BIOCHEMICAL STUDIES ON THE TISSUE CULTURE OF SOME MEDICINAL PLANTS

THESIS SUBMITED TO
THE UNIVERSITY OF KERALA
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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REGIONAL RE ORATORY
COUNCIL OF SCIENT IAL RESEARCH
TRIV 9

SEPTEMBER 1991

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This is to certify that the thesis entitled "Biochemical studies on the tissue cultures of Some Medicinal plants" submitted by **Mr. A. Jayakumaran Nair** in fulfilment of the requirements for the Ph.D. degree in Biochemistry of University of Kerala is an authentic record of research carried out by him under our supervision and guidance and that no part thereof has been presented before for any other degree.

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DECLARATION

I hereby declare that this thesis is a bonafide record of research work done by me and that no part of the thesis has been presented earlier for any degree, diploma or similar title of any other University.

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ACKNOWLEDGEMENTS

I acknowledge with a deep sense of gratitude my supervising teachers, Dr. P.R. Sudhakaran, and Dr. J. Madhusudana Rao, for the constant encouragement and inspiring and valuable guidance throughout the investigation.

I am grateful to the Director, Regional Research Laboratory, Trivandrum for providing all the necessary facilities to carry out this work and for his keen interest and encouragement. I wish to express my gratitude to Mr. S.V. Ramakrishna, Head, Fermentation Section, Regional Research Laboratory, Trivandrum for his enthusiasm in my research work.

I would like to extend my thanks to Mr. P.J. Mathew and Mr. Mathew Dan of Tropical Botanical Garden and Research Institute, Palode, Trivandrum for their kind help in obtaining the experimental plants. I acknowledge the sincere help, advice and cooperation extended by Mr. V.P. Sreedharan, Dr. K.C.M. Raja, Dr.(Mrs) Emilia Abraham, Dr.(Mrs) P. Prema, Dr. Ashok Pandey, Scientists, and Mrs. C. Kamalabai Amma, Fermentation Section of this laboratory. My sincere thanks are due to Mr. Chandra sekhara Bhat, Scientist for his help in statistical analysis, Mrs. Padmakumari Amma for HPLC analysis and all the personnel of RRL, Trivandrum who have been involved in the successful completion of this work.

I respectfully extend my thanks to Dr. A. Sasidharan Professor, Department of Botany, University College, Trivandrum for his valuable suggestions.

I owe my sincere thanks to all my friends who were a source of help, great cooperation and encourragement.

I gratefully acknowledge the award of Research Fellowship by the Council of Scientific and Industrial Research.

A. JAYAKUMARAN NAIR

PREFACE

This doctoral thesis focuses on the production of secondary metabolites by the tissue and cell cultures of a few selected medicinal plants. Callus cultures of six well known medicinal plants - *Sida cordifolia*, *Lobelia nicotianifolia*, *Tinospora cordifolia*, *Piper nigrum*, *Coscinium fenestratum* and *Rauwolfia tetraphylla* were initiated and screened for their ability to produce the respective medicinal compounds *in vitro*. Production of berberine by the cell and tissue cultures of *T. cordifolia* and *C. fenestrtum* were taken up for a detailed study since the alkaloid content was very high. The nutritional and hormonal conditions of the cell cultures of *C. fenestratum* have been optimized to get maximum berberine production. The final step in the berberine synthesis in *C. fenestratum* has been shown to be the oxidation of tetrahydroberberine.

The thesis starts with a brief review of the poduction of various secondary metabolites particularly alkaloids by plant tissue cultures, and its advantages and disadvantages. The methods employed for developing callus and cell cultures of various medicinal plants, extraction and analysis of secondary metabolites and other analytical techniques used for various enzyme assays have been given in Chapter II. The induction of callus cultures of the six experimental plants and their screening for secondary metabolites, optimization of hormone conditions for rapid callus induction and growth are described in Chapter III. Results of studies on suspension cultures of *Coscinium fenestratum* and *Tinospora cordifolia* for production of berberine and the influence of nutritional conditions and hormones on cell biomass and berberine production are given in Chapters IV and V. The effect of supplementation of amino acids and intermediates on cell biomass and berberine production by *C. fenestratum* cell cultures are given in the following Chapters. The partial purification of THB oxidase, its kinetics and modulation of its activity by different substances has been presented in Chapter VIII. The major findings and conclusions of this study have been presented in the last Chapter.

ABBREVIATIONS

BAP	Benzylaminopurine
DRG	Dragendorff reagent
DMF	Dimethyl formamide
2,4-D	2,4-Dichlorophenoxyacetic acid
GDH	Glutamate dehydrogenase
GS	Glutamine synthetase
GOGAT	Glutamate synthase
HPLC	High pressure liquid chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kinetin	6-Furfuryl amino purine
K_m	Substrate concentration at $\frac{1}{2}$ V_{max}
MeOH	Methanol
NAA	Naphthaleneacetic acid
NAD^+	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NR	Nitrate reductase
rpm	Revolutions per minute
TCA	Trichloroacetic acid
THB	Tetrahydroberberine
THB oxidase	Tetrahydroberberine oxidase

TFA	Trifluoroacetic acid
tg	doubling time
TLC	Thin layer chromatography
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
V_{max}	Maximum velocity
μ	Specific growth rate

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CHAPTER I

CHAPTER I

INTRODUCTION

This thesis presents the biochemical studies on the tissue cultures of six important locally available medicinal plants, with a view to produce medicinally important compounds under *in vitro* conditions. It incorporates results of the study on the nutritional and hormonal requirements for optimum production of an important alkaloid, berberine, and the kinetic properties and modulation of the key enzyme involved in its biosynthesis. Since the information on the production of various individual secondary metabolites by plant tissue culture *in vitro* has been reviewed repeatedly, it is not proposed to review these aspects here in detail. Only those aspects relating to the use of various pharmacologically active compounds, particularly various types of alkaloids and the general strategies being used commonly for optimal production of desired compound have been reviewed in this chapter.

1.1. PLANT KINGDOM AS A CHEMICAL RESOURCE

From prehistoric time onwards man has been using plant extracts to heal a variety of illnesses and as agents of death which range from calaber beans and hemlock

as judicial poisons to that of arrow poisons[1]. Plant kingdom has been an important source of a wide variety of speciality chemicals such as pharmaceuticals, food additives and agro-chemicals. In fact, one of the significant features of plant cell differentiation is the formation of these specific chemicals known as secondary metabolites within specialised cells or tissues. These secondary metabolites differ from the primary metabolites in that they are not generally essential for the normal life activities of the cells. But these products appear to be important in the interaction between the plant and its environment[2]. Their function may be in protecting the plant from predators, pathogens and from environmental stress or may be related to the reproductive machinery of the plant in promoting pollination.

1.1.1. Different types of commercially important natural products

The plant derived natural products range from highly pure compounds such as different medicinal compounds, complex mixtures and blends of substances such as food additives and cosmetics to bulk materials such as cocoa butter fat and tobacco. Based on the industrial importance, these products can be classified as follows.

(1) Medicinal compounds

Medicinal compounds form a major area of plant derived products. In addition to pure medicinal compounds obtained from different parts of medicinal plants, crude extracts of whole plants or individual organs, which contain large amount of different classes of compounds are also important industrially for the preparation of pharmaceutical products. The discovery and development of synthetic drugs and microbial agents led to a marked reduction in the use of plant medicinals during the period 1920-50. Even then a major part of the drugs are obtained directly or indirectly from plant sources[3]. The plant derived medicinal compounds have a variety of chemical forms and also a wide range of therapautic activities from analgesics to antifertility agents, antimicrobial and anticancerous compounds.

(2) Agro-chemicals

These are a group of plant derived chemicals such as pyrethroids which find use as antifeedants or insecticides. Pyrethroids are extremely useful plant insecticides extracted from flowers of *Chrysanthemum cinerariaefolium*. In addition to this the essential oils of certain plants such as *Phyllodendron amurense*[4], *Azaderecta indica* (Neem)[5], *Teucrium and Ajuga remota*[6] were shown to have insect repellent or insecticidal properties.

(3) Fine chemicals

Fine chemicals is a general name given to chemicals such as perfumes, flavours, aromas, colouring agents and food materials. The products range from simple compounds such as quinine to complex mixtures such as essential oils of aromas and flavours. The alkaloid quinine is used as flavouring compound in food industry whereas the aromas and flavours of most of the plants, such as that of jasmine, rose, cardamom, clove, sandal etc are mixtures of large number of chemicals. Most of them are simple monoterpenes and volatile compounds.

(4) Toxins

Some of the toxins and antimetabolites are among the most effective drugs for human diseases. Certain cytotoxic compounds such as vinblastine vincristine, colchicine etc are active against several forms of cancer. The compounds such as morphine, codeine, and products such as opium, heroin etc from *Papaver somniferum*, marijuana or hash from Cannabis, cocaine from Erythroxylon etc are good examples of narcotics, all of which find use as drugs for the treatment of certain diseases. Certain neurotoxic plant poisons are still used today by tribals in Africa and South America for hunting animals for food. Many plant poisons such as ricin from castor bean are good neurotoxins.

1.1.2. Methods of production of the natural products

The main sources for obtaining the natural products include the natural sources such as plants and microbial systems and through chemical synthesis.

(1) Source plants

A large number of medicinal compounds are still obtained from plant sources. The important drugs such as vincristine, vinblastine,colchicine, hyoscyamin, scopolamine and steroidal drugs such as diosgenin, cardiac glycosides such as digoxin etc are still obtained from natural sources. In addition to the medicinal compounds, the plant products which are complex mixtures of different types of organic compounds, such as fragrances and flavours cannot be replaced by synthetic compounds. In this traditional method of obtaining natural compounds, the source plants are cultivated in the fields and harvested after maturation and the required compounds are extracted and purified.

(2) Chemical synthesis

The natural compounds which are stereochemically more simple can easily be synthesized in the laboratory. For instance, ephedrine an alkaloid having wide use in pharmaceutical industry is now obtained by chemical synthesis.

(3) Microbial production

A large number of drugs such as antibiotics are obtained through microbial systems. The antibiotics such as penicillin, streptomycin etc are very good examples. Certain plant products which are the direct expression of a single gene, such as Thaumatin - a non-sugar sweetener isolated from *Thaumatococcus daniellii* are now obtained through microbial fermentation by genetic engineering.

1.1.3. Limitations of the conventional methods

Production of compounds, both from plants and microbial systems requires long procedures of isolation and purification. In the chemical processes also, the separation of the final product and its purification is not simple. In these cases the supply of raw materials for the synthesis of compounds affects the market supply and the cost of the products. In cases where the plants form the sources of the medicinal compounds, it requires long gestation period for the maturation of the source plants for obtaining the plant parts as the raw material for the extraction and isolation of the compound. Climatic factors, regional political and social factors can cause variations in the supply of raw materials (plants) for the extraction of compounds. Moreover, most of these medicinal plants are extinct or are nearing extinction

due to their over exploitation. In addition to all these, the yield of the compound is usually very low and raises the cost of the product.

1.2. PLANT CELL CULTURE: AN ALTERNATIVE FOR NATURAL PRODUCT SYNTHESIS

Plant tissue culture, in a broad sense, means the growing of plant cells, tissues or plant parts under aseptic conditions in a chemically defined medium. The technique of plant cell culture started about ninety years ago, when Haberlandt[7] first began experiments to culture single cells isolated from different plant tissues in simple nutrient solutions. Eventhough he was able to maintain the cells in living conditions in these nutrient solutions, cell division was not observed until much later. The tremendous progress, and rapid developments in 1950s and 1960s[8] converted the cell, tissue and organ culture from an area of academic interest into a major tool for the horticultural industry through mass propagation of horticultural and ornamental plants. Then it rapidly expanded to the area of agriculture to cover the important food and cash crops. Plant tissue culture has given a remarkable improvement in agriculture by providing improved varieties of plant genotypes through micropropagation, somaclonal variation and invitro genetic transformation through protoplast hybridization and

introduction of specific genes. Apart from this, it has now developed as a new tool for studying the basic problems in the different areas of plant sciences such as molecular biology, genetics and biochemistry including the biosynthesis of various biologically and industrially important metabolites. The recent developments in plant cell culture also have a significant impact on various sectors of chemical industry, related to the natural products. The advantages made in the cell culture technology during the early 1970s has identified the potential in plant cell culture as an enabling technology for further exploitation of the plant kingdom as a chemical resource.

1.2.1. Use of cell cultures for the production of secondary metabolites

Plant derived natural products or secondary metabolites which are stereochemically complex are expensive due to their short supply. The disadvantages of synthetic methods and short supply from the source plants, justify the increased interest in finding alternative sources of supply which avoid dependence on the whole plant and chemical synthesis. It is in this context that there is interest in using plant cell cultures in biotechnological process for the commercial production of useful secondary metabolites. There are different ways by which the plant cell cultures can be used for the secondary

metabolite synthesis. The following are some among them which can be used for the *in vitro* production of natural compounds[1].

(1) As a new route of synthesis to establish products like codeine, quinine, pyrethroids etc.

(2) As a way of synthesis to novel products from plants that are difficult to grow.

(3) As a source of a novel chemical in their own right, e.g. rutacultine from *Ruta graveolans*.

(4) As a biotransformation system, either as their own or as a part of a chemical process, e.g. digoxin synthesis.

1.2.2. Products from plant cell culture

Since the experiments of Haberland[7] more than 90 years ago and the first success of true plant tissue culture by Gautheret[9] investigations on plant cell culture have been in progress. The earliest detailed reference to plant cell culture as an industrial route to natural product synthesis is found to be patent application of Routier and Nickell in 1956[10]. Progress in this area was poor in the following years. The developments in plant cell culture during these periods were reviewed with its different problems by many authors[11-15]. In a historical perspective, Staba[16] identified about 27 review articles which appeared between 1965 to 1983 on

accumulation of secondary metabolites in cell cultures and this continues unabated. In many of these cases, the desired products were either not produced in the cultures, or the yield was extremely low. Some products are not at all produced unless there is tissue differentiation or sometimes organ formation in culture. The regenerated plants which produced both qualitatively and quantitatively the same spectrum of products as the plant, from which the culture was initiated, however served to show that the ability of the product synthesis was not lost during the culture, but because of some unknown reasons not expressed[17]. Eventhough the product yield in plant cell cultures reported during this period were very low compared to that of the whole plant, some products like diosgenine[18,19], Ginseng saponins[20], Harmin[21] and Visnagin[22] were produced in cell cultures at levels equal to or even exceeding those of the parent plant.

