the product *in situ* during culturing. It can also minimize the limitation of damage of cells due to agitation and toxicity of O_2 and other compounds. As in the case of microbial cell systems, immobilized plant cell systems offer increased volumetric productivity. As non-growth associated systems are compatible to immobilization, plant cells, where metabolite production commences after growth phase, are well suited for this system. One of the main disadvantages of immobilization often noticed is the high cost of raw materials and associated complications with regard to design of the reactor and mass transfer limitations.

The first reported immobilization of plant cells was by Brodelius and collegues in 1979 who entrapped *Catharanthus roseus*, *Morinda citrifolia* and *Digitalis lanata* in alginate beads.[5] Since then a number of reports have been published on immobilized plant cells (Table 5.1). Several reviews on immobilization methods and their effects on secondary product formation have been documented.[6,7] Immobilized plant cells have been used for a wide range of reactions, which can be divided into three groups; biotransforamtion, synthesis from precursors and the *de novo* synthesis. Entrapment of cells in gel matrix (for e.g. sodium alginate, carrageenan, agarose, etc.) and passive adsorption on support materials such as gelatine, fiberglass, polyurethane foam, polystyrenes, etc. are the most extensively adopted methods in plant cell systems.[8-12] Rho *et al.* (1990)[13] have introduced novel surface-immobilization techniques using non-woven polyester fibers for *C. roseus* cells. The polyester fibers were placed in the

reactor over the stainless steel structures and inoculated with cells. Highly efficient immobilization (95%) of *C. roseus* cells was observed within 12 hours of inoculation and good biofilm was developed within few days. The authors have claimed excellent growth characteristics of the cells

Table 5.1 Production of plant secondary metabolites through immobilization

Plant species	Immobilized Substratum	Product	References
Catharanthus roseus	Calcium alginate	Ajmalicine	Brodelius *et al.* 1979[6]
C. roseus	Hollow fibers	Ajmalicine	Asada&Shuler, 1989[14]
C. roseus	Fiberglass mats	Alkaloids	Facchini& Dicosmo, 1990[38]
Taxus cuspidata	Glass fiber mats	Taxol	Fett Neto *et al.* 1992[37]
Capsicum frutescens	Reticulate Polyurethane	Capsaicin	Lindsey *et al.* 1984[39]
C. annuum	Calcium alginate	Capsaicin	Johnson *et al.* 1991[29]
Papaver somniferum	Calcium alginate	Codeine	Furuya *et al.* 1984[40]
P somniferum	Polyester fibre	Sanguinarine	Kurz *et al.* 1990[41]
Digitalis lanata	Calcium alginate	Digitoxin	Alfermann *et al* 1980[42]
Mucuna pruriens	Calcium alginate	L-DOPA	Wichers *et al.* 1983[43]
Atropa belladonna	Reticulate Polyurethane	Tropane Alkaloid	Collinge & Yeoman, 1986[44]
Lithospermum erythrorrhizon	Reticulate Polyurethane	Shikonin	Park *et al.* 1990[17]

Thalictrum rugosum	Fiberglass mats	Protoberberine	Facchini DiCosmo, 1990[38]
Solanum aviculare	Cell aggregate	Solasodine	Tsoulpha & Doran, 1991[45]
Hyoscyamus muticus	Calcium alginate	Solavetivone	Ramakrishna *et al.* 1993[46]
Apium graveolens	Chitosan	Alkaloids	Beaumont *et al* 1989[47]
Nicotiana tabacum Oxide coated with Poly-L-Lysine	Polyphenylene alkaloid	Tropane	Vankova *et al* 1990[48]
Dioscorea deltoidea	Polyurethane foam	Diosgenin	Robertson *et al.* 1989[49]
Coleus blumei	Adhesion on Luffa	Rosmarinic acid	Martinez & Park, 1994[27]
Artemisia annua	Agarose	Artemisinin	Chem *et al.* 1993[50]

comparable to suspension cultures. Several workers have reported that the immobilized cells of *C. roseus* accumulate higher levels of indole alkaloids, relative to free cells, when immobilized in alginate.[14,15] Koge *et al* (1992)[16] immobilized *Coffea arabica* cells on various porous matrices of various shapes and porosity and reticulated polyurethane foam. It was found that the

production of caffeine was not affected in all these cases. Park *et al.* (1990)[17] has claimed that single stage culture of *Lithospermum erythrorrhizon* cells in polyurethane foam matrices yielded higher productivity than conventional two-stage culture system. Vanek, *et al.*[18] studied the influence of immobilization using alginate, pectate and carrageenan gels, in polyurethane foam and on the surface of polyphenylenoxide on the biotransformation of verbenol by *Solanum aviculare* cells. Authors suggested that during the preparation of immobilized biocatalysts, alteration occurs in the induction of expression (or repression) of different enzymes involved in cell metabolism. The positive physiological effects of alginate entrapment of cells are due to calcium ion content and low phosphates in the medium.[9] Cell to cell contact is increased by spatial confinement and cell growth is limited by the matrix mechanical resistance.[20] In addition, alginate can also act as elicitor of secondary metabolism in cultured plant cells.[19]

Although, the immobilized cell system have the potential to improve the economic feasibility of processes for producing secondary metabolites, but, it fail to apply in all plant cell systems. In many plants, products remained intracellular or only partially secreted or degraded very fast in the extracellular conditions.[21] Some strategies to overcome these limitations have been tested. These include pH cycling, use of permeabilizing agents and ultrasonic permeabilization.[22-24] The cell permeabilization to release the metabolites into the medium has been tried in several cases and its advantages and disadvantages have been clearly accounted in the Chapter No. III. Permeabilization of cells encapsulated in gel matrix may protect the cells from permanent damage. Continuous production of secondary

metabolites can possibly be accomplished using permeabilized cells in immobilized culture condition. Among many permeabilizing agents, dimethyl sulfoxide (DMSO) has been a popular choice for the reversible permeabilization of immobilized cells.[25] Knorr and Berling (1987)[26] have examined the effect of permeabilization and immobilization on amaranthin production by entrapping the *Chenopodium rubrum* cells in a complex Ca-alginate-chitosan/DMSO gel system. Amaranthin content was highest for immobilized cultures treated with dimethyl sulfoxide or dissolved chitosan than the free cells. Park and Martinez *et al.* (1994)[27] also used permeabilized *Coleus blumei* cells in immobilized-fed batch culture. Preconditioned *C. blumei* with 0.1% DMSO and then immobilized in fibrous skeleton of mature fruit of *Luffa cylindrica* yielded rosmarinic acid half that obtained in cell suspension cultures. Berlin et al. (1989)[28] reported that immobilized cell cultures treated with permeabilizing agents could be used as tools for biotransformation studies. He has observed the negative effects of permeabilizing agents in free tobacco cell cultures. N-propanol used as permeabilization agent has caused severe growth inhibition and bioconversion efficiency of the cells decreased considerably.

Another important strategy related to increasing secondary metabolite production is fungal elicitation. Addition of cell wall constituents of microorganisms, enzymes, or heavy metals, may induce specific biosynthetic pathways. A brief account on various elicitation mechanisms and their usefulness has been described in Chapter No. IV Many studies have proved the efficiency of immobilized cells followed by elicitation.[14,29]

Another strategy that has been used to improve the productivity of plant cell culture system is by adsorption of the product *in situ*. A wide range of adsorbents like XAD-2, XAD-4, XAD-7, IRA-958, and some solvents like n-hexadecane etc. were used in various systems. Several groups have proposed that the use of adsorbents for *in situ* product removal to reduce some of the unique problems associated with plant cell bioprocesses.[14,30-32] Berlin *et al.* (1984)[33] employed a water insoluble triglyceride phase to retain volatile hydrocarbons produced by *Thuja occidentalis*. Brodelius *et al.* (1979)[6] and Rhodes *et al.* (1986)[34] observed that *in situ* removal could be used to enhance alkaloid secretion by immobilized cells and by hairy roots, respectively. Parr *et al.* (1987)[35] used ion exchange resins to remove nicotine alkaloids formed during growth of tobacco plant cells and found 3-fold increase in extracellular nicotine production. Robins and Rhodes (1986)[30] have reported that addition of polymeric adsorbents stimulated 15-fold increase in anthraquinone production in *Cinchona ledgeriana* cell culture. Payne and Payne (1988)[32] have used XAD-7 for *in situ* product recovery of indole alkaloids from *Catharanthus roseus* cultures. Kim and Chang (1990)[36] were able to enhance Shikonin production by employing *in situ* extraction and cell immobilization. Buitelaar et al. (1992)[51] also have used XAD-7 in *Tagetus patula* cultures. Addition of XAD resin raised the excretion to 50% of the total thiophenes produced. The probable mechanism behind the increase in product accumulation may be either preventing the feedback inhibition by adsorbing the products or the protection of products from degradation.[30]

The utilization of technique viz. Immobilization, elicitation and permeabilization coupled to the use of an adsorbent, seems an effective strategy to improve the production of secondary metabolites in plants. In

the present attempt we have studied the synergistic effect of immobilization, permeabilization or elicitation and in situ extraction of products using polymeric adsorbent, XAD-7 We have treated the immobilized *P roseus* cells with permeabilizing agents such as DMSO and CTAB for 15 minutes and then added with polymeric adsorbent, XAD-7 The immobilized cells of *P rosea* were treated with optimal concentrations of mycelial hydrolysates of two fungi viz. *Rhizopus nigricans* and *Aspergillus niger* for 72 hours along with non-ionic XAD-7 resins.