During the period after 1973, there are a large number of reports on cell cultures, which has given reasonable yields of desired specific compounds[17,23]. In addition, the range of substances observed in cultures of all types (i.e. callus, tissue and organ) has widened dramatically[24-26].

The secondary metabolites which are reported from the cell and tissue cultures of various plants belong to major group of compounds such as quinones, phenyl propanoids, flavanoids, alkaloids and other similar products.

Formation of various types of anthraquinones and naphthaquinones in the callus and cell cultures of various plants have been reported by several authors[27-32]. Biosynthesis of Shikonin derivatives were extensively studied and an industrial process for the commercial production with cell cultures of *Lithospermum erythrorhizon* after optimizing the media and other culture conditions was developed[33-38].

There are a large number of reports regarding the accumulation of the plant pigment anthocyanin in the callus and cell cultures of various plants such as *Daucus carota* and *Beta vulgaris*[39-46]. Flavanoids such as quercetin also have been reported from a large number of plant cell cultures[47-51]. The cell cultures of *Nicotiana tabacum* accumulated different types of hydroxy cinnamic acid derivatives[52-54]. *Pyrus malus* has also been shown to contain chlorogenic acid and its derivatives[55]. Another widespread and important conjugate, rosmarinic acid has been produced in relatively higher amounts by the cell

cultures of *Coleus blumei*[56], *Rosmarinus officinalis*, *Salvia officinalis* etc[57]. *Lithospermum erythrorhizon* also produced rosmarinic acid under conditions which supresses the synthesis of Shikonin. Cultures of *Rehmania glutinosa* produced verbascoside and other related compounds in relatively higher quantities[58].

A number of isoprenoids have been reported to be accumulated in the tissue cultures of several plants. Cultures of *Jasminum officinale* and *Rosa damascena*[59,60], accumulated very low levels of monoterpenes although the enzymes of isoprenoid biosynthesis were shown to be active in these tissues. The induction of sesquiterpenoid phytoalexins in cell cultures has continued to attract interest particularly in species of the Solanaceae when elicited with microbial agents[61-63].

The suspension cultures of *Gossypium hirsutum* produced gossypol[64] and different species of Dioscorea produced diosgenin and other related saponins in the callus and cell cultures[65-68]. Saponins were also detected in suspension cultures of *Trigonella foenum-greacum*[69] and *Agave wightii*[70] as well as in calli of *Tribulus tirrestris*[71]. The physiological properties of the Ginseng saponins have motivated a number of studies for the production of ginsenoside in cultures of *Panax ginseng*. The same array

of eleven saponins that is found in ginseng root has been observed in root differentiated and undifferentiated callus cultures[72].

There are a few examples of the formation of highly oxygenated and modified triterpenes such as Ecdysteron in the calli of *Trianthema portulacastrum*, Physalin in the calli of *Physalis minima*[73-75] and quassin in the cultures of *Picrasma quassioides*[76]. The dark grown cell cultures of *Lycopersicon esculentum* accumulated carotenoids with higher percentage of lycopene[77].

A variety of compounds coming under different groups of natural products including alkaloids that are produced by the callus and cell cultures of different plants are listed as appendix. A number of reviews are available describing the use of plant tissue culture for the production of various classes of compounds and their merits and demerits[8-16]. Therefore, a detailed review of the production of all the different classes of compounds by cell culture is not attempted here. Since alkaloids form a major group of interest, the literature regarding the production of alkaloids by the cell cultures of different plants has been reviewed later, separately in this chapter.

1.2.3. Production of Novel compounds

In addition to the use of plant tissue cultures as an alternate route for the synthesis of established products it can also be used as the source of novel compounds which are not observed in the parent plant; for instance,, production of rutacultin by cultures of *Ruta graveolans* and sesquiterpene lactones by cultures of *Andrographis paniculata*[25]. Jalal et al[50] reported the formation of six new flavones in the callus cultures of *A. paniculata* which are not detected in the plants.

1.2.4. Biotransformation

Biotransformation is another means through which the synthetic ability of the plant cells may be utilized in commercial process. Biotransformation can be carried out using either plant cells or isolated enzymes. A number of reports are available on the biotransformation of different compounds, mainly as part of organic synthesis. The most advanced industrial application being the hydroxylation of α-methyldigitoxin to α-methyldigoxin[78]. Biotransformation of digitoxigenin by Ginseng hairy root cultures have been reported[79]. Vanek et al[80] reported the biotransformation of 2-(4-methoxy benzyl)-1-cyclohexanone to its glucoside by the cell cultures of *Dioscorea deltoidea*.

1.2.5. Use of plant cell culture in the study of metabolism

In addition to the use of plant cell culture as a powerful technique for the production of commercially important metabolites, micropropagation and genetic modifications of crop plants, it forms a valuable tool for the basic biochemical studies. The cell cultures of higher plants can be used instead of microorganisms to study the problems related to photosynthesis, respiration, protein synthesis and regulation of various metabolic pathways. It can also be used instead of the whole plant for the study of the biosynthetic pathways of various compounds including secondary metabolites and their degradation. It can be used as a system for studying the molecular and biochemical basis of responses towards different types of stress conditions like temperature, light, microbial attack, heavy metals and other type of toxic compounds.

The biosynthesis of secondary metabolites can be studied by supplementing radioactive precursors and intermediates and also by the assay of enzymes of the biosynthetic pathway. For example, the biosynthetic pathways of berberine have been fully elucidated at enzyme level using the cell cultures of berberis sps[81] and *Coptis japonica*[82,83]. Schumacher et al[84] observed the formation

of a novel enzyme upon elicitation in the cell cultures of *Eschscholtzia californica*. They have isolated, purified and characterized the enzyme. Yamamoto et al[85,86] studied the mechanism of berberine secretion using the cell cultures of *Thalictrum flavum*. Schmelzer et al[87] tried to find out the molecular mechanisms responsible for the altered enzyme activities and secondary metabolism followed by elicitation in the cell cultures of *Petroselinum crispum* by fungal extracts. Thus the plant cell cultures form efficient systems for studying the biochemical, biophysical and molecular mechanism behind various cellular phenomenon.

1.3. PRODUCTION OF ALKALOIDS BY PLANT CELL CULTURES

Alkaloids form an important group of naturally occurring compounds with diverse properties, functions and are being widely used in pharmaceuticals. They are generally grouped into different classes such as Indole alkaloids, Isoquinoline alkaloids, Pyridine alkaloids, Tropane alkaloids, Purine alkaloids etc based on their common structural features. Since these are important secondary metabolites, a number of attempts had been made to study the synthesis of these compounds as well as to optimize their production in cell cultures of different plants. A brief review of literature in this area is given below.

(1) Indole alkaloids

Indole alkaloids are a group of alkaloids derived from the aromatic amino acid tryptophan characterized by the presence of indole ring. Reports on this class of alkaloids have been dominated by studies on cultures of *Catharanthus roseus*. This species is best known for its ability to produce the antineoplastic dimeric indole alkaloids vincristine and vinblastine. The literature concerning the formation of various indole alkaloids in cell and tissue cultures of *C. roseus* has been reviewed by Van der et al[88]. Several aspects involved in the formation of secondary metabolites such as regulation of secondary metabolism, environmental factors influencing the secondary metabolism, biosynthesis and enzymology of the products, analysis of product formation, immobilization of cultured cells and stability of cell lines etc are discussed by them. Eventhough the cell cultures of *C. roseus* could produce a large number of different types of indole alkaloids in culture, it is still not possible to produce the most important dimeric indole alkaloids vinblastine and vincristine by suspension cultures. But two novel dimeric indole alkaloids Voafrine A and B have been isolated from another Apocynaceae plant *Voacanga sp.*[89].

Suspension cultures of the related species *C. ovalis* have been reported to contain an array of alkaloids similar to that found in cultures of *C. roseus*. In addition to the usual alkaloids it showed the presence of apparicine and alstonine. The alkaloids that are found in the cell suspension cultures of *Rauwolfia serpentina* have been examined in detail. The major component was vomilenine, along with smaller amounts of ajmaline, reserpine, ajmalicine, serpentine, yohimbine, alstonine, sarpagine, vinorine etc[90,91]. Shoot cultures of *R. serpentina* contained more amount of alkaloids than plant levels[92,93].

Suspension cultures of *Tabernaemontana divaricata* produced different types of indole alkaloids such as apparicine, conoflorine, coronaridine, tubotaiwine, catharanthine and vinervine. Some other related species such as *T. iboga* and *T. elegans* also produced some of the above mentioned alkaloids in callus cultures[94,95].

A number of other Apocynaceae plants such as *Stemmadenia tomentosa*, *Voacanga africana*[96], *Ochrosia elliptica* etc also produced indole alkaloids in cultures[97]. Bioassay screening of suspension cultures of *Picralima nitida* led to the isolation of another new alkaloid pecricine along with pericalline[98]. The cell cultures of *Ailanthus altissima* produced β-carboline alkaloids in comparatively higher

quantities[99,100]. The same type of alkaloids were also accumulated in the heterotrophic cultures of *Peganum harmala* which include harmine, harmaline, harmol and harmalol[101]. The formation of harman and norharman was also observed in suspension cultures of *Cinchona ledgeriana*[102]. The main interest of Cinchona relates however to the production of quinine and quinidine by *C. ledgeriana* and *C. succirubra*[103]. In addition to quinine and quinidine, cinchonidine and cinchonine were also observed, and these form the main indole alkaloid content of the cell cultures of both species[104,105]. Recently, formation of canthine-6-one alkaloids in the cell cultures of *Brucea javanica* has been reported by Liu et al[106].

(2) Isoquinoline alkaloids

A number of studies were carried out to produce the pharmacologically active morphine alkaloids by the cell cultures of *Papaver bracteatum* and *P. somniferum*. The extensive studies indicated that neither species appear to produce measurable amount of morphine or codeine in cultures unless extensive shoot differentiation is induced[107,108]. The root organ cultures of *P. somniferum* have been found to accumulate thebain. Sangunarine was the main alkaloid in the calli of *Corydalis ophiocarpa*, where it was accompanied by protopine; a new alkaloid

of the corypalline type[109]. Jatrorrhizine was found as the main alkaloid in the calli of *Dioscoreophyllum cumminisii* along with magnoflorine and palmatine[110]. These alkaloids were also produced in the callus cultures of *Plagiorhegma dubium* and *Berberis stolonifera*[111]. Small amounts of berberine and columbamine were also found in the alkaloid fraction of *B. stolonifera* callus cultures. Berberine was the major component in other systems. The suspension cultures of *Coptis japonica* produced large amounts of berberine[112,113]. Smaller amounts of jatrorrhizine and coptisine were also formed. The calli and suspension cultures of *Thalictrum minus* produced a complex mixture of isoquinoline alkaloids dominated by berberine[114]. The berberine that was produced in the suspension cultures was largely released into the medium, where it crystallised as berberine nitrate or chloride[115,116]. Calli of *Nandina domestica* have also been found to secrete berberine gradually into the medium during long term culturing[117].

A different profile of isoquinoline alkaloids was observed in suspension cultures of *Fumaria capreolata* where the major components were protopine, magnoflorine and reticultine[118]. Suspension cultures of *Escholtzia californica* produced benzophenanthridine alkaloids, mainly dihydrochelirubine, dihydrosangunarine, dihydromacarpine

and dihydrochelerythrine[119]. Suspension cultures of *Macleaya microcarpa* have been reported to secrete sanguinarine and protopine into the culture medium[120].

(3) Quinolizidine alkaloids

A series of legume species have been extensively examined for their ability to produce quinolizodine alkaloids in culture. Although each species (Lupinus, three species of Cytisus, *Laburnum alpinum, Baptisia australis, Sarothamus scoparius)* displayed a characteristic alkaloid profile in its aerial tissues, only very low levels were produced in culture and this consisted almost exclusively of lupanine[121,122].

(4) Tropane alkaloids

The pharmacological importance of tropane derivatives and the responsiveness of solanaceous species in culture have made the study of this group of alkaloids a popular theme. In most cases, however the levels of production have been extremely low. Neither differentiated nor undifferentiated calli of *Duboisia leichnardtii* contained any tropane alkaloids[123]. Suspension cultures of *Datura innoxia* contained 0.0016 percent of their dry weight as scopolamine[124]. A cell line of *Hyoscyamus niger* contained 0.01 to 0.02 percent of its dry weight as hyoscyamine.[125]

Cultures of *Duboisia leichhardtii*[126,127], *Duboisia myoporoides*[127,128], *D. hopwoodii*[127], *Hyoscyamus muticus*[129], *H. niger*, *H. albus*[130] and *Atropa belladona*[131] all showed alkaloid production response as a result of root differentiation.

(5) Pyridine alkaloids

The production of nicotine in Nicotiana and related genera has been examined in numerous studies. In general, it has not been difficult to find modest levels of nicotine in cell cultures of *Nicotiana tabacum* and this can be increased by screening and by optimization of medium[132,133]. Cultures of different species of Duboisia have been found to contain nicotine in addition to the tropane alkaloids[126-128].

Trigonelline has been shown to occur widely in suspension cultures of gymnosperms and angiosperms[134]. The cultures of *Phaseolus aureus* and suspension cultures of *Trigonella foenum-graecum* produced Trigonelline and was much higher in the latter[134,135].