5.2 Materials and methods

Plant material

The origin and growth conditions of the *P. rosea* culture used for this experiments was same as in the previous chapters. Fourteenth day old suspension culture maintained on MS medium was used for all the experiments.

Adsorbent

The neutral polycarboxylic ester, XAD-7 was purchased from Aldrich chemical company. Prior to use, the resin was soaked in methanol for 24 hours and then washed with several volumes of distilled water in a Buchner funnel. The washed resin was air dried in the Buchner funnel by applying vacuum on the filter. One gram of dried resin was weighed into a dialysis bag and tied properly These bags were autoclaved in culture medium for 15 minutes to minimize the reduction of undesired adsorption of medium components.

Immobilization of cells:

P rosea cells were harvested from the culture medium on 14th day of incubation by filtration and washed once with sterilized distilled water. Fifteen gram fresh biomass was suspended in sterilized 3% (w/v) of sodium alginate in MS basal medium, mixed well and then dropped into sterile 50mM $Cacl_2$ $5H_2O$ solution containing 1% sucrose (Kim and Chang, 1990).[36] Each bead was approximately 0.5mm in diameter and 2 g bead contains approximately 1g cells. Beads were washed thrice with the MS medium and resuspended in fresh MS medium.

Followed by immobilization, beads were treated with permeabilizing agents viz. Dimethylsulfoxide and cetyltrimethylammoniumbromide at a concentration of 0.01% (v/v) and 0.001ppm for 15 minutes. Beads were rinsed with sterilized culture medium and resuspended in fresh MS medium. The beads were added at a concentration of 2.0g per flask containing 40 ml liquid medium and incubated for 7 days. All flasks except control were supplied with a dialysis bag on seventh day of incubation and incubated for seven more days. Plumbagin content of beads, medium and resin was determined.

Elicitation of cells

Fungal elicitors were prepared from *Rhizopus nigricans* and *Aspergillus niger* as described in Chapter No. IV Fungal mycelial hydrolysate at a concentration of 1.0 % (v/v) was added to each flask containing 2 g. immobilized cells of *P rosea* and were incubated for 72 hours. Beads were harvested and washed for thrice with distilled water. Experiments with XAD-7 was carried out by adding one resin bag into

flasks containing seven day old cultures and incubated for another seven days. Plumbagin content from the cells, medium and resin was extracted and determined by HPLC analysis.

Adsorption studies with Plumbagin

To determine the adsorption behaviour of plumbagin on XAD-7, 0.75mg of resin was added to 15 ml of solutions of plumbagin in concentration ranging from 0 to 150μM/l. The shake flasks containing the solutions were placed on a shaker for 2 days and the amount of plumbagin adsorbed onto the resin was determined.(Fig. 5.2)

Extraction and analysis of Plumbagin in XAD

The plumbagin content of the XAD was determined by adding 50 ml. of methanol to a dialysis bag and extracted the XAD for 24 hours on a shaker. The methanol was evaporated under vacuum and the residue was dissolved in 1 ml. methanol and used for HPLC analysis.

Plumagin present in the cells and medium was determined as described in Chapter III.

5.3 Results and discussion

The use of several product-enhancing strategies together has been found to be highly beneficial in the case of plant cells. There are few reports where multiple treatment protocols have enhanced the secondary metabolite yield several folds. In the present investigations, we have tried two types of protocols where immobilized cells of *P. indica* have either been elicited or permeabilized and in all the cases, XAD resin has been used to adsorb

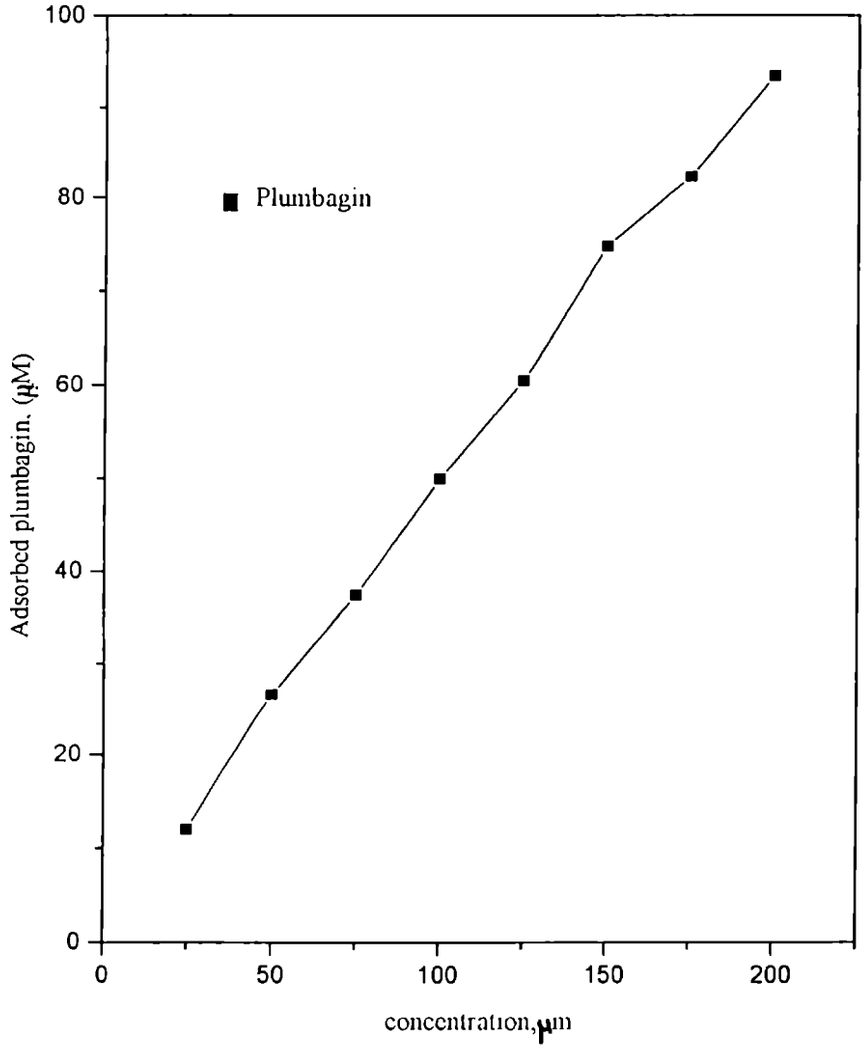

Fig.5.1 Adsorption of Plumbagin on XAD-7

Table 5.2 Synergistic effect of immobilization, permeabilization and *in situ* extraction

Type of cells	Plumbagin (cells) (mg/g D.Wt.)	Plumbagin (medium) (mg/g D. Wt.)	Total Plumbagin (mg/g D Wt.)	% increase over control
Untreated cells	2.906 (±0.11)	0.073 (±0.02)	2.979 (±0.17)	00.00
Permeabilized cells (free cells)	6.073 (±0.071)	0.473 (±0.043)	6.546 (±1 12)	119.74
Immobilized cells	5.464 (±0.44)	0.289 (±0.063)	5 752 (±0.81)	99.60
Permeabilized & Immobilized cells	5.77 (±0.21)	0.798 (±0.018)	6.568 (±0.062)	120.48
Permeabilized, Immobilized & Resin treated cells	8.66 (±0.09)	Nil (±0.009)	9.828* (±0.34)	229.91

Permeabilizing agent used: CTAB at a concentration of 0.001 ppm

Cells Immobilized in 0.25% Sodium alginate (Sigma) with 50 mM Calcium chloride solution, Resin used: XAD – 7

* Plumbagin recovered from the resin _ 1 168 mg/g D Wt.

Data represents the average of 3 replicates. Data in parenthesis indicates ± SD

Table 5.3 Synergistic effect of immobilization, permeabilization and *in situ* extraction

Type of cells	plumbagin (cells) (mg/g. D.Wt.)	plumbagin(medium) (mg/g.D Wt.)	Total plumbagin (mg/g.D Wt)	% increase over control
Untreated cells	2.814 (±0.009)	0.083 (±0.017)	2.897 (±0.04)	00.00
Permeabilized cells	8.118 (±1.05)	0.202 (±0.01)	6.32 (±1 13)	118.16
Immobilized cells	5.464 (±0.31)	0.289 (±0.008)	5 752 (±0.73)	99.6
Permeabilized & Immobilized cells	8.613 (±0.03)	0.598 (±0.1)	9.211 (±2.07)	217.95
Permeailized, Immobilized & Resin treated cells	7.605 (±0.051)	0.121 (±0.065)	9.579* (±0.083)	230.65

Permeabilizing agent used: DMSO at a concentration of 0.01% (v/v);

Cells immobilized in 0.25% sodium alginate (Sigma) with 50 mM calcium chloride solution.

* Plumbagin recovered from the resin-1 853 mg/g D Wt

Data represents the average of 3 replicates Data in parenthesis indicates ± SD

secreted metabolites. Table 5.2 and 5.3 indicates synergistic effect of immobilization, permeabilization and in situ adsorption of metabolites during synthesis. In the first set of experiments, the alginate entrapped cells of *P rosea* were permeabilized with 0.001 ppm CTAB for 15 minutes. Subsequently, the permeabilized gel beads were cultured in fresh medium containing one bag of (1g. per 40ml. medium) XAD-2. It can be seen that the untreated cells produced 2.98 mg/g. D. Wt. of plumbagin and the majority of the product is inside the cells (2.9 mg/g. D. Wt.). Whereas the immobilized cells have produced 5.752 mg/g D. Wt. of cells which was nearly 100 per cent higher than the control (Table 5.1). The permeabilized gel beads produced 6.546 mg/g D. Wt. of cells and which was nearly 120% higher yield in comparison to control. Surprisingly, permeabilized free cells were also produced the same amount of metabolite. The combined treatment of permeabilization, immobilization and resin added cells produced 9.83 mg/g D. Wt. of plumbagin, which was 230 per cent higher than the control. These results indicate that the multiple treatment strategy has enhanced the product yield substantially But, permeabilization and immobilization alone as individual treatment has enhanced product yield 120% and 100%, respectively. The combined treatment of the above two did not result in cumulative benefit. Similarly, when the cells were permeabilized with DMSO [0.01% (v/v)] the response of the cells is similar. The combined treatment of permeabilization and immobilization and resin addition for in situ product adsorption has resulted in significant increase in total plumbagin production (9.579 mg/g. D. Wt.) which was 230 per cent higher than the control cells (Table 5.3). In these experiments also, it was noticed that permeabilization and immobilization as individual treatment alone has enhanced the product yield 118.16 % and 99.6 % higher than

Table 5.4 Synergistic effect of elicitation, immobilization and *in situ* extraction

Type of cells	Plumbagin (cells) (mg/g.D.Wt.)	Plumbagin(medium) (mg/g.D.Wt.)	Total PLB (mg/g.D.Wt.)	% increase over control
Untreated cells	2.814 (±0.08)	0.069 (±0.01)	2.882 (±0.63)	00.00
Immobilized cells	5.463 (±0.11)	0.289 (±0.09)	5.752 (±0.44)	99.58
Elicited free cells	5.54 (±1.34)	0.221 (±0.03)	5.761 (±0.021)	99.89
Immobilized & Elicited cells	6.43 (±0.47)	0.323 (±0.076)	6.753 (±0.53)	134.32
Immobilized, Elicited and Resin treated cells	6.214 (±0.058)	0.113 (±0.01)	7.918 * (±0.0612)	174.74

Elicitor used: *Rhizopus nigricans* mycelial hydrolysate at a concentration of 1.0 %, (v/v)

Cells immobilized in 0.25% sodium alginate (Sigma) with 50 mM calcium chloride solution; Resin used: XAD-7

* Plumbagin recovered from the resin – 1.591 mg/g D. Wt.

Data represents the average of 3 replicates. Data in parenthesis indicates ± SD

Table 5.5 Synergistic effect of Immobilization, elicitation and *in situ* product recovery

Type of cells	Plumbagin(cells) (mg/g D Wt.)	Plumbagin(medium) (mg/g D. Wt.)	Total PLB (mg/g D. Wt.)	% increase over control
Untreated cells	2.813 (±0.95)	0.069 (±0.061)	2.882 (±0.046)	00.00
Immobilized cells	5.464 (±0.067)	0.289 (±0 11)	5.752 (±0.081)	99.6
Elicited cells	4 11 (±0.036)	0.196 (±0.007)	4.306 (±0.025)	49.4
Immobilized & Elicited cells	6.403 (±0.11)	0.344 (±0.016)	6.747 (±1 73)	134.11
Immobilized, Elicited & Resin treated cells	6.983 (±0.48)		8.47* (±0.57)	193.89

Elicitor used: *Aspergillus niger* mycelial hydrolysate at a concentration of 1.0 %, (v/v)

Cells immobilized in 0.25% sodium alginate (Sigma) with 50 mM calcium chloride; Resin used. XAD-7

- Plumbagin recovered from the resin – 1 487 mg/ g D Wt.
- Data represents the average of 3 replicates. Data in parenthesis indicate ±SD

control respectively. Whereas the combination of above these two protocols only resulted 217.95 % higher yield.

In the second set of experiments, the cells were elicited instead permeabilization. The results obtained by individual treatments or by combinations were presented in Table 5.4 and 5.5. When the cells were elicited with *Aspergillus niger* (1.0%, v/v) about 50% enhancement in product yield was noticed. Whereas with immobilization, alone 100% higher yield over control was noticed. The multiple treatment protocol have enhanced the metabolite yield by 134.11 % whereas resin added cells along with above two treatments accounted 193.89 % higher yield of plumbagin Similar results were obtained when cells were elicited with *Rhizopus nigricans* at a dosage of 1.0% (v/v).

From the above experiments, it is clear that the multiple treatment strategies do synergize for enhanced accumulation of the metabolites. However, it was noticed that the maximum amount of plumbagin accumulated remains in the range of 7.98 to 9.83 mg/g. D. Wt. Earlier, it was noticed that either elicitation or permeabilization at optimal conditions had also resulted in 9-10 mg/g. D. Wt. of plumbagin accumulation. This trend shows that either by individual treatments at optimum level or combined treatments will only accumulate nearly the same amount of metabolite.

Multiple treatment strategies have greatly enhanced the ajmalicine production in *Catharanthus roseus* (Asada and Shuler, 1989)[14] Such synergistic effects have not been noticed in the present study. The maximum

amount of plumbagin accumulated was nearly same either in the case of individual treatments or treatments in combination. This observation lead us to speculate that in the case of stress related compounds (plumbagin), the stress causing events either individual or multiple result in same effect on the cell. In case of non-stress related compounds, such as ajmalicine, the combined treatments may act synergistically. Once the cells attain the thresh hold level of stress to stimulate enhanced product accumulation, further attempt to increase the stress may not result in higher product yield.

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CHAPTER VI

SECONDARY METABOLITES FROM CALLUS CULTURE OF *PLUMBAGO ROSEA*, LINN.

INTRODUCTION

Many compounds have been reported form different parts of *P rosea*, L.[1,2,3] Recently, the chemistry of naphthoquinone of the genus *Plumbago* has been reviewed by Dinda et al.[4] An up-to-date list of compounds isolated from these plants along with their physical constant, and spectral data, methods of isolation, structure elucidation, synthetic studies, biogenetic studies and biological activities have been discussed in this paper. However, no effort was until now made on to investigate the secondary metabolic constituents of *in vitro* culture of *P. rosea* except plumbagin. In the course of further studies on the secondary metabolites of the *P rosea* callus culture, a compound, [CH_3-$(CH_2)_{12}$-CH_2-CH_2-COOH], was isolated and characterized which was not reported earlier from this plant species.

5.2 Experimental

Dried callus culture (100g) was extracted with 100% methanol in a soxhlet apparatus. The reddish brown extract (1 lit.) was concentrated under vacuum and the last traces of the solvent removed under pressure. The dark residue (10 g), thus obtained shown four prominent spots in the solvent system, Hexane:ethyl acetate, 5:5. In addition to these spots, the TLC plate showed dark colour at the solvent front and base.