(6) Purine alkaloids

Cell cultures of *Coffea arabica* accumulated low levels of caffeine and theobromine with part of the product being released into the medium[136,137]. Applying a saline stress and growing the cells in the light, enabled

to increase the production of these alkaloids to a higher level, particularly in the case of Caffeine[138]. The production of various types of alkaloids by different plant cell cultures was reviewed extensively by Hay et al[139].

1.4. STRATEGIES FOR IMPROVING PRODUCT YIELD IN CELL CULTURES

The accumulation of various medicinal compounds in the cell cultures clearly indicates that plant cell cultures can be used as an efficient system for the synthesis of pharmaceutically important compounds. Of the pharmacologically active principles found in plants, alkaloids are the most important compounds. Only a few of these alkaloids are now produced by chemical synthesis, i.e. Caffeine and Ephedrine. A great deal of interest has been focused on the indole and isoquinoline alkaloids which represent the two major groups of pharmaceutically important plant products.

One of the main factors which has prevented cell cultures from being considered as a serious rival to whole plant as well as chemical synthesis has been their relatively low yields of secondary metabolites. Over the last ten years, particularly with improved understanding of cultural requirements and introduction of cell selection, there have been many examples where the level of secondary

metabolites in cultures approaches and in some cases exceeds that in the whole plant[140]. The production of desired compounds in amounts equivalent to or higher than that in plants from which they are derived is important, particularly for the commercial production of the specific product with the help of suitably designed bioreactors. A number of experimental strategies have been adopted to achieve maximum product formation.

Improvements in the understanding of nutritional and hormonal requirements for better growth and product yield led to the optimization of nutritional and other cultural requirements for the improved yield of required compounds by the cell cultures. Several aspects involved in the formation of secondary metabolites such as regulation of secondary metabolism, environmental factors influencing secondary metabolism, biosynthesis and enzymology of products, analysis of product formation, immobilization of cultured cells and stability of cell lines for the improvement of alkaloids production by the cell cultures of *C. roseus* were reviewed by Van der et al[88]. A number of attempts have been made to enhance the production of pyridine alkaloids by manipulating the culture medium[141-143]. The highest yield of nicotine that has been reported so far (5.3 percent of the dry weight)

was obtained in a photomixotropic suspension culture of *N. tabacum* at the end of an optimized three stage protocol of manipulating the medium[144]. The biosynthesis of Shikonin derivatives was extensively studied for optimizing the nutritional and hormonal conditions[33,34,37,38]. Various studies regarding the optimization of culture conditions and influence of different hormonal types on anthocyanin production have been conducted in the cell cultures of *Daucus carota*[39,145,146] and also in a number of other systems[40-44].

Another approach for improving the product yield in cell cultures is to select high yielding cell lines by adopting different selection procedures. In the production of secondary metabolites, the variability within cell cultures, called somaclonal variation[147] has been used for selection of high producing cell lines[44,132,148-152]. After selection of high yielding cell lines followed by standardization of nutritional components, it enormously increased the product formation in the cell cultures of *Lithospermum erythrorhizum* and in 1983, the process was commercialized by Mitsui Petrochemicals in Japan[153]. High yielding cell lines were also selected as part of improving the product yield or to find out cell lines capable of producing specific products in a large number of plant cell cultures such as *Catharanthus*

roseus[44,154]. Sato and Yamada[113] established high berberine producing cell lines of *Coptis japonica*.

Suzuki et al[155] isolated four high berberine producing cell lines of *T. minus* by adopting a novel method of selection, eventhough the cell lines were not showing stability with respect to the berberine production during successive subculturing. Fontanel et al[156] have studied the somaclonal variation in the berberine producing capability of different cell culture strains of *Thalictrum minus* to find out a high producing culture strain with stability in the quantity of berberine production.

In many cases the product formation occurred in the cell cultures or increased when the cultures were subjected to various stress conditions. There are a lot of reports on the production of alkaloids and other compounds by the elicitation with appropriate microorganisms such as fungal/bacterial extracts[157-161]. Suspension cultures of *Daucus carota* produced 6-methoxymellein when treated with live fungus *Chaetomium globosum*[162]. Various types of stress induced isoflavanoid production have been reported in some legumes[163-165]. Suspension cultures of *Gossypium hirsutum* produced gossypol during normal growth, but increased dramatically by challenging them with conidia or extracts of the wilt pathogen *Verticillum*

dahliae[166]. Wink et al[167] showed the influence of various environmental stresses on a transient increase in the alkaloid production in *Lupinus polyphyllus*. Treatment of suspension cultures of *Papaver somniferum* with homogenate of fungus Botrytis increased the Sangunarine content[168,169].

In many cases the formation of the required product was closely related to the tissue organisation and differentiation[170]. For example, the differentiated cultures of *Apium graveolens* produced flavour components which consists of phthalide derivatives[171]. Organogenesis in cultures of *Sarothamnus scoparius* has been shown to lead to increased levels of alkaloid accumulation[172]. A requirement for differentiation has also been observed in cultures of *Heimia salicifolia* which produced no alkaloids in undifferentiated state[173].

Root organ cultures of *Hyoscyamus niger* accumulated scopalamine in excess to those found in roots and leaves of the plant[174]. A number of other plant cell cultures also showed an alkaloid production response as a result of root differentiation[126-131].

In those cases where the product formation is linked with the root formation in cultures, the hairy root cultures induced by *Agrobacterium rhizogenes* can be applied successfully. A large number of research reports are

available on the production of alkaloids by the hairy root cultures of different species which are otherwise difficult to produce in cell cultures. Hairy root cultures of *N. rustica* produced nicotine and anatabin in modest levels[175,176]. Yonemitsu et al[177] recently reported the production of lobeline by the hairy root cultures of *Lobelia inflata*. In the case of *Atropa belladona* also the hairy root cultures induced by *A. rhizogenes* increased the atropine content upto 0.37 percent of their dry weight[178]. In addition to alkaloids there are a number of reports on the production of pigments[175,179-183]. Recently Sato et al[184] reported the production of Anthraquinones by the hairy root cultures of *Rubia tinctoria*.

1.5 ADVANTAGES AND DISADVANTAGES OF PLANT CELL CULTURE SYSTEMS

Plant cell and tissue culture appears to be an alternative for a constant supply of homogenous material when compared to the chemical synthesis of natural products and extraction from natural sources. Fig.1 compares the conventional methods of obtaining natural products which include the cultivation of the plant, followed by extraction, isolation and purification of the desired compound with that of the plant cell culture techniques, using both free cells and immobilized cells for continuous or semicontinuous production of a desired compound.

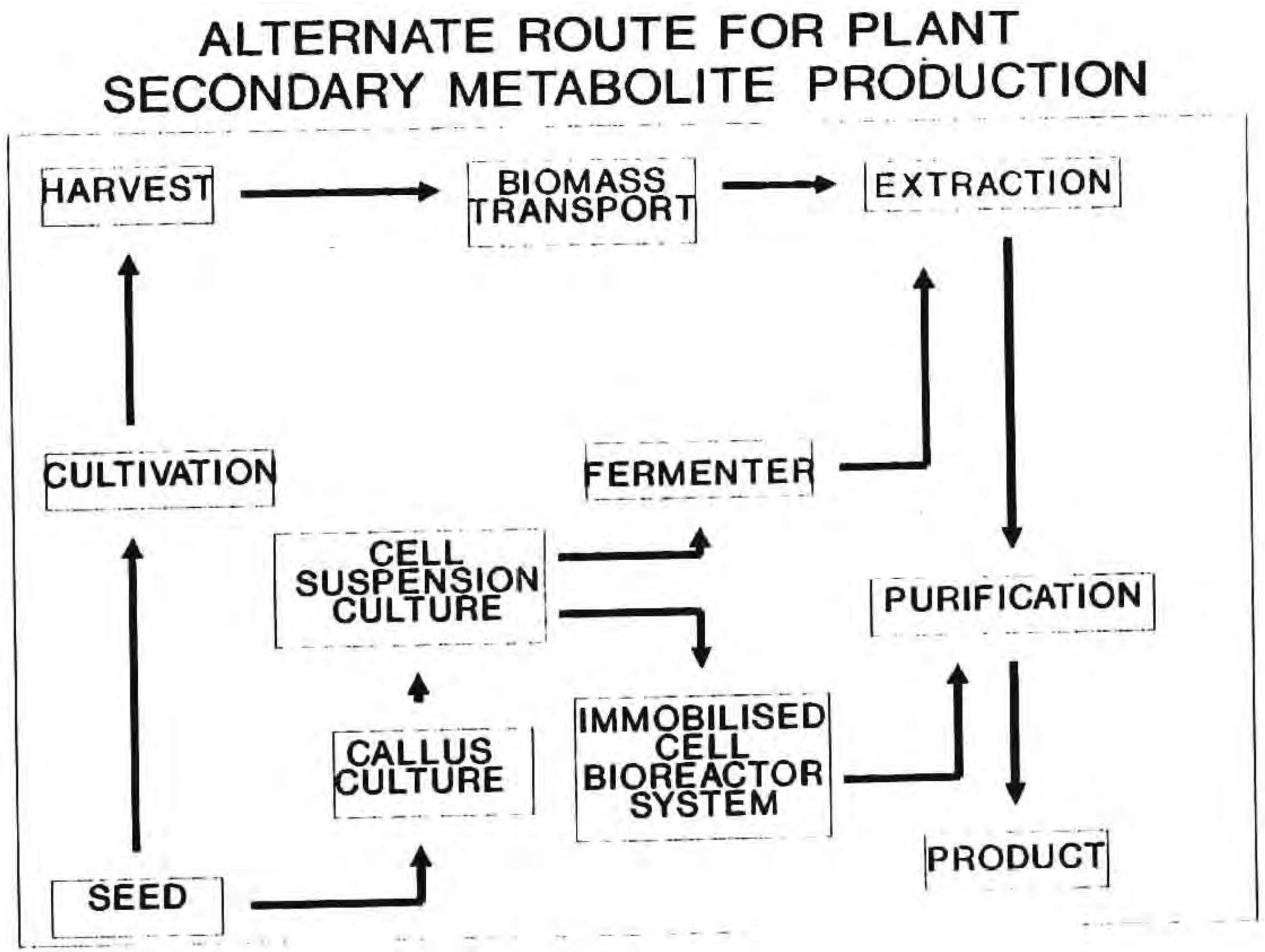

ALTERNATE ROUTE FOR PLANT
SECONDARY METABOLITE PRODUCTION
HARVEST
BIOMASS TRANSPORT
EXTRACTION
CULTIVATION
FERMENTER
CELL SUSPENSION CULTURE
PURIFICATION
CALLUS CULTURE
IMMOBILISED CELL BIOREACTOR SYSTEM
SEED
PRODUCT

Fig.1.1

This alternate route for natural product synthesis has got many advantages. The cell culture system will be free from the influence of various environmental factors, including climate, pests, disease, geographical and seasonal constraints.

There will be a close control over the market supply because the cell culture system provides a defined production system and the product can be obtained as and when required, in desired quantities. In addition to these, there will be a close control on the quality and yield of the product.

It will reduce the land use. The land used for the cultivation of source plants can be used for the cultivation of food crops and other cash crops.

1.6. OBJECTIVES OF THE PRESENT STUDY

India particularly Kerala, being a tropical region with very good agroclimatic conditions, has a vast wealth of medicinal plants estimated to be around 2000 species. Most of the drugs prescribed in native systems of medicine and some of the modern medicines have their origin from these plants. Plant derived drugs dominate the prescription of Ayurveda, the well known system of Indian medicine. There is massive demand for medicinal plants for extraction

of biologically active compounds and also for use in native medicines. Over exploitation of these medicinal plants coupled with ecological degradation and large scale destruction of forests, medicinal plant resources become increasingly scarce, and some of them are now in the endangered list. *Coscinium fenestratum, Kaempferia rotunda, K. galonga* etc are some of the endangered plant species which are used extensively for different medicinal purposes. Therefore efficient biotechnological strategies are essential for protecting the plant species and make available these important medicinal plants or the products for the chemical and pharmaceutical industry.

In the present investigations it is proposed to study the invitro production of some important medicinal compounds using the cell cultures of six well known medicinal plants which are available locally. The plants selected for the studies include *Rauwolfia tetraphylla* (Apocyanaceae), *Piper nigrum*(Piperaceae), *Tinospora cordifolia* (Menispermaceae), *Sida cordifolia* (Malvaceae), *Lobelia nicotianifolia*(Lobeliaceae, earlier Campanulaceae) and *Coscinium fenestratum* (Menispermaceae). The proposed study aimed at establishing suitable protocol for the production of cell cultures of these plants and thereby to use this for the optimal production of pharmacologically active compounds. Further, berberine is an important alkaloid which has a number of pharmacological and other properties.

It is synthesised by different pathways in different plants. It is not known how berberine is synthesised by *T. cordifolia* or *C. fenestratum*; the plants which contain berberine. Therefore this study also aimed to identify the major pathway through which berberine is synthesised by these plants as well as their cell cultures by studying the key enzymes involved in its biosynthesis. In the present work, it has been possible to produce berberine, lobeline and reserpine alkaloids by plant tissue culture method. By studying the effect of growth hormones and other nutrients on cell growth, berberine biosynthesis and various enzyme activities it has been possible to develop a two stage culture system for maximum production of berberine.

CHAPTER II

CHAPTER II

MATERIALS AND METHODS

2.1. SOURCE OF PLANTS

The Medicinal plants selected for the *in vitro* studies were collected from Tropical Botanical Garden and Research Institute, Palode, Thiruvananthapuram. All the experimental plants were reared in the garden attached to Regional Research Laboratory, Thiruvananthapuram, under green house conditions.

2.2. SELECTION OF EXPLANTS AND SURFACE STERILIZATION

Various parts of the experimental plant, such as young and mature internodes, young leaves and root tissues were selected as the explants. The excised plant parts were thoroughly washed with a mild detergent in tap water and surface sterilized by immersing in 70 percent ethyl alcohol for 30 seconds and washed with sterilized water. The explants were then kept in 0.01 percent mercuric chloride for five minutes. The surface sterilized tissues were washed repeatedly with sterile distilled water and transferred to a petridish cut into small pieces of tissues approximately 5 mm length or diameter and washed again with sterilized distilled water. These explants were inoculated into appropriate culture media under aseptic conditions.

2.3. PREPARATION OF PLANT TISSUE CULTURE MEDIA

Different types of tissue culture media such as Murashige and Skoog (MS)[185], Gamborg's B_5 (B_5)[186] medium, Modified B_5 (E1)[187] medium, and Linsmaier and Skoog (LS)[188] medium were used for various experimental purposes. The composition of each of the above media is given in the Table 2.1. Stock solutions of suitable concentrations of each were prepared by dissolving appropriate quantity of required chemicals in double distilled water. For media preparation the necessary ingredients were weighed and dissolved in double distilled water, the stock solutions were added and pH was adjusted by dilute HCl or NaOH. Before autoclaving the medium was distributed in containers such as culture tubes or Erlenmeyer flasks. For solid media 7 gm of agar (tissue culture grade) was added to one litre media. The media were autoclaved at 120°C and one kg/cm² pressure for 15 to 20 minutes. The containers with media after sterilization were removed for cooling as soon as possible. The stock solutions for the media were prepared as follows and stored under refrigeration.

2.3.1. Stock solutions

The composition of the stock solution of various nutrients and salts prepared for each of the media is as given below.

Table 2.1 : Inorganic salt composition of plant tissue culture media

Ingredients	Amount (mg/litre)			
	MS	B_5	LS	EI
Macronutrients				
$MgSO_4.7H_2O$	370	250	370	400
KH_2PO_4	170	-	170	250
$NaH_2PO_4.H_2O$	-	150	-	-
KNO_3	1900	2500	1900	2100
NH_4NO_3	1650	-	1650	600
$CaCl_2.2H_2O$	440	150	440	450
$(NH_4)_2.SO_4$	-	134	-	-
Micronutrients				
H_3BO_3	6.2	3	6.2	3
$MnSO_4.H_2O$	15.6	10	15.6	10
$ZnSO_4.7H_2O$	8.6	2	8.6	2
$NaMoO_4.2H_2O$	0.250	0.250	0.250	0.250
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025	0.025	0.025
KI	0.83	0.75	0.83	0.80
$FeSO_4.7H_2O$	27.8	27.8	27.86	27.8
Na_2EDTA	37.3	37.3	37.26	37.3
Sucrose (g)	30	20	30	25
Vitamins				
Thiamine-HCl	0.5	10	0.4	10
Pyridoxine-HCl	0.5	1	-	1
Nicotinic acid	0.05	1	-	1
Myo-inositol	100	100	100	250
pH	5.8	5.5	5.7	5.5

Abbreviations : MS - Murashige and Skoog (1962)
B_5 - Gamborg et al (1968)
LS - Linsmaier and Skoog (1965)
EI - Gamborg et al (1983)

A. Stock solutions for MS medium

	Quantity (mg/100ml)
1. Micronutrient stocks	
$MnSO_4.4H_2O$	2230.00
$ZnSO_4.4H_2O$	860.00
H_3BO_4	620.00
KI	83.00
$Na_2MoO_4.2H_2O$	25.00
$CuSO_4.5H_2O$	2.50
$CoCl_2.6H_2O$	2.50
2. Iron/EDTA stock	
$FeSO_4.7H_2O$	557.00
$Na_2.EDTA.2H_2O$	745.00
3. Vitamin stock	
Glycine	200.00
Nicotinic acid	50.00
Pyridoxine HCl	50.00
Thiamine HCl	10.00

B. Stock solutions for B_5 Medium

1. Micronutrient stock	
$MnSO_4.H_2O$	1000.00
H_3BO_4	300.00
$ZnSO_4.7H_2O$	200.00

	Quantity (mg/100ml)
$Na_2MoO_4.2H_2O$	200.00
$CuSO_4.5H_2O$	2.50
$CoCl_2.6H_2O$	2.50

2. Calcium chloride stock

$CaCl_2.2H_2O$	15.00g/100ml

3. Potassium iodide stock

KI	75.00mg/100ml

4. Vitamin stock

Nicotinic acid	100.00
Thiamine HCl	1000.00
Pyridoxine HCl	100.00
Myo-inositol	10000.00

C. Stock solutions for E1 Medium

1. Micronutrients

Stock solution of B_5 medium

2. Potassium iodide

KI	80.00

3. Vitamin stock

Nicotinic acid	100.00
Thiamine HCl	10000.00
Pyridoxine HCl	100.00

D. Stock solution for LS Medium

	Quantity (mg/100ml)
1. Vitamin stock	
Thiamine HCl	100.00
Nicotinic acid	100.00
Pyridoxine HCl	100.00
Myo-inositol	10000.00

E. Stock solution of growth hormones

E.I. Auxins

The stock solutions (1 mM) of the various auxins were prepared by dissolving the appropriate amount in 2 to 5 ml of ethanol and gradually diluting to 100 ml in a volumetric flask using double distilled water. The auxins used were (1) 2,4-Dichlorophenoxy acetic acid (2,4-D) 22.40 mg/100 ml, (2) Naphthalene acetic acid (NAA) 18.62 mg/100ml (3) Indole-3-acetic acid (IAA) 17.52 mg/100 ml (4) Indole-3-butyric acid (IBA) 20.32 mg/100 ml (5) 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) 22.55 mg/100 ml.

E.II. Cytokinins

The stock solutions of Cytokinins (Kinetin and BAP) of 1 mM concentrations were prepared by dissolving the appropriate amount in a small volume of 0.5N HCl and gradually diluting to 100 ml with double distilled water.

The cytokinins used were (1) 6-benzyl amino purine (BAP) 22.50 mg/100 ml (2) 6-furfuryl amino purine (Kinetin) 21.52 mg/100 ml.

2.3.2. Preparation of media

The different media were prepared by mixing the stock solution of different nutrients and salts as detailed below.

	Quantity/litre
MS media	
NH_4NO_3	1650.00 mg
KNO_3	1900.00 mg
$MgSO_4.7H_2O$	370.00 mg
$CaCl_2.2H_2O$	440.00 mg
KH_2PO_4	170.00 mg
MS micronutrients (stock solution)	1.00 ml
Iron/EDTA (MS stock solution)	5.00 ml
Vitamins (MS stock solution)	1.00 ml
Sucrose	30.00 g

All the above components were added to about 800 ml of double distilled water and made upto 1000 ml. pH was adjusted to 5.7 with 0.2N NaOH or 0.2N HCl.

	Quantity/litre
2.3.3. Preparation of B_5 medium	
NH_4SO_4	134.00 mg
KNO_3	2500.00 mg
NaH_2PO_4	150.00 mg
$MgSO_4.7H_2O$	250.00 mg
Sucrose	20.00 gm
Iron/EDTA (MS stock solution)	5.00 ml
$CaCl_2.2H_2O$ (B_5 stock solution)	1.00 ml
Micronutrients (B_5 stock solution)	1.00 ml
Vitamins (B_5 stock solution)	1.00 ml
pH was adjusted to 5.5.	
2.3.4. Preparation of E1 medium	
NH_4NO_3	600.00 mg
KNO_3	2100.00 mg
KH_2PO_4	250.00 mg
$MgSO_4.7H_2O$	400.00 mg
$CaCl_2.2H_2O$	450.00 mg
Myo-inositol	250.00 mg
Sucrose	25.00 gm
Iron/EDTA (MS stock solution)	5.00 ml

	Quantity/litre
Micronutrients (B_5 stock solution)	1.00 ml
Potassium iodide (EI stock solution)	1.00 ml
Vitamins (EI stock solution)	1.00 ml

pH was adjusted to 5.5 as in the above cases.

2.3.5. Preparation of LS medium

Composition and procedure for the preparation of LS medium was exactly similar to that of MS medium except the components of vitamin stock. A separate vitamin stock (LS vitamin stock) was prepared as described earlier. One ml of the stock solution was added to the final volume of one litre medium as in the above cases. pH of the media was adjusted to 5.7 before autoclaving.

2.3.6. Types of media used

Both solid media and liquid culture media were used for different experiments. For the preparation of solid media, 7 gm of agar (Tissue culture grade) was added to one litre liquid media and heated to 90°C in a water bath, until the agar was melted and uniformly distributed. When the agar was melted, the media was dispensed into tubes or flasks and autoclaved as in the case of liquid media.

Solidified agar media were used for the studies with callus cultures and liquid media were used for various experiments with cell suspension cultures. Different concentrations and combinations of auxins and cytokinins were used for different experiments and its details are given in respective chapters.

2.4. DETERMINATION OF CELL GROWTH

The growth of cells in callus cultures and cell suspension cultures were estimated by determining the fresh weight and dry weight of the tissues and cells in the cultures.

The callus tissue fragments were separated from the culture media, and the fresh weight determined using the Mettler analytical balance. The tissue was kept in hot air oven at 60°C for 24 hours and the dry weight was determined as above.

In the case of cell suspension cultures, the cells were separated by centrifugation, washed twice with distilled water and determined the fresh weight. The cells were kept at 60°C for 24 hours in a hot air oven and determined the dry weight as described above.

2.5. EXTRACTION OF ALKALOIDS

2.5.1. From Callus cultures

The callus cultures, after two months of incubation were taken out, dried under reduced pressure, powdered and extracted with methanol using a Soxhlet extractor for 24 hours until all the colour was leached out from the tissue. The methanol extract was acidified with 1N HCl and the excess acid was neutralised with liquid ammonia. This was extracted repeatedly with dichloromethane, mixed and concentrated in a Rotary Flash evaporator. The concentrated samples were used for chromatographic analysis.

2.5.2. Extraction of berberine from the cells of suspension cultures

The cells from the suspension cultures were separated by centrifugation at 10000 rpm for 10 minutes and were suspended in hot methanol. The cells were homogenized with the help of a mortar and pestle in hot methanol medium and repeated two or three times until all the colour leached out from the homogenate. The homogenate was centrifuged at 10000 rpm and the supernatant was used for various qualitative and quantitative studies.

2.5.3. Extraction of berberine from the medium of cell suspension cultures

Berberine present in the medium of cell suspension cultures were extracted according to the method of Nakagawa

et al[115]. The berberine containing medium was passed through a column (20x1.5 cm) packed with neutral Amberlite XAD-2. Berberine was then eluted with methanol. The methanol eluate was concentrated and used for the various analyses.

2.6. THIN LAYER CHROMATOGRAPHY (TLC)

The crude alkaloid extract from the callus and cell suspension cultures were analysed by analytical TLC using the following solvent systems for each desired alkaloid.

1. Piperine

The methylene chloride extract from the callus cultures were analysed by TLC using chloroform as solvent system and compared with an authentic sample of piperine.

2. Reserpine

The following solvent systems were used for the analysis of reserpine in the extract by TLC.

(1) Toluene:ethyl acetate:diethylamine[189] (70:20:10)

(2) n-Heptane:ethylmethyl ketone:methanol[190] (58:34:8)

2. Ephedrine

Dichloromethane extract of *Sida cordifolia* was analysed for the presence of ephedrine by TLC using the following solvent system.

Toluene:ethyl acetate:diethylamine[191] (70:20:10)

4. Lobeline

The same solvent system described above was used for the analysis of Lobeline in the methylene chloride extract of the callus cultures of *Lobelia nicotianifolia*.

5. Berberine

Methylene chloride extract from the callus cultures and cell suspension cultures of *Tinospora cordifolia* and *Coscinium fenestratum* were analysed by using the following solvent system.

(1) n-Propanol:formic acid:water[191] (90:1:9)

(2) Toluene:ethyl acetate:diethylamine[191] (70:20:10)

(3) n-Butanol:acetic acid:water[112] (70:20:10)

(4) Chloroform:methanol:acetic acid[112] (25:10:1)

TLC plates were prepared using silica gel G (E. Merck) as the adsorbent. A slurry of silica gel was prepared by mixing silica gel with distilled water in the ratio 1:2 (w/v) and spread on glass plates (20x20 cm and 20x10 cm) with help of a TLC applicator which was adjusted to a thickness of 1.5 mm.

Rectangular glass chromatography chambers (30x20x25cm) were lined with filter paper to provide a saturated atmosphere. The chromatographic run was carried out at room temperature.

Standard compounds and samples were applied on TLC plates, 2 cm from the bottom of the plates, using separate

capillary tubes. When the spots were thoroughly dried, the plates were placed in the chromatographic chambers and sealed tightly. The solvent was allowed to run and when the solvent front came to the top of the plate, the chromatogram was removed, the solvent front was marked and allowed to dry.

The authentic samples of alkaloids such as piperine, reserpine, ephedrine, lobeline and berberine were used to detect the presence of respective alkaloids on TLC. The authentic samples were procured from Sigma and Aldrich Chemical Companies, USA.

2.6.2. Detection of alkaloids

(a) UV-detection

Alkaloids such as berberine and reserpine were detected under UV (345 nm). These alkaloid spots appeared as bright yellow and blue fluorescent spots respectively.

(b) Dragendorff reagent (DRG)

Berberine and other alkaloid spots were also detected by this spray reagent. DRG was prepared according to Munier and Machboeuf[192] by dissolving (a) 850 mg of basic bismuth nitrate in a mixture of water and acetic acid (4:1) (b) 8 gm of potassium iodide in 20 ml of water and mixing (a) and (b) in equal volumes. This stock solution was stored in brown coloured glass bottles. At the time

of spraying 1 ml stock solution was mixed with 2 ml acetic acid and 10 ml water.

The chromatographic plates after development were sprayed with dragendorff reagent and observed the characteristic spots of alkaloids.

(c) Methanol-sulphuric acid reagent

This spray reagent was prepared by mixing methanol with concentrated sulphuric acid in the ratio of 9:1 carefully. This reagent was also used as above for the detection of alkaloid spots.