5.2.1 Chromatographic separation of the crude extract:

The dark-brown residue (10 g) was dissolved in chloroform (25 ml) and silica gel was added. The solvent was removed under pressure and the powder was transferred to a column of silica gel (100 g). The column was eluted successfully with hexane, 10; hexane: ethyl acetate, 9:1; hexane ethylacetate, 8:2; hexane ethyl acetate, 6:4; hexane ethyl acetate, 5:5; hexane: ethyl acetate, 3:7; hexane: ethyl acetate, 1:9; ethyl acetate, 10 and ethyl acetate Methanol, 9.9:0.1. Fractions of 50 ml. were collected and concentrated. Each fraction was checked by TLC and the fraction containing similar spots were pooled together. Fractions were grouped as shown in Table 5.1.

Table 5.1 showing the characteristics of fractions obtained after column chromatography from the crude methanol extracts of *P. rosea* callus culture

Eluants	Fraction No.	Group No.	Compounds
Heaxane	1-8	I	Nil
Hexane:EtoAc (9:1)	9-15	II	Nil
Hexane:EtoAc (8:2)	16-25	III	A
Hexane:EtoAc (6:4)	26-35	IV	Nil
Hexane:EtoAc (5:5)	36-45	V	B
Hexane:EtoAc (3:7)	46-55	VI	C
Hexane:EtoAc (1:9)	56-65	VII	Nil
Hexane:EtoAc (10)	66-75	VIII	Nil
Hexane:EtoAc (9.9:.1)	76-85	IX	D

Total fractions were categorized into 9 groups based on the similarity of spots seen in TLC.

Group I

Fractions 1-8 were combined after seeing the individual TLC and were evaporated. No compounds were obtained in this group.

Group II

The yellow residue obtained from these fractions resisted crystallization and suggested waxy nature. It was not examined further.

Group III

The fractions 16-25 were combined and concentrated after seeing the individual TLC of each fraction. The reddish orange crystals were analyzed by TLC with a solvent system of hexane Ethyl acetate, 6:4. It showed a bright orange spot on TLC (Rf Value 0.63). It was denoted as compound A. The residue was run along with authentic plumbagin (Sigma) and suggested as plumbagin. The residue was dissolved in ethyl acetate and a small amount of silica gel was added. The powder obtained after removal of the solvent was further purified by running a silica gel (100-120 mesh size) column (50 g) set up with hexane ethyl acetate, 9:1. The column was eluted with hexane: ethyl acetate, 8:2 and fractions were collected. Fractions containing the single orange spots were mixed. The orange residue was crystallized from ethyl acetate and further, the

structure was analyzed by NMR spectrum, IR spectrum and MASS spectrum along with the standard plumbagin.

Group IV

No compound was obtained from this fraction, hence discarded.

Group V

The fractions from 36-45 yielded a yellowish crystalline solid on evaporation. The residue was collected and crystallized again in ethyl acetate. It was designated as compound B

Group VI

The residue from 46-55 were concentrated and a reddish crystalline substance was obtained. It was recrystallized and designated as compound C.

Group VII & Group VIII

No compounds were obtained from fractions 56-75.

Group IX

The fractions 76-85 yielded reddish crystalline solid on evaporation of solvent, which was further purified by column chromatography with the same solvent system.

All the single spotted fractions were subjected to NMR spectrum and MASS spectrum. It was designated as compound D.