(d) Iodine reagent

About 10 gm solid iodine was spread at the bottom of an air tight chromatography tank. The TLC plates after developing with solvent were exposed to the iodine vapour present in the chromatographic chamber. The alkaloid spots developed yellowish or dark brown colour.

2.6.3. Purification and quantitation of berberine by TLC

A known volume (50 to 100 µl) of concentrated sample was applied on a chromatographic plate (20x20 cm) using a microlitre syringe and developed with one of the solvent systems mentioned above. The bright yellow fluorescent band corresponding to the R_f of standard berberine was scraped and eluted with methanol. The amount of berberine in this methanol solution was determined by measuring

absorbance in a Hitachi spectrophotometer at 345 nm. The concentration of berberine was calculated from a standard graph. The standard curve was constructed using standard berberine of various concentrations which were run in the TLC and eluted as above. In each separate experiment a known quantity was also run along with the sample to check the reproducibility of the extraction. The recovery was always more than 99 percent. Pure methanol was used as the blank.

2.7 HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

HPLC analysis was carried out on a Waters Associates liquid chromatography system equipped with UV-absorbance detector Model 440, U 6K injector and a Omniscribe recorder (Houston Instrument). A Bondapak C-18 column was used for the alkaloid (Lobeline and berberine) analysis. Mobile phase suitable for Ion pair chromatography was used for berberine analysis. The mobile phase consists of 1 mM tetrabutyl ammonium phosphate in double distilled water adjusted to pH 2.0 with phosphoric acid and acetonitrile (60:40)[193]. The solvent was degassed before use and was prepared freshly for each experiment.

In lobeline analysis, the solvent system acetonitrile and 0.1 percent trifluoro acetic acid (TFA) in the ratio 3:7 was used[177]. Flow rate was adjusted to 2 ml/minute. A chart speed of 1 cm/minute, UV-detector wave length

of 254 nm at sensitivity 0.005 and column temperature was 25°C..

2.7.1. Preparation of sample

The crude dried alkaloid extract was dissolved in absolute methanol, vortexed for three to five minutes,centrifuged and the supernatant was removed and stored in stoppered vial at -10°C until use.

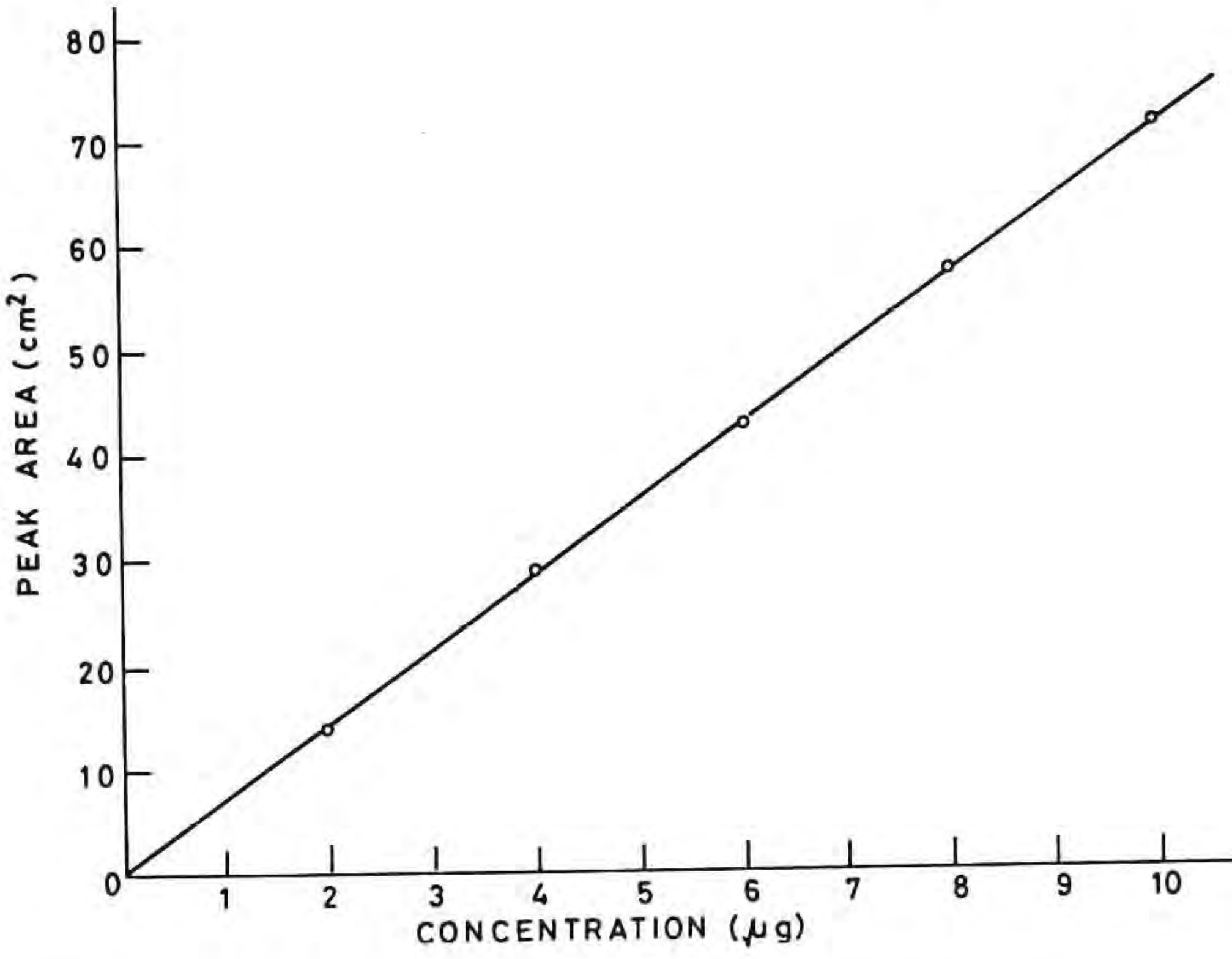
2.7.2. Standard solution and calibration of the instrument

The standard solution of berberine and lobeline were prepared by dissolving 1 mg in 10 ml of absolute methanol in volumetric flasks. Standard solutions of different concentrations from 0.1 mg/ml to 0.5 mg/ml were prepared and were kept in stoppered vials at -10°C. Using standard solutions of different concentrations (20 µl from the above stock solutions) a standard graph relating concentration to peak area was obtained, both for berberine and lobeline.

2.7.3. Qualitative and quantitative estimation

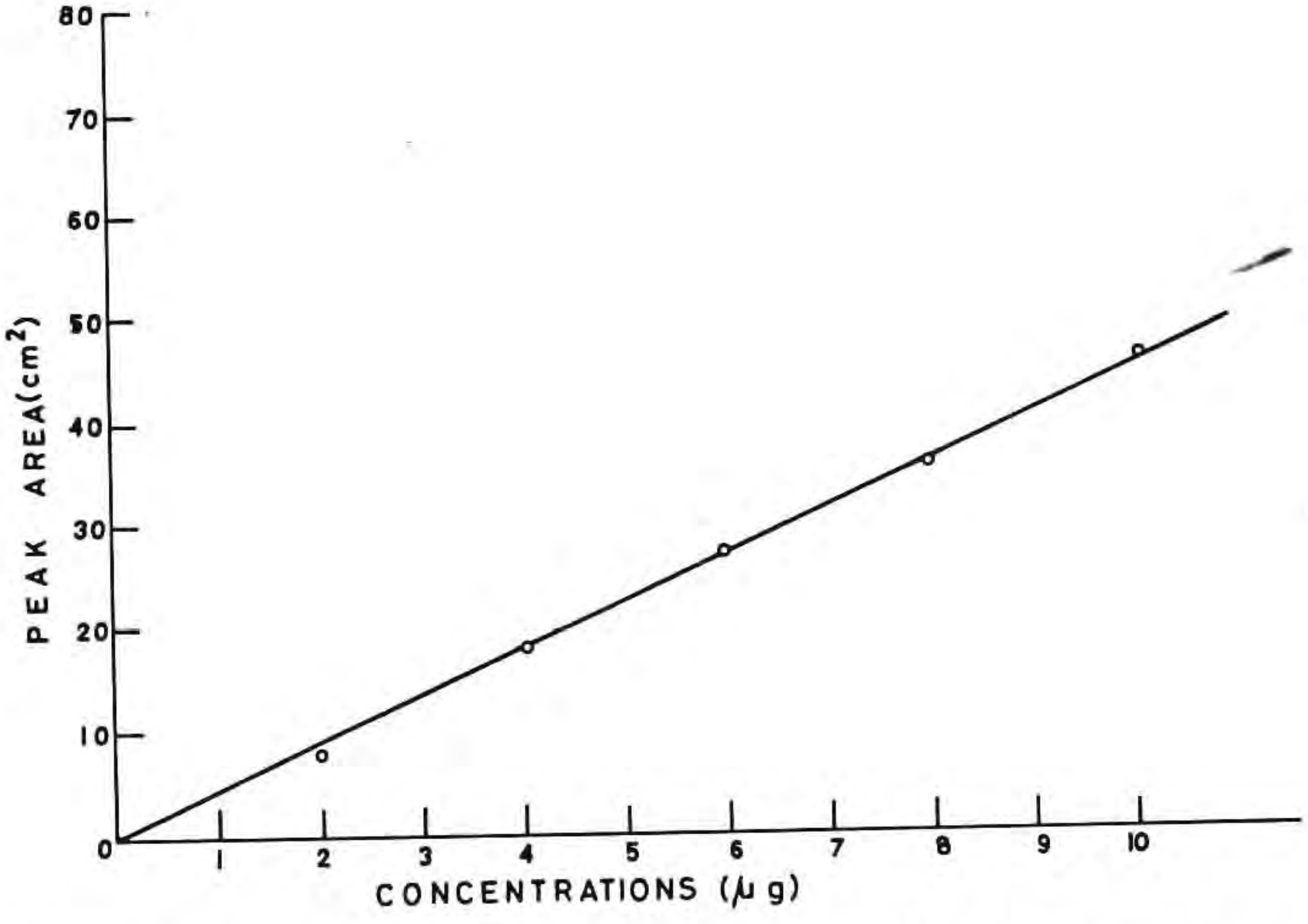
About 20 µl of suitably diluted sample in methanol was injected into the column to find the retention time (20 minutes). Berberine present in the sample was identified by comparison of the retention time of the unknown peak with that of the standard. The concentration of berberine in the sample was obtained from standard graph

Fig.2.1. Calibration curve for berberine



Different concentrations of berberine in a total volume of 20 µl was applied to HPLC and the area under berberine peak was measured and plotted against concentration of berberine.

Fig.2.2. Calibration curve for lobeline



Different concentrations of lobeline in a total volume of 20 µl was applied on the HPLC and the area under lobeline peak was measured and plotted against concentration of lobeline.

(Fig.2.1). Total quantity of berberine was calculated and expressed as mg/culture or percentage of berberine per dry cell biomass.

The lobeline content of the crude alkaloid extract was also determined and expressed as above with the help of a standard graph (Fig.2.2).

2.8 PURIFICATION AND QUANTIFICATION OF RESERPINE

The crude alkaloid extract was loaded on a silica gel column chromatography and preparative TLC and quantified gravimetrically.

2.8.1. Column chromatography

The crude alkaloid extract was loaded on a silica gel column of 50 cm x 3 cm and eluted with dichloromethane and different fractions were collected. The fractions were tested for the presence of reserpine. The fourth to eighth fractions containing reserpine were mixed and concentrated in Rotary flash evaporator and purified by preparative TLC.

The TLC plates after development were visualized under UV light. The blue fluorescent band corresponding to the standard sample was scraped and eluted with dichloromethane. All the eluants were mixed together and taken in a pre-weighed vial. The solvent was removed under vacuum and was weighed again. The final weighing was repeated to

get constant values and the quantity of reserpine was calculated and expressed as µg/gm of dry callus tissue.

2.9 GROWTH KINETICS

Growth kinetics were studied by calculating the kinetic parameters such as specific growth rate and generation time during the growth cycle of callus and cell suspension cultures[194].

(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

$$\mu = \frac{(\log \frac{x_t}{x_o}) \; 2.303}{t}$$

where x_o is the initial biomass of logarithmic phase and x_t is the biomass at time 't'.

(b) Generation time (tg)

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

$$tg = \frac{0.693}{\mu}$$

2.10 KINETICS OF BERBERINE PRODUCTION

The kinetic parameters of product formation such as rate of berberine production (product formation rate),

yield, productivity and specific product formation rate were calculated as described below.

(a) Rate of berberine production (dp/dt)

The rate of berberine production was calcualated from the logarithmic phase of product formation of the product formation curve using the equation.

$$\frac{dp}{dt} \quad \text{where } dp = P_t - P_o;$$

P_o is the product at the initial stage of the logarithmic phase of product formation curve and P_t is the product at time 't'. dp/dt gives the rate of product formation during the active stages of prduct formation.

(b) Specific product formation rate

Specific product formation rate was calculated using the formula

$$\frac{\text{Total product}}{\text{Total biomass}} \text{ / time}$$ and was expressed as the berberine formed per mg of dry biomass per day.

(c) Yield

Yield is the product formed per dry weight of the biomass of cultured cells. It was calculated by using the formula

$$\text{Yield} = \frac{\text{Total product}}{\text{Total biomass}}$$

(d) Productivity

It is the product formed per time and was determined using the formula

$$\text{Productivity} = \frac{\text{Total product}}{\text{Time (days)}}$$

and was expressed as mg of berberine formed per day.

2.11. ENZYME ASSAY

2.11.1. Enzyme extraction

About 400 mg of the cells were filtered from the medium and was homogenised with 10 mg of insoluble PVP and extracted with Tris-HCl buffer containing 1 mM dithioeruthritol (0.1 M pH 0.5) at 0 to 5 °C. The homogenate was centrifuged at 15000 rpm for 30 min at low temperature. The supernatant was used as the crude enzyme source for assay.

2.11.2 Nitrate reductase (NADPH: Nitrate oxidoreductase EC. 1.6.6.2)

The Nitrate Reductase (NR) activity was assayed following the method developed by Kadam et al[195] with slight modification. A known quantity of the cultured cells, filtered from the medium was taken in a small stoppered tube, containing potassium phosphate buffer (0.1 M pH 7.5), 20 mM sodium nitrate and 0.3 mM NADH in a final volume of 2 ml. The tubes were evacuated for two minutes to facilitate the infiltration of the substrate and the co-factor into the cells. The evacuated tubes were incubated at 30°C

for 30 minutes in the dark. At the end of the incubation period, the vacuum was released and the tubes were placed in a boiling water bath for five minutes, for complete extraction of nitrate. The contents of the tubes were centrifuged and nitrate was assayed in a suitable aliquot of the supernatant by adding one ml each of one percent sulphanilamide in 1N HCl and 0.01 percent N-(1-naphthyl)-ethylene diamine dihydrochloride. Absorbance was recorded at 540 nm after 10 minutes. The enzyme activity was expressed as µ moles of nitrite formed/hr/gm fresh weight.

To rule out the possibility of further nitrite utilization by nitrate reductase a control was used where sodium nitrate was replaced by sodium nitrite (20 mM) in the reaction mixture mentioned above. It was observed that under the experimental conditions used for the assay of nitrate reductase, no further utilization of nitrite occurred.

2.11.3. Glutamate dehydrogenase (L-glutamate:$NADP^+$ oxidoreductase (deaminating) (GDH) (EC.1.4.1.3)

In vitro assay of glutamate dehydrogenase was carried out according to the method of Pahlich and Joy[196]. The activity was assayed, following the decrease in absorbance at 340 nm due to the oxidation of NADH. The reaction mixture consists of 100 mM Tris-HCl buffer (pH 7.5) 10 mM α-ketoglutarate, 100 mM ammonium chloride and 0.1 mM NADH per ml. The reaction was started by adding the enzyme

extract. Specific activity was expressed as μ moles of NAD^+ formed/min/mg protein.

2.11.4. Glutamine synthetase (GS) (EC. 6.3.1.24)

Glutamine synthetase was assayed based on the biosynthetic procedure developed by Boyer et al[197]. The reaction mixture contained Tris-HCl buffer (0.1 M pH 7.0); 50 mM NH_4Cl, 10 mM ATP, 50 mM $MgCl_2$ and 100 mM glutamate in a final volume of 0.2 ml. Reaction was started by the addition of enzyme extract to the reaction mixture and incubated for 15 minutes at 37°C. Reaction was stopped by the addition of 1.8 ml of 1.1 percent $FeSO_4$ in 0.3N H_2SO_4. Colour was developed by adding 0.15 ml of 6.6 percent ammonium molybdate in 7.5N H_2SO_4 and absorbance was recorded at 540 nm. A blank was also used which consisted of all the above components except the glutamate. The specific activity was expressed as μ moles of phosphate released/min/mg protein.

2.11.5. Glutamate synthase (GOGAT) (L-glutamate:$NADP^+$ oxidoreductase (transaminating) (EC. 1.4.1.13)

The assay of GOGAT was carried out following the method developed by Sodek and De Silva[198]. The activity was measured by following the decrease in absorbance at 340 nm due to NADH oxidation during the reaction. The reaction mixture contained Tris-HCl buffer (0.1 M, pH 7.5), 0.02 M 2 -ketoglutarate, 0.05 M glutamine and 0.2 mM NADH

per ml of the final volume. Reaction was started by adding the enzyme. Specific activity was expressed as moles of NAD^+/min/mg protein.

2.11.6. ATPase ACTIVITY

About 500 mg of cells were homogenised in about five ml of histidine HCl buffer (pH 6.7) along with polyvinyl pyrrolidone and neutral glass powder. The homogenate was filtered and centrifuged at 10000 rpm for 20 minutes. The supernatant obtained was again centrifuged at 18000 rpm for 60 minutes. The pellet containing mainly the plasma membrane was resuspended in 10 ml of buffer[199].

Plasma membrane ATPase activity was assayed by following the release of inorganic phosphate from ATP[200]. The reaction system contained 1 mM ATP, 40 mM histidine HCl-buffer (pH 6.7), 50 mM $MgCl_2$. The test tube containing the reaction mixture was placed in a water bath at 37°C for five minutes and reaction was started by adding the enzyme. After 30 minutes of incubation at 30°C, the reaction was stopped by the addition of 2 ml of a solution containing 2 percent v/v H_2SO_4, 0.5 percent ammonium molybdate and 0.5 percent sodium lauryl sulphate. The detergent was included to avoid the development of turbidity. The phosphomolybdate was reduced with 20 µl of 10 percent (w/v) ascorbic acid and allowed to develop the colour over 5 minutes at 30°C. Two types of blanks were prepared, one without

substrate (ATP) and the other without enzyme. The absorbance of the solution was measured at 750 nm.

One unit of enzyme activity was defined as that amount of enzyme which releases 10 µ moles of inorganic phosphate per mg protein per hour.

2.11.7. Synthesis of tetrahydroberberine

The substrate for the assay of tetrahydroberberine oxidase-tetrahydroberberine (THB) has been synthesized from berberine following the method of Mirza et al[201]. Berberine was reduced to tetrahydroberberine by sodium borohydride ($NaBH_4$) in a medium containing 90 percent methanol and kept overnight. The reaction mixture was analysed by TLC and it showed complete conversion of berberine to THB. It was extracted into dichloromethane and purified by crystallisation from methanol. It showed UV absorption maxima at 280 nm ($UV\ \lambda_{max}^{MeOH}$ nm 280). The product obtained was confirmed as THB by comparing IR, UV spectra and melting point. The purity of the compound was also checked by TLC.

2.11.8. Assay of tetrahydroberberine oxidase

Assay of THB oxidase was carried out following the method of Okada et al[202] with slight modification.

The substrate (THB) solution was prepared in phosphate buffer (0.06 M, pH 7.5) containing 10 percent dimethylformamide (DMF), by dissolving 75 mg of THB in 1000 ml.

The reaction mixture consists of 0.5 ml enzyme solution and 1 ml substrate solutions. The assay mixture was kept for one hour at 30°C in a water bath. The reaction was stopped by immersing the reaction tubes in boiling water bath for three minutes. The assay mixture was centrifuged at 10000 rpm for 10 minutes and the supernatant was collected.

The rate of berberine formation by the enzyme action was measured by UV spectrophotometry by recording the optical density at 345 nm. Analysis of the product by HPLC as described earlier showed that berberine was the only product.

Two blanks were used for the enzyme assays. One with substrate and denatured enzyme (by heating) and the other with substrate and buffer instead of enzyme. This was for giving correction for a possible autooxidation of tetrahydroberberine under the above experimental conditions.

2.12. EXTRACTION AND ESTIMATION OF TOTAL DNA FROM THE CULTURED CELLS

The callus tissue or the cells filtered from the suspension cultures were homogenized and extracted with 10 percent trichloroacetic acid (TCA) by heating in a boiling water bath for two hours. The TCA extraction was carried out after removing all the pigment compounds from the tissue

by extracting with acetone. The TCA extract was centrifuged at 10000 rpm for 10 minutes and the supernatant was used for the estimation of DNA, and the pellet was used for the extraction of protein.

DNA was estimated by the diphenylamine reaction[203]. One gm of diphenylamine was dissolved in 100 ml of glacial acetic acid and 2.5 ml of concentrated sulphuric acid. This reagent was freshly prepared for each experiment. Four ml of diphenylamine reagent was added to one ml of the test solution and the tubes were incubated in a boiling water bath for 10 minutes, cooled and measured the optical density at 595 nm, against a blank consisting of water and reagent. The DNA content of the sample was calculated using a standard graph.

2.13. EXTRACTION OF TOTAL PROTEIN

The residue obtained after the removal of TCA extract for the estimation of DNA, was dissolved in 1N NaOH and warmed in a water bath for one hour. The extract was centrifuged at 10000 rpm and the supernatant was removed and used for protein estimation.

The protein content of the above sample and other samples such as enzyme extract were estimated according to Lowry's method[204].

2.13.1. Protein estimation

(a) Reagents

(1) Alkaline sodium carbonate solution

Alkaline sodium carbonate solution was prepared by dissolving 20 gm of Na_2CO_3 in one litre of 0.1 M NaOH.

(2) Copper sulphate-sodium potassium tartarate solution

This reagent was prepared by dissolving 5 gm of $CuSO_4.5H_2O$ in one litre water containing 10 gm of sodium potassium tartarate.

(3) Folin-Ciocalteau reagent (2 N)

The commercially available reagent was diluted with equal volume of distilled water at the time of use. This reagent is the solution of sodium tungstate and sodium molybdate in phosphoric acid and hydrochloric acid.

(4) Alkaline solution of copper sulphate

This was prepared at the time of use by mixing 50 ml of reagent (1) and 1 ml of reagent (2).

The protein extract was suitably diluted and 5 ml of the alkaline solution was added to one ml of the test solution. It was thoroughly mixed and kept at room temperature. After 10 minutes, 0.5 ml of diluted Folin-Ciocalteau reagent was added and rapidly mixed with the help of a vortex. It was kept at room temperature for 10 minutes and measured optical density against the appropriate

blank at 700 nm. Distilled water or buffer was used instead of protein sample in the blank.

Protein concentration of the unknown sample was calculated using a standard graph.

2.14. STATISTICAL ANALYSIS

The values given in the tables are the mean of the number of experiments conducted as given in the respective tables ± standard error of the mean. The statistical significance of the compared values were carried out using student 't' test[205], by using the following formula

$$\frac{\bar{x}_1 - \bar{x}_2}{\frac{n(\sigma x_1^2 + \sigma x_2^2)}{2(n-1)}}$$

where $\bar{x}_1$ and $\bar{x}_2$ are the mean values to be compared.

σx_1 - Standard error of the value $\bar{x}_1$

σx_2 - Standard error of the value $\bar{x}_2$

n - No. of experiments

2.15. SOURCE OF CHEMICALS AND BIOCHEMICALS

The chemicals used were from different sources.

Various salts for media preparation and other purposes, solvents such as methanol, dichloromethane, silica gel for TLC and column chromatography, reagents such as diphenylamine and trichloro acetic acid were obtained from E. Merck.

Various vitamins, amino acids, myo-inositol, ATP, NADH, ADP, NAD^+, sulphanylamide, N-(1-naphthyl)ethylene diamine dihydrochloride, tris-HCl, Histidine-HCl, tetra-butyl ammonium phosphate, trifluoroacetatic acid, and standard berberine-HCl were obtained from Sigma Chemical Company, USA.

The column packing materials such as Amberlite resin XAD-2, and CM Sephadex, the standards of Lobeline etc were obtained from Aldrich Chemical Company, USA.

CHAPTER III

CHAPTER III

INDUCTION OF CALLUS CULTURES AND ANALYSIS OF ALKALOIDS

3.1. INTRODUCTION

As described in the previous chapter, plants such as *Sida cordifolia*, *Lobelia nicotianifolia*, *Piper nigrum*, *Tinospora cordifolia*, *Coscinium fenestratum* and *Rauwolfia tetraphylla* are the medicinal plants selected for the present study. A brief description of these plants and the major pharmacologically active compounds from them is given below.

(1) *Sida cordifolia* Linn.

Sida cordifolia (Malvaceae) is distributed in moist places throughout the tropical and subtropical India. Roots, leaves and seeds are slightly bitter in taste and are used in traditional medicines. Its various pharmaceutical uses have been documented. The alkaloid ephedrine is the main pharmacologically active component of the plant, particularly in leaves and roots. It is a very effective biologically active compound and is used extensively in pharmacological preparations.[206]

(2) *Lobelia nicotianifolia* Heynę.

L. nicotianifolia is a large biennial or perennial herb found in Western ghats. It is the Indian substitute

for obtaining the alkaloid lobeline, the active ingredient of the plant. The lobeline content of *L. nicotianifolia* is 1 to 1.8 percent[207]. The leaves and seeds have been used in traditional medicine. The plant has got various therapeutic and biological activities such as antibacterial , antifungal, antiprotozoal, antiviral, LNS and CVS effects[208].

(3) *Tinospora cordifolia* (wild)

T. cordifolia (Menispermaceae) is a woody climber distributed in the moist tropics of Asia, Africa and Australia. This species is present in the tropical India. This important medicinal plant is mentioned in Ayurvedic literature as a constituent of several compound preparations used in general disability, dyspepsia, fevers and urinary diseases. Various therapeutic properties have been ascribed to this plant including antiviral activity against Ranikhet disease in poultry. The main active components of this plant include berberine, a pharmaceutically important alkaloid [209].

(4) *Piper nigrum* Linn.

Piper nigrum (Piperaceae) a perennial climbing shrub, mostly cultivated in the hot and moist parts of India, Ceylon and other tropical countries. *Piper nigrum* or Black pepper is an important spice crop and is valued for its berries. The dried and processed berries are

used in food preparation as a condiment or flavouring ingredient. The alkaloid piperine is a main component of the piper berries, which is responsible for the pungent taste of the berries. This compound is absent in other parts of the plant. It is now used as a flavouring compound in food preparations and liquors. It is also used as an efficient insecticide, more effective than pyrethrums[210].

(5) *Coscinium fenestratum* Colebr.

C. fenestratum (Menispermaceae), a woody climber is endemic to South India. The stem of the plant is yellowish brown externally and yellow internally. The yellow coloured extract of the stem has been used in South India and Ceylon as a yellow dye and as a bitter tonic. The plant as such has got various therapeutic activities. The stem contains berberine as the main component upto 3.5 percent of its dry weight[211].