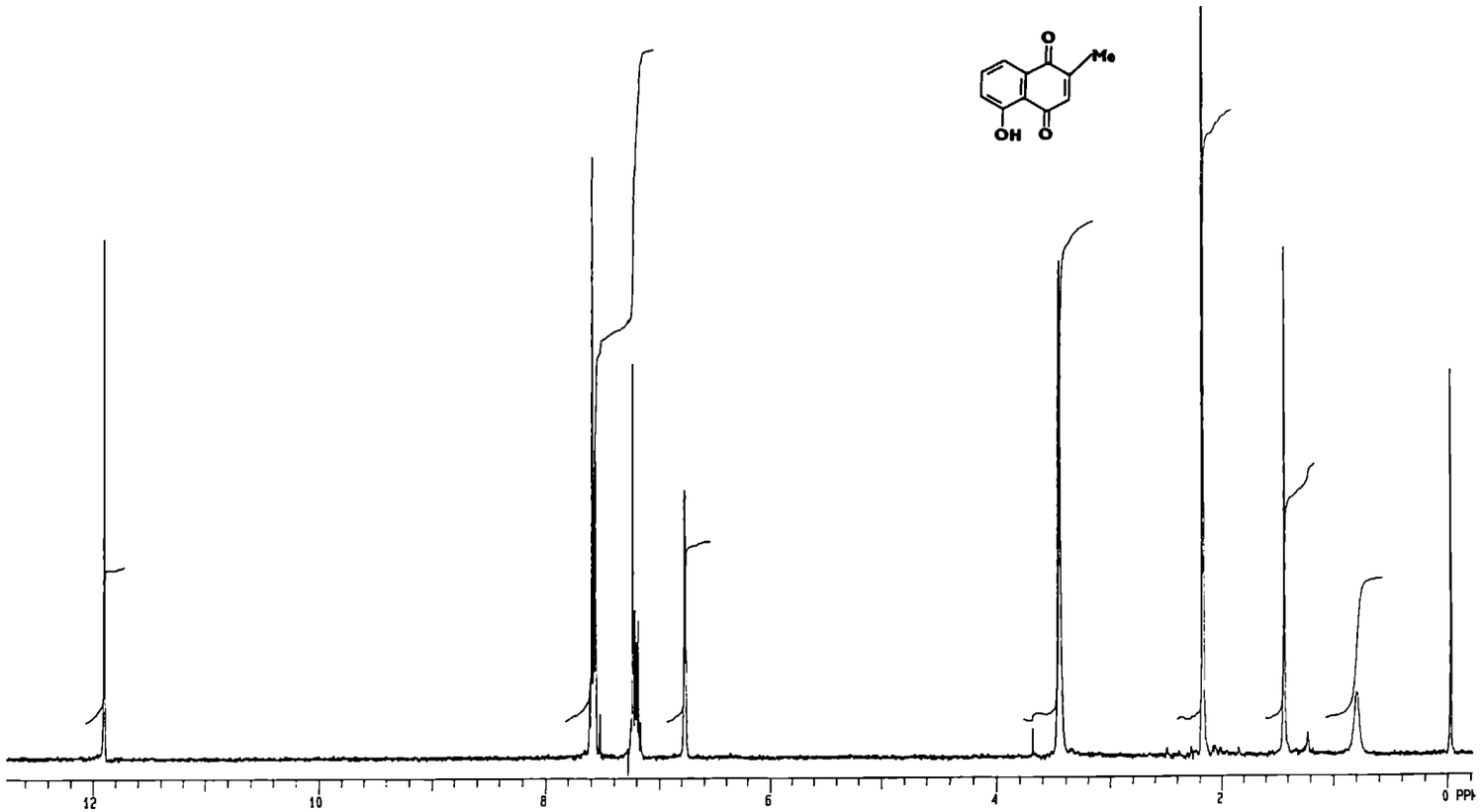

FIG. 6.1 ^{1}H-NMR SPECTRUM OF PLUMBAGIN

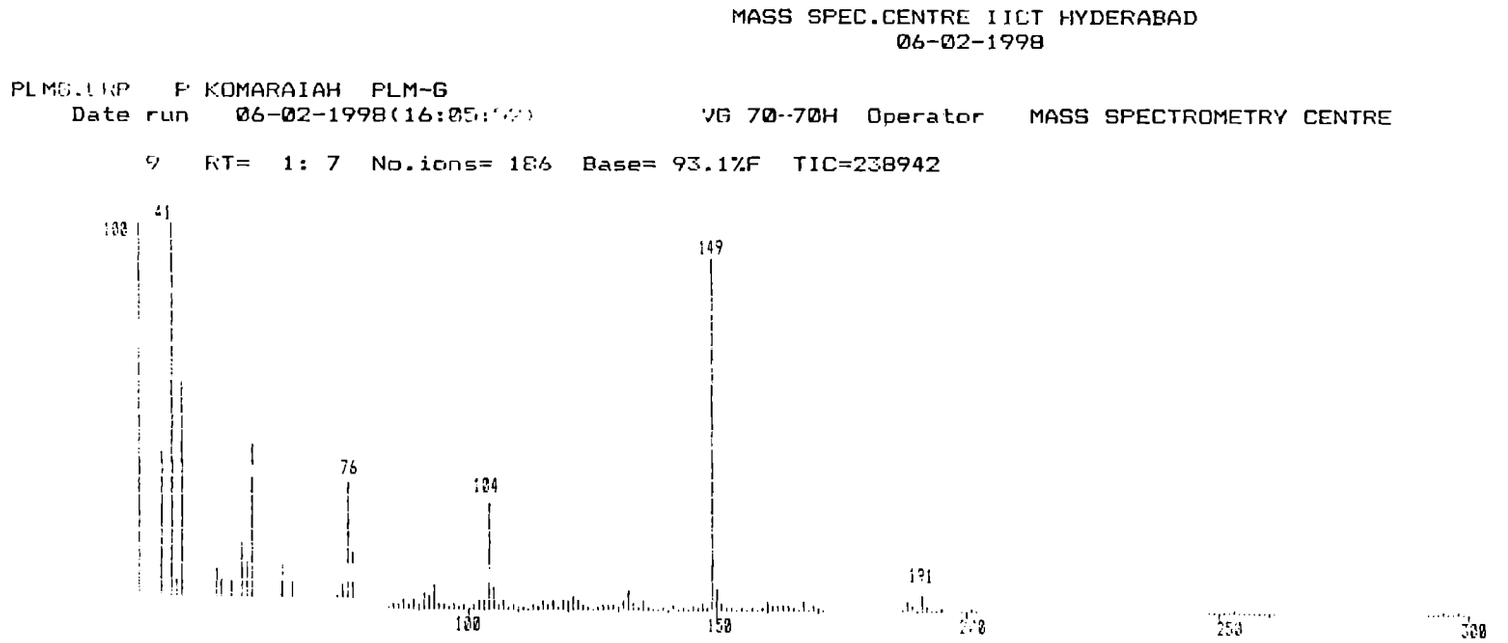

FIG. 6.2 MASS SPECTRUM OF PLUMBAGIN

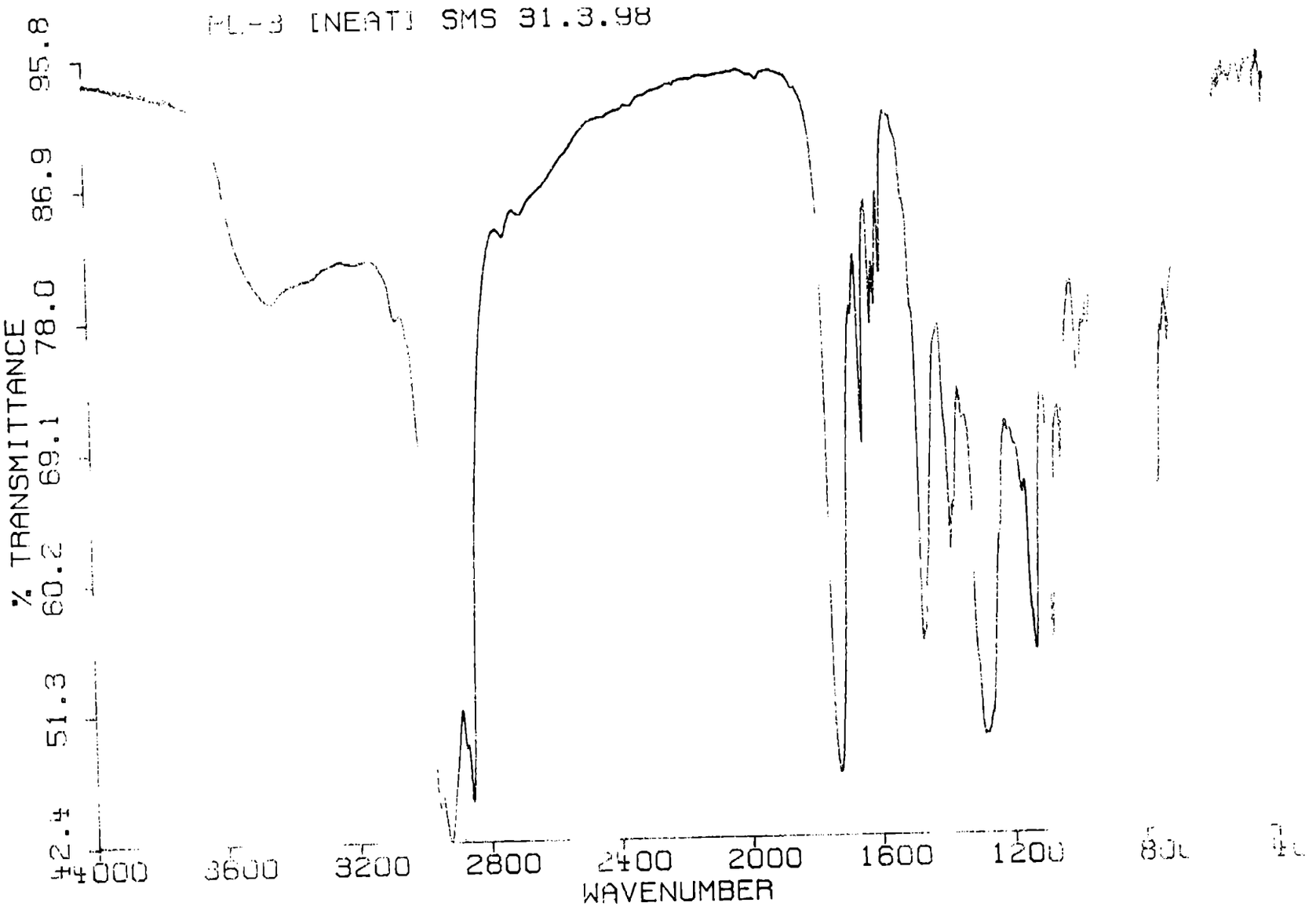

FIG. 6.3 IR SPECTRUM OF PLUMBAGIN

Compound A:

Compound A was crystallized from ethyl acetate as reddish orange needles, m. p. 78°C. and it was identified as plumbagin from spectral analysis. NMR spectrum, I R spectrum and MASS spectrum of plumbagin is given in separate pages.

Compound B:

CH_3-$(CH_2)_{12}$-CH_2-CH_2-COOH (**1**)

The structure of the compound B (**1**) was settled from its spectral data.

The molecular formula C_{16} H_{32} O_2 was assigned for the compound from its elemental analysis and mass spectrum (M^+ 256). The 200 MHz ^{1}H NMR spectrum of the compound (Fig. 5.4) provided precious information on the structural aspects of the compound (Table 5.2).

Table 5.2 200 MHz ^{1}H-NMR spectral data of 1 (in $CDCl_3$)

Chemical Shifts	Corresponding proton No.	Multiplicity signals	Assignment
2.26	2	t (J=7.0 Hz)	$-CH_2-$ (adjacent to –COOH)
1.54	2	m	$-CH_2-$
1.22	24	brs	$(CH_2)_{12}$
0.80		t (J=7.0 Hz)	$-CH_3-$

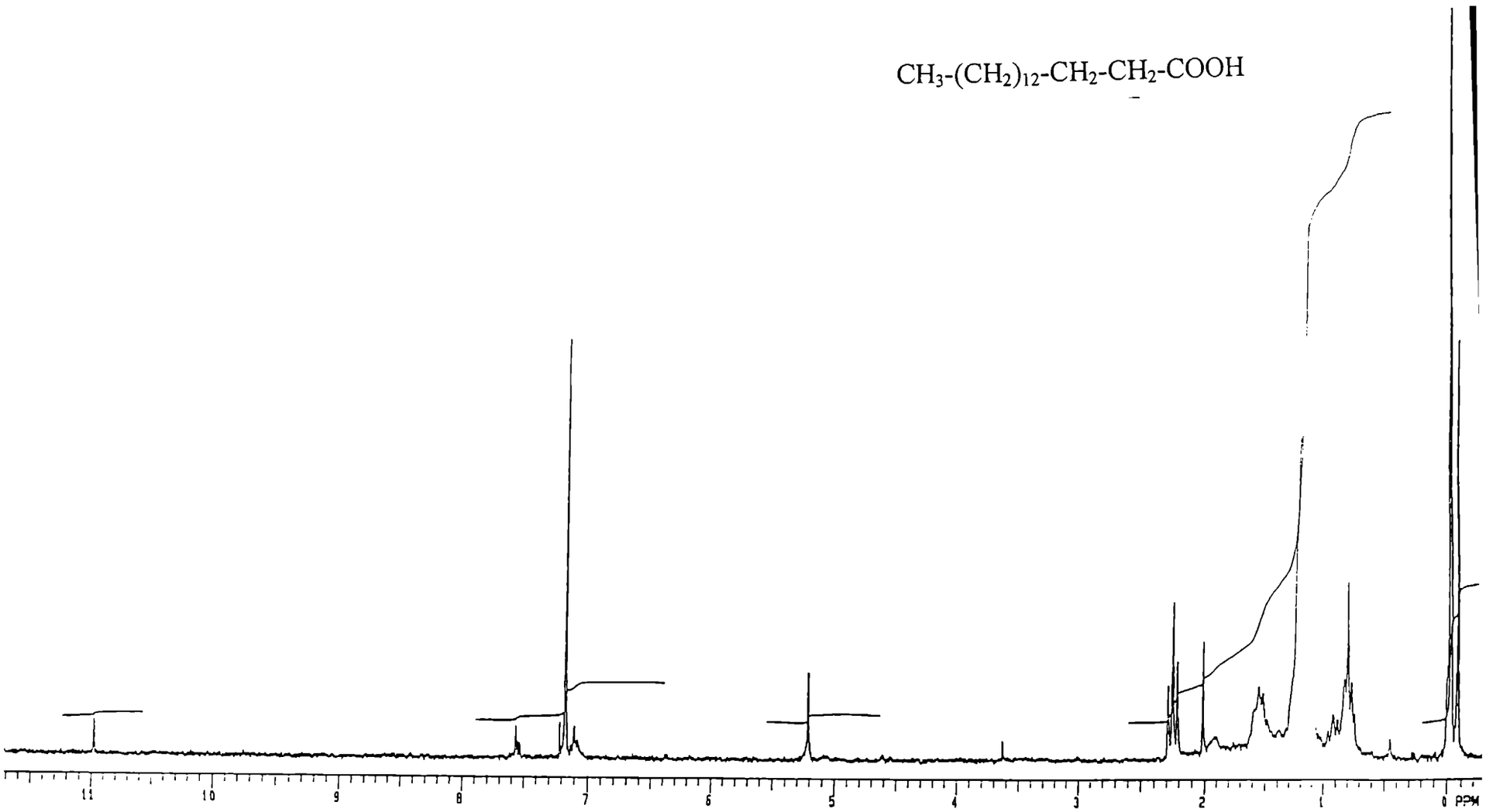

FIG. 6.4 ^{1}H-NMR SPECTRUM OF HEXADECANOIC ACID

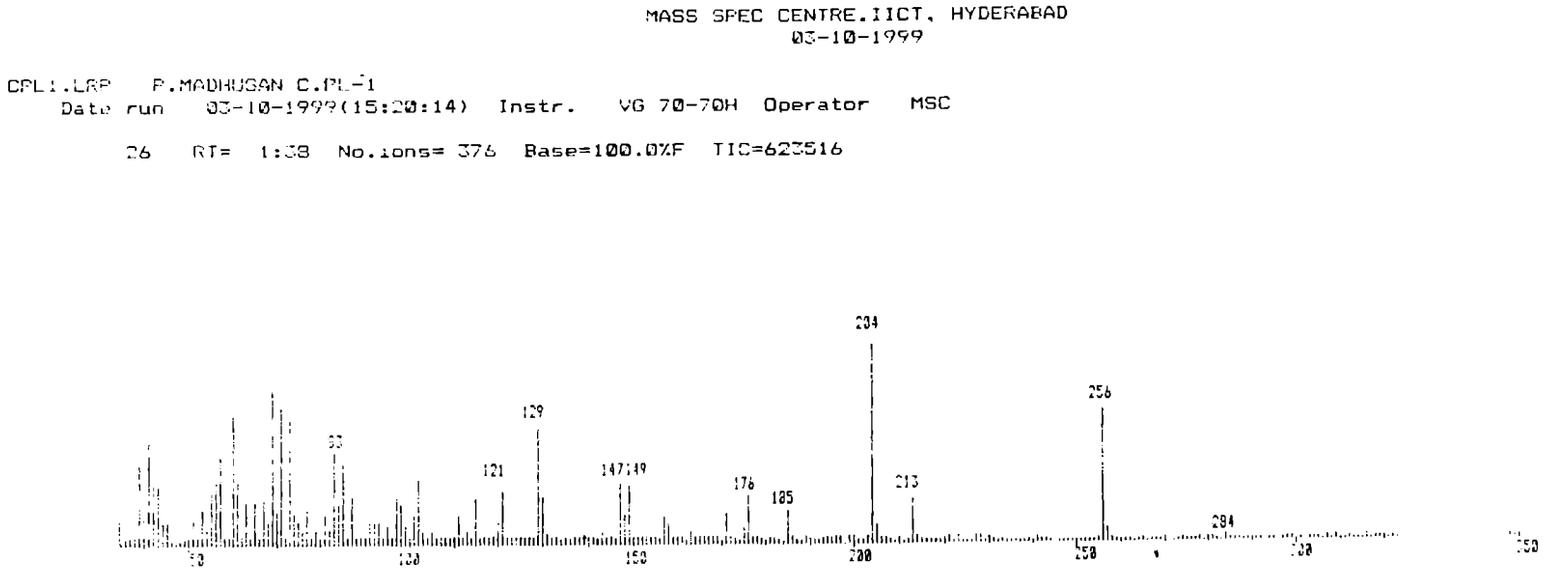

FIG. 6.5 MASS SPECTRUM OF HEXADECANOIC ACID

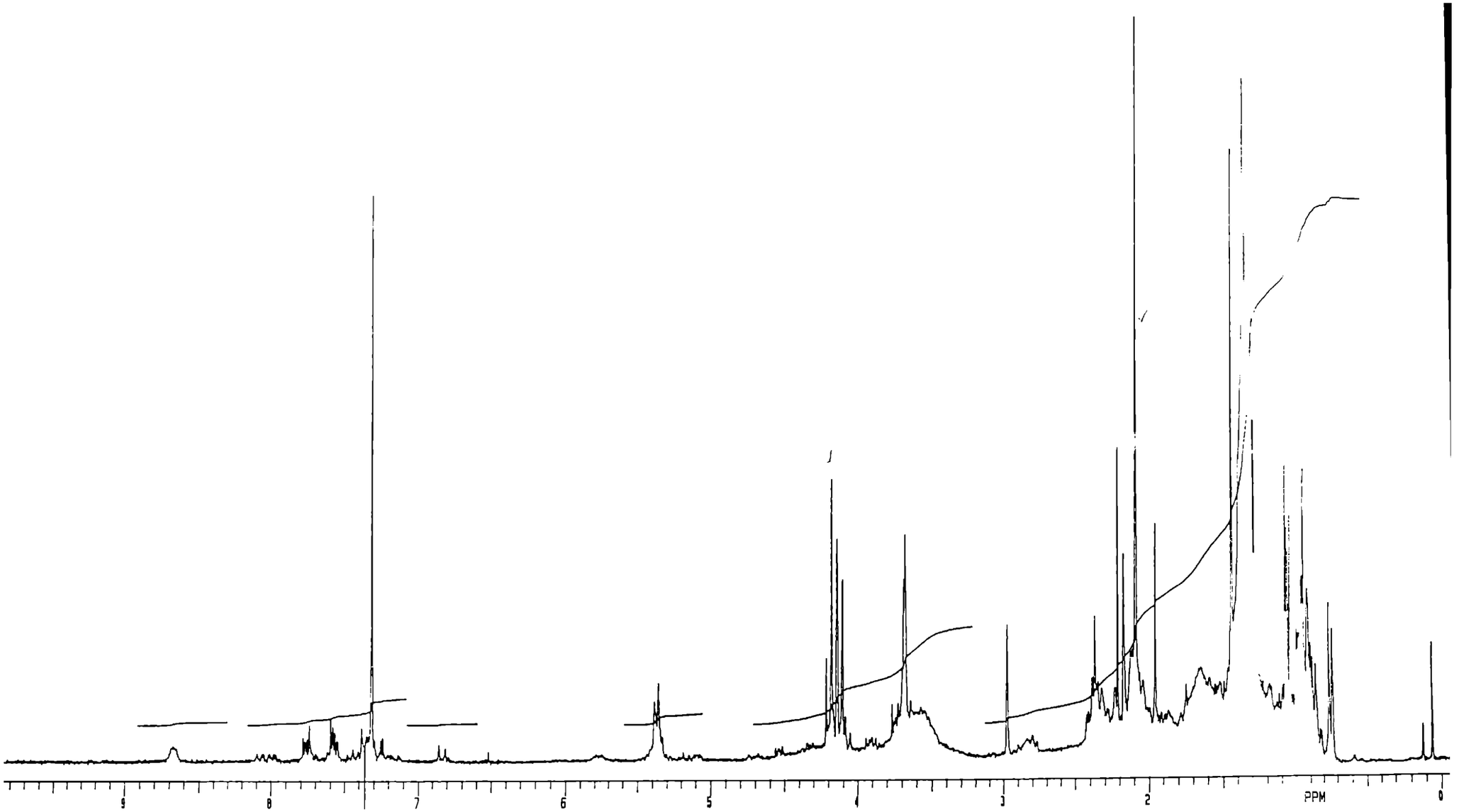

FIG. 6.6 ^{1}H NMR SPECTRUM OF COMPOUND C

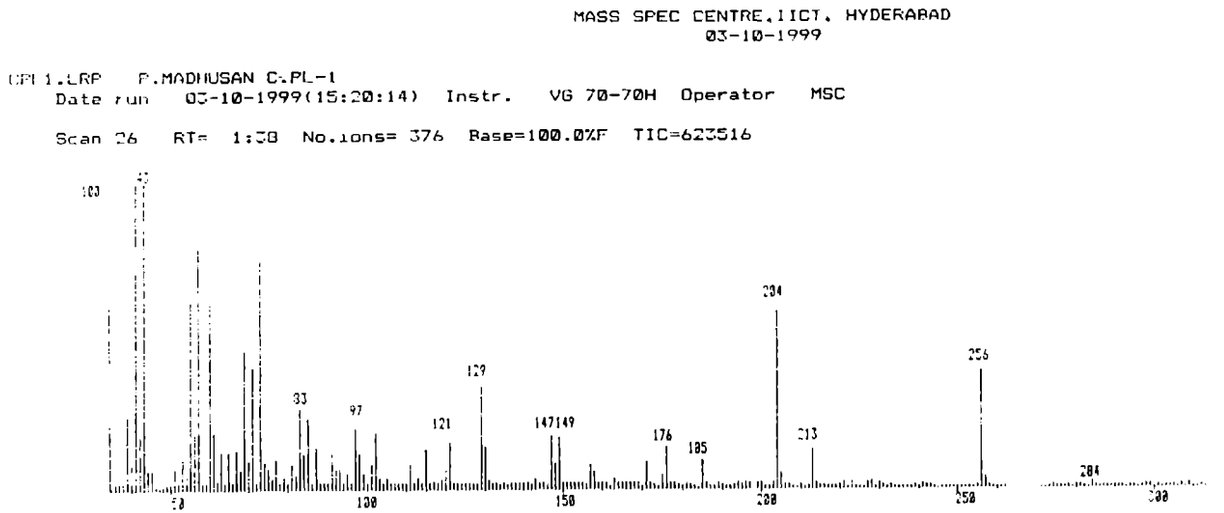

FIG. 6.7 MASS SPECTRUM OF COMPOUND C

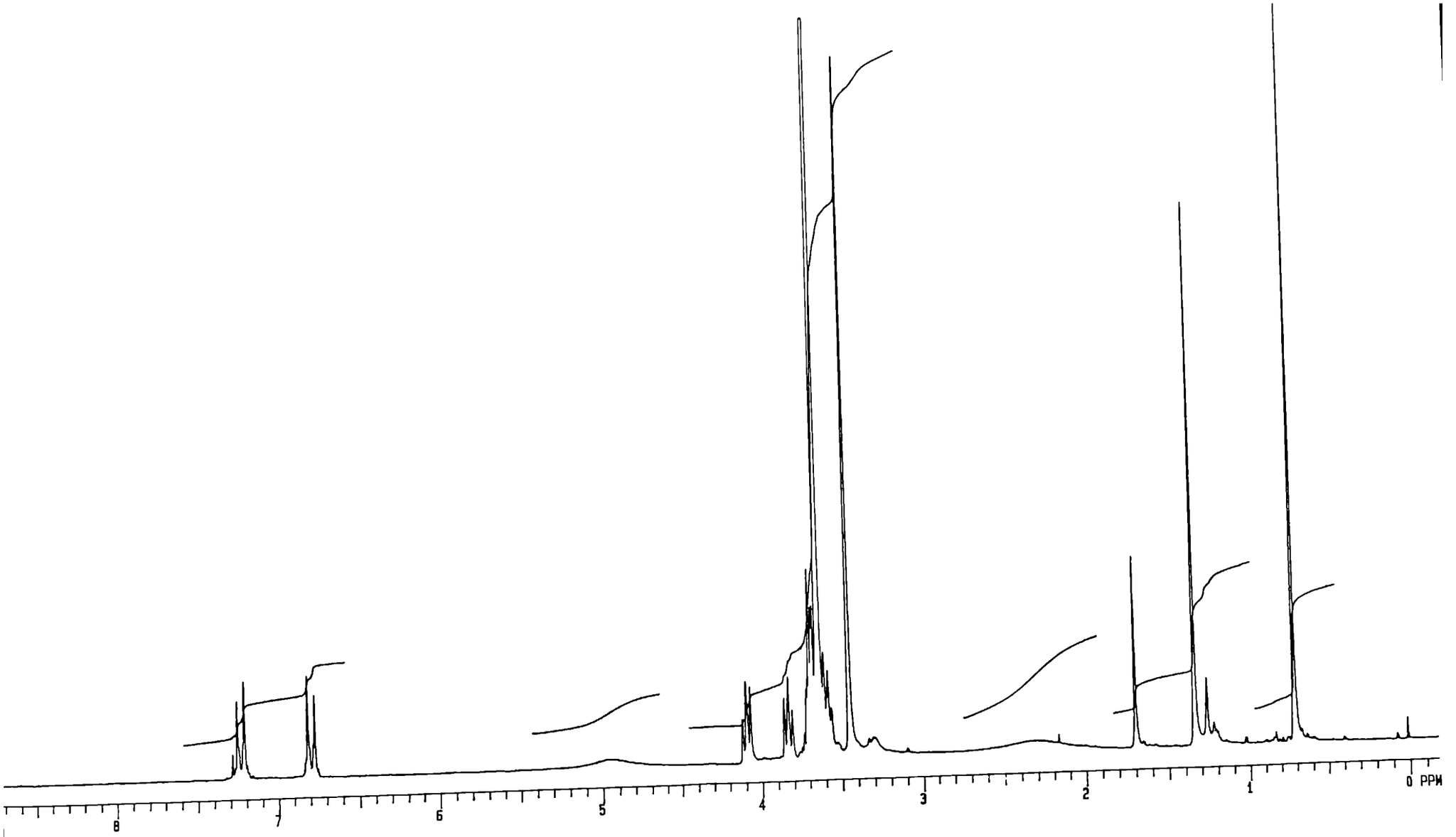

FIG. 6.8 ^{1}H NMR SPECTRUM OF COMPOUND D

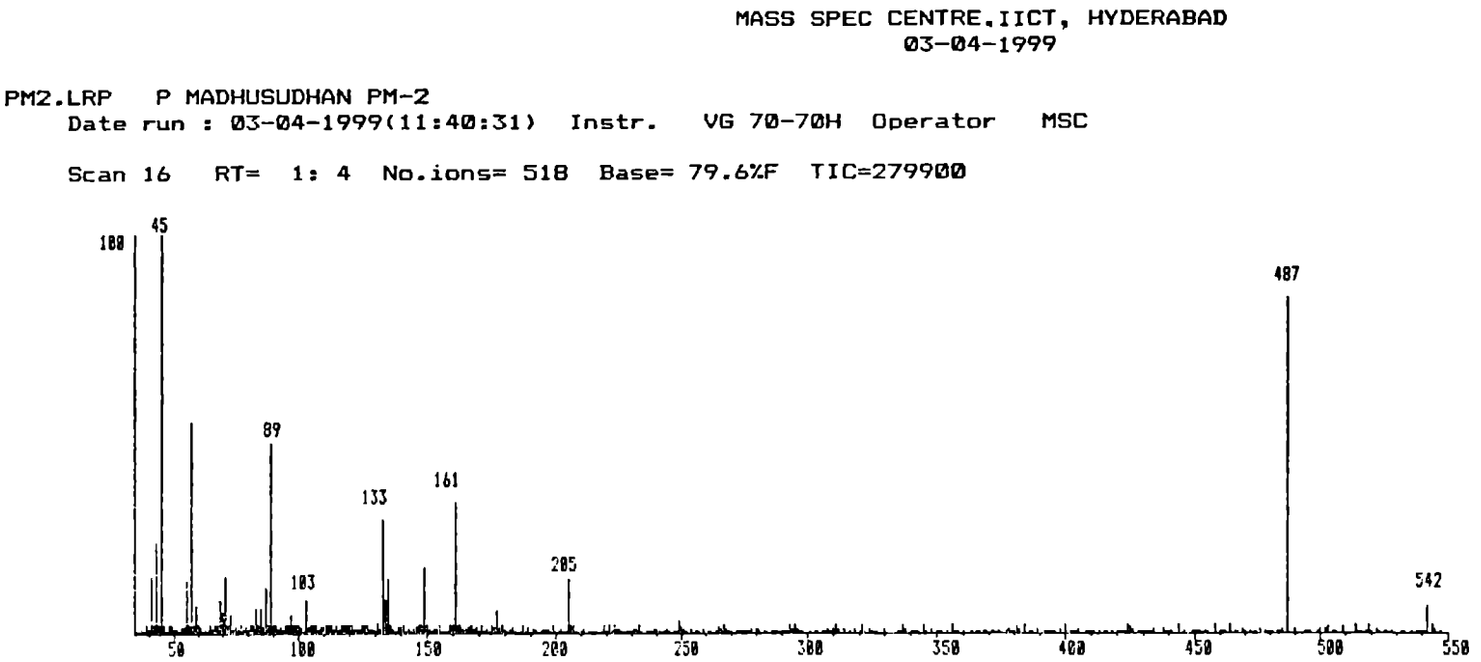

FIG. 6.9 MASS SPECTRUM OF COMPOUND D

MS: (Fig. 5.5)

M^+ 256, 213, 204, 185, 176, 149, 147, 129, 121, 97, 83, 73, 55, 43

The ^{1}H-NMR data clearly indicated the structure of the compound as:

CH_3-$(CH_2)_{12}$-CH_2-CH_2-COOH ≡ CH_3-$(CH_2)_{14}$ – COOH

The mass spectrum (M^+ 256) also supported the above structure.

Compound C & D

Structure of both of these compounds were not fully interpreted. The ^{1}H NMR and Mass spectra are given separately. Compound C is expected to be the ester of Octadecanoic acid.

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Summary

P rosea syn. Indica belong to the family of *plumbaginaceae*, is an important medicinal plant, cultivated widely in India. The roots of these plant are generally used for medicinal purposes mainly as diuretic, germicidal, vessicant, and abortifacient. It is also used for anaemia, diarrhea, leprosy and common wart. The bark of the root contains orange yellow pigment named plumbagin, a crystalline substance, belongs to the class of naphthoquinone. Its chemical structure is 5-hydroxy 2-methyl 1,4-naphthoquinone. Apart from *P rosea, P zeylanica, P europea, Drosera and Drosophyllum* also contains plumbagin. The most exploited source of plumbagin is, of course, *P.rosea* roots. The roots contain 0.9mg/g D.Wt. of plumbagin in the roots. These plants grow very slowly and the roots suitable for plumbagin extraction can be obtained only after several years of growth. The productivity of the plant is also rather poor.

The focus of the present study was to develop alternative strategies to obtain plumbagin. The tissue culture of P rosea for micropropagation has been studied. However, reports on plumbagin accumulation is scanty. The tissue culture route to obtain plumbagin appears to be attractive and technically feasible. There are several reports on various treatments to stimulate the secondary metabolism in plant cells. The major objectives of this study are,

1 To optimize the media conditions for growth of callus culture of *P rosea* and plumbagin accumulation.

2.Studies on hairy root culture of *P rosea.*

3.To study the influence of various permeabilizing agents such as CTAB, DMSO, Triton X-100 and Chitosan on release of the metabolite in to the bulk medium.

(1) To study the effect of various biotic elicitors prepared from bacterial and fungal cultures for enhanced accumulation of plumbagin.

(2) To apply combination of treatment methods for synergistic influence on plumbagin accumulation.