(6) *Rauwolfia tetraphylla* Linn. (R. cannescens)

R. tetraphylla (Apocyanaceae) resembles in appearance *R. serpentina* which has been extensively used in Ayurvedic medicines. *R. tetraphylla* is distributed in the hotter regions of India. The roots of these plants are often used as substitute for those of *R. serpentina*.

This plant species contains all alkaloids present in *R. serpentina*, such as ajmalicine, ajmaline, reserpine, yohimbine etc in addition to tetraphyllicine and tetraphylline, which are not detected in *R. serpentina*. Among the various Rauwolfia alkaloids, reported from different species, reserpine is pharmacologically the most potent alkaloid. Various therapeutic activities have been attributed to this alkaloid. One of the important pharmacological effects of reserpine is its antihypertensive and sedative effects. The roots of *R. tetraphylla* contain about 0.05 percent reserpine, on a dry weight basis[212].

The formation of callus cultures an undifferentiated mass of parenchymatous cells marks the beginning of successful plant cell cultures. The induction of callus cultures requires the selection of suitable explants from the source plant, an appropriate nutrient medium and aseptic culture conditions. In order to develop cell culture systems of medicinal plants to study the production of pharmacologically active compounds and to use it as a continuous system for optimal production of the different secondary metabolites, the first step would be to induce the growth of callus cultures from the suitable explants of the medicinal plants[213]. Once the callus is induced, this can also be used to study the metabolism of medicinal compounds and can be manipulated to regenerate into whole plants.

As discussed in the introduction, plant cell cultures are efficient systems for the study of the metabolism of medicinal compounds and for their *in vitro* production. Callus cultures from suitable explants of the above mentioned plants have been raised and studied for the growth characteristics and tested for *in vitro* production of the secondary metabolite of commercial importance. In order to find out a suitable combination of growth regulators their effect on callus induction and the biomass formation has been studied and the results are discussed.

3.2. MATERIALS AND METHODS

3.2.1. Aseptic preparation of explants

All the medicinal plants used in the present study were collected from Tropical Botanical Garden and Research Institute, Palode, Thiruvananthapuram and they were reared in the garden attached to the Regional Research Laboratory, Thiruvananthapuram. Different parts of the plants such as young stem internodes, leaves, petioles and root segments were selected and used as the explants for callus initiation. The explants were surface sterilized. After disinfection, the plant parts were carefully trimmed by removing the blackened and dead tissues. Small pieces of tissues approximately 5 mm in length or diameter were excised and inoculated separately into culture tubes containing nutrient agar medium.

3.2.2. Nutrient media and culture conditions

Murashige and Skoog (MS) basal medium supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D), Naphthalene acetic acid (NAA) and Benzyl amino purine (BAP) at various concentrations were used for callus initiation from the explants in all the cases. The media and other supplements were prepared as detailed in Chapter II.

The surface sterilized explants were implanted into the media and were incubated at 25±2°C, in continuous darkness or in 16 hours photoperiod (1200-1500 lux) in a culture room.

When callus was induced, it was excised and transferred to the fresh media of the same composition. Subculturing was carried out every two to three weeks maintaining uniformity in callus proliferation. The effect of hormone combinations on biomass accumulation of calli derived from different plants was monitored. It was done by measuring the difference of the fresh and dry weight of the tissue in the initial and final phase of the experiment with a view to determine a suitable hormonal combination for biomass accumulation.

3.2.3. Kinetics of callus growth

In order to investigate the actual pattern of growth and metabolic processes, growth kinetics of the callus

cultures of each plant was carried out in the media having highest biomass accumulation. A preweighed callus fragment was inoculated into the culture tubes containing 20 ml of solid agar medium and incubated. The final fresh and dry weight of the calli were recorded periodically every seven days, upto a period of seventy days. The dry biomass weight of the callus cultures were plotted against time and the kinetic parameters such as specific growth rate (μ) and doubling time (tg) were calculated from the growth curve using the equations given in Chapter II under general methodology.

Total DNA and protein of the callus tissues were extracted and estimated in each case along with the biomass determinations to study the variations at different stages of growth. The extraction and estimation of DNA and protein from the cultured cells were carried out as explained earlier (Chapter II).

3.2.4. Extraction, isolation and identification of secondary metabolites

Two months old callus cultures of each plant were subjected to extraction using suitable solvent system and were analysed qualitatively and quantitatively using Thin Layer Chromatography (TLC) and High Pressure Liquid Chromatography (HPLC) with authentic samples of the respective compounds as standard. The compounds were isolated in pure form and identified by melting point

and TLC. The structural confirmation of these compounds were also obtained using IR and ^{1}H NMR spectra. The details of the procedure of extraction and analytical techniques employed were given under the general methodology.

3.3. RESULTS

3.3.1. Initiation of callus cultures

The results obtained in the experiments of callus initiation and accumulation of biomass by the individual plants have been presented separately.

3.3.1.1. *Sida cordifolia*

Callus induction was observed in all the explants after two weeks as small protuberances of tissues from the cut ends of the stem explants and from the surface and margins of the leaf explants. Young stem internodes and young leaf tissues were found to be the most suitable explants for the callus induction. Stem internodes or leaf segments were used for frequent callus production for different experimental purposes. Internodal segments from the young shoots of similar size were used for studying the effect of different combination of growth regulators on callus induction. Murashige and Skoog medium supplemented with various combinations of growth regulators were used for this purpose. The results are presented in Table 3.1. The explants cultured in all the different

Table 3.1 : Effect of different combinations of growth hormones on callus induction in different plants

Auxins µM	BAP µM	Percent of callus induction											
		Sida cordfolia[1]		*Lobelia nicotianifolia*[2]		*Tinospora cordifolia*[3]		*Piper nigrum*[4]		*Coscinium fenestratum*[5]		*Fauwolfia tetraphylla*[6]	
		2,4-D	NAA	2,4-D	NAA	2,4-D	NAA	2,4-D	NAA	2,4-D	NAA	2,4-D	NAA
10	0.00	65	70	95	90	80	75	80	80	70	65	90	95
10	0.25	90	90	95	95	85	80	85	80	60	50	95	95
10	0.50	90	80	98	90	95	78	85	60	50	50	90	95
10	1.00	85	70	95	95	90	84	85	50	50	50	93	95
10	5.00	80	70	98	80	75	70	60	20	0	0	70	80
5	5.00	50	40	95	70	60	55	30	45	0	0	30	20
5	0.25	75	55	95	90	90	90	80	60	10	0	80	70
5	0.50	60	50	98	90	85	70	65	70	0	0	72	65
5	1.00	60	40	100	90	80	76	60	60	0	0	50	55
0	1.00	30	25	0	0	0	0	0	0	0	0	30	30
20	0.50	--	--	--	--	--	--	--	--	70	20	--	--
30	0.50	--	--	--	--	--	--	--	--	40	60	--	--

* Explants: 1. Stem internodes
2. Young leaves (Data collected on 35th day of inoculation)
3. Young leaves
4. Stem internodes
5. Leaf pulvinus (basal part of leaf patiole - Data collected on 60th day of inoculation)
6. Stem internodes

*Percentage was calculated using the mean value of 20 independent determinations, based on the total number of explants inoculated. Data collected on 40th day of inoculation (except 2 and 5).

hormone combinations have produced calli and both 2,4-D and NAA were found to be equally effective in callus induction in this case. The percentage of callus formation was higher with higher auxin concentration. It was maximum in media with 10 µM 2,4-D and 0.25-0.5 µM BAP; and also in 10 µM NAA and 0.25 µM BAP. Direct organogenetic response was not seen in any of these media.

The callus cultures of the plants were maintained by subculturing the callus after every two to three weeks period. The effect of hormone combinations on biomass accumulation by the callus cultures was calculated by recording the initial and final fresh and dry weight of the callus cultures. The results are given in Table 3.2. Maximum biomass was obtained in the media supplemented with 10 µM 2,4-D and 5 µM BAP, after incubation for a period of 40 days. Same concentration of NAA along with 5 µM BAP also showed the highest biomass accumulation when compared to the other concentrations studied. Higher concentration of auxins both 2,4-D and NAA have resulted in increased fragility of the callus tissue and the situation was found more in the former case. Higher concentration of BAP or lower concentration of 2,4-D and NAA favoured the formation of compact calli. Another unique feature of this callus culture was its green colour even in the presence of 2,4-D. In the absence of BAP however

Table 3.2 : *Sida cordifolia* callus cultures - Effect of different hormone combinations on biomass accumulation

Sl. No.	2,4-D/NAA (µM)	BAP (µM)	2,4-D		NAA	
			Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)
				A		B
1	10	0.00	3125(±5.60)	658(±2.25)	3520(±5.08)	805(±3.80)
2	10	0.25	3270(±5.00)	670(±1.16)	4260(±5.00)	887(±3.50)
3	10	0.50	4152(±5.25)	891(±1.90)	6080(±6.50)	1240(±6.30)
4	10	1.00	5860(±5.18)	1165(±1.70)	6325(±4.75)	1280(±5.50)
5	10	5.00	7520(±6.00)	1507(±2.80)	6820(±7.00)	1338(±2.90)
6	5	5.00	4657(±5.00)	950(±4.10)	3900(±5.20)	810(±4.20)
7	5	1.00	5920(±4.25)	1210(±3.11)	4360(±4.30)	927(±2.60)
8	5	0.50	6425(±4.25)	1302(±2.60)	3573(±5.72)	776(±1.30)
9	5	0.25	5760(±5.58)	1203(±2.35)	3252(±3.80)	702(±2.50)
10	0	1.00	2170(±5.60)	590(±1.90)	2140(±4.55)	473(±5.20)

Initial biomass : Fresh weight : 380(±4.57) mg
Dry weight : 83(±1.57) mg

Data collected after 40 days of incubation
Each value is the mean SEM of 20 independent determinations
All values compared with those of Sl.No.1. In all cases $p < 0.05$

't' values to Table 3.2
(Comparison of dry weight)

't' value between	't' values	't' value between	't' values
1A and 2A	6.702	8A and 9A	39.943
2A and 3A	140.376	1B and 2B	22.443
3A and 4A	151.964	3B and 4B	6.763
4A and 5A	147.630	4B and 5B	13.190
8A and 9A	39.943	6A and 7B	33.491

it became pulpy white and when transferred to media supplemented with auxin and BAP it developed yellowish green colour.

3.3.1.2. *Lobelia nicotianifolia*

In *L. nicotianifolia*, all the explants produced calli and the response was almost identical in all the cases except the root explants where the percentage of callus induction was only 30 to 35. In all the other cases it was 95 to 100 percent. The effect of different combinations of auxins and BAP on the callus induction is shown in Table 3.1. Young leaf fragments of equal size were used as explants. Higher concentration of both 2,4-D and NAA caused higher percentage of callus induction and was low in the media supplemented with 5 µM NAA and 5 µM BAP compared to the other hormone combinations. Callus formation was not observed in media containing BAP alone at a concentration of 1 µM.

The effect of hormone combinations on biomass accumulation by the callus cultures were carried out and the results are summarised in Table 3.3. Highest biomass accumulation was recorded in the medium containing 10 µM 2,4-D and 5 µM BAP . In media supplemented with NAA it was obtained with a concentration of 10 µM along with 1 µM BAP.

Table 3.3 : *Lobelia nicotianifolia* callus cultures - Effect of different hormone combinations on biomass accumulation

Sl. No.	2,4-D/NAA (µM)	BAP (µM)	2,4-D		NAA	
			Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)
				A		B
1	10	0.00	2700(±1.33)	614(±0.38)	2921(±2.10)	599(±3.74)
2	10	0.25	4270(±1.60)	718(±1.03)	3150(±1.92)	674(±2.60)
3	10	0.50	4892(±1.52)	969(±1.92)	4120(±1.80)	840(±1.50)
4	10	1.00	5268(±2.11)	1056(±1.14)	6768(±3.10)	1458(±2.58)
5	10	5.00	7821(±1.36)	1603(±5.85)	6154(±5.25)	1290(±2.44)
6	5	5.00	3250(±1.25)	678(±3.52)	5650(±1.40)	1021(±2.97)
7	5	1.00	6210(±1.86)	1242(±5.24)	7250(±5.00)	1610(±2.66)
8	5	0.50	5655(±0.85)	1100(±1.67)	5860(±2.80)	1158(±1.96)
9	5	0.25	4265(±0.72)	845(±0.88)	5110(±3.20)	1088(±1.68)
10	0	1.00	3405(±5.60)	710(±4.38)	3260(±6.58)	738(±2.12)

* Initial biomass : Fresh weight : 378(±3.85) mg
Dry weight : 77(±1.24) mg

Data collected after 40 days of incubation

Each value is the mean ±SEM of 20 independent determinations
All values compared with those of Sl.No.1. In all cases $p < 0.05$

't' values of Table 3.3
(Comparison of dry weight)

't' value between	't' values	't' value between	't' values
1A and 2A	133.947	2B and 3B	78.197
3A and 4A	55.092	4B and 5B	66.896
7A and 8A	36.509	5B and 6B	98.956
9A and 10A	42.728	6B and 7B	54.439
1B and 2B	23.282	7B and 8B	38.342

The callus tissues cultured in media with 2,4-D was yellowish white and that raised in media with NAA was yellowish green with dark green spots. Increased compactness and green colour of the callus tissue was obtained in relatively higher concentration of BAP. The callus cultures induced in the medium supplemented with 5 µM BAP and 5 µM NAA produced shoot buds when cultured in the same medium, after the first subculturing. Hence studies were carried out to investigate the organogenetic ability of the callus cultures and also with a view to regenerate plantlets.

Regeneration of plantlets

The callus cultures (Fig.3.1) induced in the medium supplemented with 5 µM NAA and 5 µM BAP produced shoot bud primordia in the same medium after the first subculturing. Similar response was not observed in the case of callus cultures grown in other media, when subcultured in the media of above combination. But all the callus cultures produced shoot primordia when cultured in MS media with 2.5 µM NAA and 5 µM BAP, during second or third subculturing (Fig.3.2). Table 3.4 summarises the effect of different combination of NAA and BAP on morphogenetic ability of the callus cultures.