(3) To optimize selected treatment methodologies for enhancement of plumbagin production.

To achieve the above aims, systematic studies have been planned to develop callus and hairy root cultures of *P rosea.*

The callus cultures of *P. rosea* were developed in Murashige and Skoog, Schenk and Hildebrandt and Gamborg's medium. The composition of growth hormones were standardized with various concentrations of auxins (IAA, NAA, 2,4-D) and cytokinins (BAP and Kinetin) It was observed that maximum biomass accumulation was developed in MS medium containing 1 mg/L IAA, 0.5mg/L of NAA and 0.3 mg/L of BA. It was also noticed that 5 fold increase in dry weight was obtained in 30 days of culturing and the plumbagin content has doubled during this period. Embryogenic callus cultures were obtained after regular subculturing and screening for more than two years. The plumbagin content in embryogenic callus cultures were found to be double to that of the non-embyrogenic callus on 30th day of incubation. Cent per cent shoot regeneration was obtained using 1.0 mg/l IAA and 1.0 mg/l BA. The doubling time of the callus was found to be 9 days in MS medium, 14 days in B5 medium and 18.5 days in SH medium and accordingly highest specific growth rate of 0.0767 day^{-1} was noticed in MS medium. The callus cultures were maintained for a period of 5 years by

subculturing every 2 weeks and incubated at 25± 2°C at 10/14 photoperiod. An attempt has been made to develop hairy root culture of *P rosea* by infecting the shoots and leave discs. The hairy roots obtained were analyzed for plumbagin content. It was noticed that both doubling time of the hairy roots was more and plumbagin content was found to be lower than callus cultures. Hence, further studies were confined to with callus cultures only.

The accumulation of plumbagin at various dosage levels of permeabilizing agents such as CTAB, DMSO, Trition X-100 and Chitosan were studied. Both intracellular and extracellular plumbagin was estimated. It was noticed that cell damage was very severe in case of chitosan, and CTAB whereas DMSO and Triton-X-100 at lower dosage level and shorter contact time could alter the cell membrane integrity effectively, to release the metabolite into the medium without damaging the cells. It was observed that CTAB and Chitosan cannot be used as permeabilizing agents but very effective to release the metabolites.

P rosea cells were challenged with elicitor compounds prepared using fungal and bacterial cultures. The fungal cultures used were *Aspergillus niger, Rhizopus nigricans* and the bacteria used was *Bacillus cereus*. *Saccharomyces cerevisiae* was also tried for elicitation purposes. Dose response profiles were generated with each elicitor. In addition to hydrolysed biomass, spent culture media was also used as elicitors. Among all the microbial strains, the elicitor prepared from Bacillus was given lowest response (39.4-62.98%). The mycelial hydrolysate of *R. nigricans* has exhibited 200 to 236 % higher accumulation of the metabolite. It is significant to note that the spent culture medium of *A. niger* has found to be very effective as elicitor. The accumulation of metabolite was nearly 3 fold over control.

The synergistic effect of permeabilization, elicitation, immobilization and *in situ* product removal, when used in combination were studied. In one set of experiments, the cells of *P rosea* were immobilized in sodium alginate and subsequently pemeabilized with 0.001ppm CTAB or with 0.01% (v/v) DMSO for short duration. The treated immobilized cells were cultured in fresh medium containing XAD-2, a nonionic adsorbent. Parallelly, experiments were carried out using individual treatments only. It was noticed that the combination of treatments, immobilization and resin addition has enhanced the plumbagin accumulation by 230 percent over control (9.83 mg/g.D.Wt.). Similarly, experiments were carried out with elicitation instead of permeabilization. The elicitor used was derived from *A. niger* or *R. nigricans* (1.0 % v/v). It was found that multiple treatment strategies (immobilization, elicitation and resin addition) has resulted in nearly 200 percent higher yield of plumbagin over control. Similar results were also obtained when the cells were treated with 1.0% (v/v) R. nigricans derived elicitors.

From these studies, it can be concluded that the increase in metabolite yield was found to be 200 to 250 percent higher than the control when the cells were subjected to either individual treatment such as permeabilization and elicitation or combination of treatments. This has led us to speculate that in case of stress related metabolites (such as naphthoquinones) the synergistic effect of combined treatment is negligible due to saturation effect of the treatment used and beyond a levels of these stress applied to stimulate the product accumulation, the cell response was poor. The thresh hold level of stress to the cells to trigger the enhanced product accumulation can be achieved either by individual treatment or by sequential treatments. In all

the treatment strategies we have attempted, the increase in metabolite yield was found to be nearly 3 fold irrespective of the treatment used.

The present research activity conclusively demonstrates the potential of callus cultures of *P rosea* as a source of plumbagin. Employing various treatments viz. elicitation, permeabilization and immobilization could enhance the product accumulation in the callus cultures. The same procedure could be applied for the production of compounds belonging to the group of naphthoquionones as most of these are found produced during stress conditions. Further work pertaining to large scale culturing is needed before evaluating this methodology on techno-economical basis.