When the auxin quantity was reduced, the frequency of shoot bud formation increased. However the complete

Fig.3.1 : Induction of callus and regeneration of shoots in the same medium

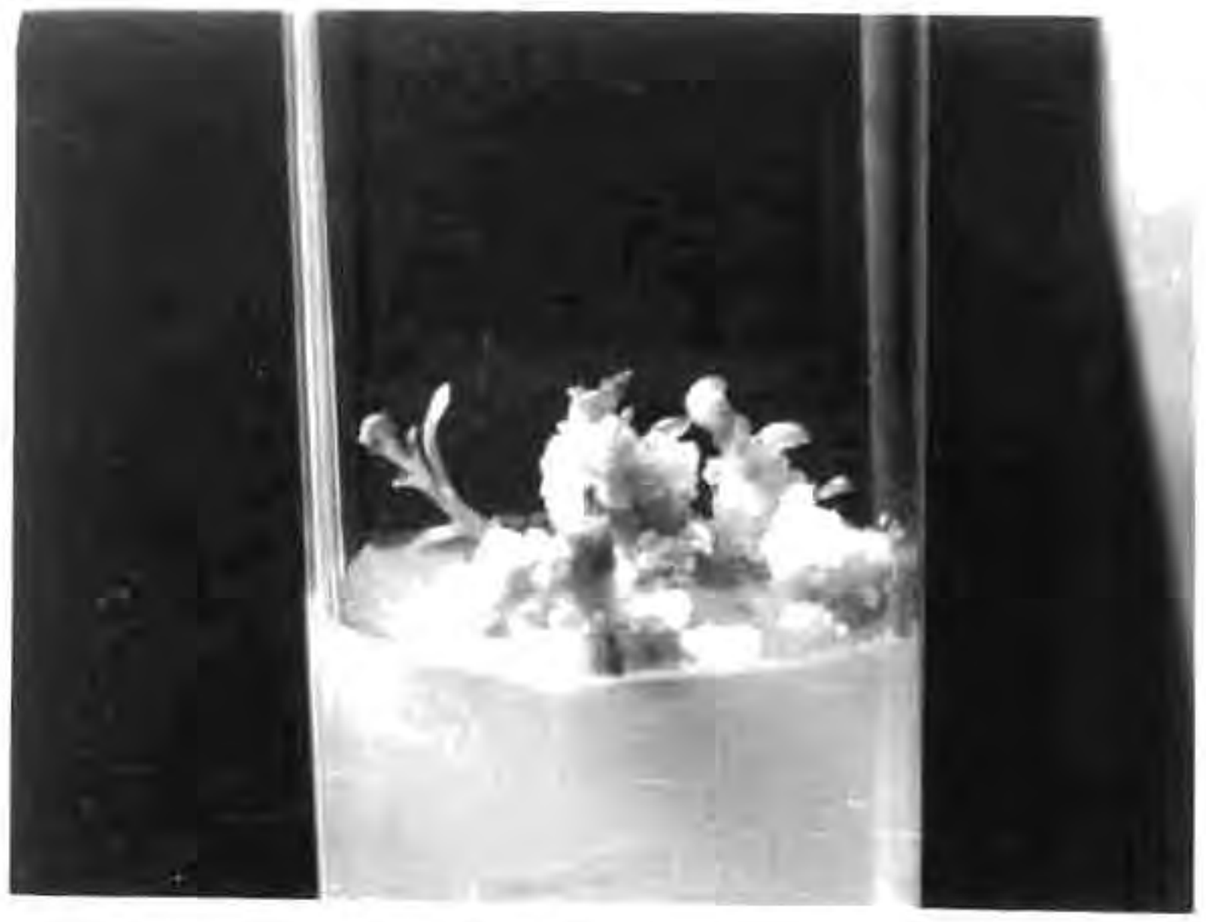


Fig.3.2 : Regeneration of shoots in the callus Culture when transferred to the regeneration medium

Table 3.4 : Effect of various combinations of NAA and BAP on shoot regeneration in the callus cultures of *Lobelia nicotianifolia*

Hormones (µM)		Percent of frequency of shoot regeneration	Mean ±SEM of shoot buds formed per 400 mg callus
NAA	BAP		
10.0	5.0	Nil	-
5.0	5.0	70	22.8(±0.4)
2.5	5.0	80	34.0(±0.5)
0.5	5.0	65	6.7(±0.7)
0.0	5.0	Nil	-

Data collected after 28 days of incubation

Each value is the mean ±SEM of 16 independent determinations

Percent frequency of shoot regeneration was calculated from the number of regenerations based on the total number of experiments.

elimination of auxins (NAA) was not advantageous, as it did not promote the elongation of bud primordia and also it results in the differentiation into compact green mass.

Rooting of shoots

For root initiation, the shoots were separated and subcultured individually in half strength MS medium supplemented with 2.5 µM NAA (Fig.3.3). Different concentration of auxins were tried for root initiation and media with 2.5 µM NAA gave 80 percent rooting (Table 3.5). Higher concentration of auxins promoted callus formation from the regenerated shoots. The rooted plantlets were successfully transferred to the soil.

3.3.1.3. *Tinospora cordifolia*

In *T. cordifolia* callus was initiated from the young leaf and stem internodes. The young stem internodes were found to be most suitable among the explants tried. However highest percentage of callus induction was observed in the media supplemented with 10 µM 2,4-D and 0 .5 µM BAP and 5 µM 2,4-D and 0.25 µM BAP. The results of the effect of different hormone combinations are summarised in Table 3.1.

The callus induction was found as small protuberances of tissues from the cut surfaces and sides of the stem

Fig.3.3 : Root formation in the regenerated shoots when transferred to the rooting medium

Table 3.5 : Effect of different concentrations of IAA/NAA along with BAP on rooting of *L. nicotianifolia*

BAP (µM)	NAA (µM)			IAA (µM)		
	5.0	2.5	0.5	5.0	2.5	0.5
0.00	55% +	80% -	7% -	15% -	30% -	5% -
0.50	+	+	+	+	+	+

+ Callus formation - No callus formation

Percent was calculated from the mean value of 16 independent determinations.

and leaf explants. The callus cultures were yellowish in colour and they could be subcultured every three weeks in the same media in which they were initiated. The accumulation of biomass in all the above media were studied by recording the initial and final fresh weight and dry weight of callus tissue. The results are given in Table 3.6. Biomass accumulation was highest in the media supplemented with 2,4-D at a concentration of 10 µM along with 1 µM BAP In NAA supplementation also, 10 µM concentration has given highest biomass but along with 0.5 µM BAP.

The calli were fragile when the concentration of BAP supplementation was low or absent. Presence of this hormone at higher concentration reduced the fragility and increased the brown colour. The callus cultures did not show any sign of organogenetic response under any of the hormone concentrations tried above.

3.3.1.4.*Piper nigrum*

In *Piper nigrum*, callus was induced from the stem explants and leaf petiole segments as small protuberances after 30 to 40 days of incubation. Young leaves and terminal buds failed to induce calli as it blackened and died after three weeks. The stem internodes were selected as the explants for frequent callus induction. The results are presented in Table 3.1. Maximum percentage

Table 3.6 : *Tinospora cordifolia* callus cultures - Effect of different hormone combinations on biomass accumulation

Sl. No.	2,4-D/NAA (µM)	BAP (µM)	2,4-D		NAA	
			Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)
				A		B
1	10	0.00	2617(±6.32	417.23(±1.55)	1620(±3.52)	324(1.03)
2	10	0.25	2650(±3.58)	509.61(±1.50)	1360(±1.66)	312(±1.13)
3	10	0.50	2907(±1.52)	570.53(±1.82)	2544(±3.37)	480(±1.60)
4	10	1.00	3130(±7.60)	636.00(±2.30)	1642(±4.82)	335(±2.08)
5	10	5.00	2830(±3.88)	533.96(±1.22)	1538(±5.72)	328(±0.98)
6	5	5.00	1592(±3.63)	325.00(±1.85)	820(±2.92)	165(±2.83)
7	5	1.00	1650(±1.38)	334.00(±2.38)	1224(±1.73)	240(±1.38)
8	5	0.50	2170(±5.22)	452.08(±1.54)	1318(±3.55)	282(±1.56)
9	5	0.25	2010(±3.37)	392.30(±2.22)	1450(±2.81)	360(±3.11)
10	0	1.00	1896(±2.11)	371.76(±2.68)	766(±6.00)	132(±2.56)

Initial biomass : Fresh weight : 320(±3.26) mg
Dry weight : 86(±1.55) mg

Data recorded after 40 days of incubation

Each value is the mean ±SEM of 20 independent determinations
All values compared with those of Sl.No.1. In all cases $P < 0.05$

't' values to Table 3.6
(Comparison of dry weight)

't' value between	't' values	't' value between	"t' values
1A and 2A	60.559	1B and 2B	11.097
2A and 3A	36.523	2B and 3B	121.274
6A and 7A	4.221	3B and 4B	78.130
8A and 9A	31.285	4B and 5B	4.304
9A and 10A	8.345	6B and 7B	33.682

of callus induction was recorded in media containing 10 µM 2,4-D along with 0.25 µM to 1.0 µM BAP. Callus initiation was not observed in media containing 1 µM BAP alone without auxin. Of the combinations of NAA tried, the highest percentage was obtained with 10 µM of NAA alone and also with same concentration of NAA with 0.25 µM BAP.

The callus tissues induced were separated from the explant and transferred to the fresh media of the same composition. The initial and final biomass of the callus tissues were recorded to study the effect of hormones on the biomass accumulation. The results are presented in Table 3.7. Maximum biomass accumulation was observed in the media supplemented with 10 µM 2,4-D and 0.25 µM BAP. Among the media supplemented with NAA, the highest biomass accumulation was recorded at a concentration of 10 µM along with 0.25 µM BAP. The absence of BAP and complete elimination of auxins have reduced the biomass accumulation considerably.

The calli were fragile in the media with higher concentration of auxins particularly with 2,4-D. The tissues were greyish white in colour, but with high concentration of BAP, the brown or grey colour increased. The compactness of calli were more in the media with NAA than with 2,4-D. There was no organogenetic response

Table 3.7: *Piper nigrum* callus cultures - Effect of different hormone combinations on biomass accumulation

Sl. No.	2,4-D/NAA (µM)	BAP (µM)	2,4-D		NAA	
			Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)
					A	B
1	10	0.00	3214.85(±3.78)	618.07(±3.72)	2130.00(±1.80)	438.56(±1.22)
2	10	0.25	3936.91(±5.25)	675.68(±3.72)	2845.80(±1.50)	587.84(±1.34)
3	10	0.50	3016.43(±7.20)	508.75(±2.89)	2136.00(±3.58)	430.00(±1.56)
4	10	1.00	2140.00(±6.24)	412.33(±2.66)	1880.90(±2.82)	393.67(±1.38)
5	10	5.00	1897.30(±3.58)	371.45(±1.85)	1635.00(±2.50)	347.20(±1.42)
6	5	5.00	948.00(±2.91)	197.50(±3.25)	1148.62(±1.37)	236.77(±1.22)
7	5	1.00	1324.80(±5.33)	319.40(±3.49)	1250.80(±1.82)	231.35(±0.90)
8	5	0.50	1647.00(±3.43)	357.25(±2.80)	1320.73(±2.80)	281.85(±0.99)
9	5	0.25	1435.87(±2.58)	370.75(±1.35)	1426.20(±2.60)	365.09(±1.30)
10	0	1.00	1026.00(±3.79)	199.33(±2.55)	1120.00(±1.68)	225.38(±1.55)

Initial biomass : Fresh weight : 320(±5.86) mg
Dry weight : 81(±2.33) mg

Data collected after 40 days of incubation

Each value is the mean ±SEM of 20 independent determinations
All values compared with those of Sl.No.1. In all cases $p < 0.05$

't' values of Table 3.7

(Comparison of dry weight)

't' value between	't' values	't' value between	't' values
1A and 2A	15.484	9A and 10A	35.000
3A and 4A	34.710	4B and 5B	33.184
4A and 5A	17.840	8B and 9B	14.503
7A and 8A	11.961	9B and 10B	27.755

in the callus cultures under any of the hormone combinations tried for the biomass accumulations. Presence of chlorophyll or any other pigmented cells was not observed during the course of investigation.

3.3.1.5. *Coscinium fenestratum*

In *Coscinium fenestratum*, all the explants used for the callus induction except the leaf petiole bases failed to produce callus in all the hormone combinations tried. The pulvinus region of the leaf petiole started to induce calli after 40 to 50 days of incubation. Callus induction was more rapid in dark than in the light. Callus initiation from the leaf petiole base (pulvinous) under different hormone combinations were studied and the results are presented in Table 3.1. Higher concentration of auxins upto 20 µM and 30 µM both in the case of 2,4-D and NAA promoted callus initiation.

The calli initiated were excised and subcultured in the respective fresh medium. The effect of hormone combinations on growth were studied by recording the fresh and dry weight of inoculum and tissues after 40 days of incubation. The results are presented in Table 3.8. Biomass accumulation was very low when compared to the other plants particularly *T. cordifolia* which belongs to the same family. Medium supplemented with 10 µM 2,4-D and 1 µM BAP has given the highest biomass accumulation

Table 3.8 : *Coscinium fenestratum* callus cultures - Effect of different hormone combinations on biomass accumulation

Sl. No.	2,4-D/NAA (µM)	BAP (µM)	2,4-D		NAA	
			Fresh wt. (mg/culture)	Dry weight (mg/culture)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)
				A		B
1	10	0.00	927(±2.60)	208(±1.80)	512(±1.60)	98(±0.96)
2	10	0.25	1142(±2.11)	321(±1.25)	765(±1.26)	112(±1.52)
3	10	1.00	1260(±1.22)	385(±1.06)	1216(±1.55)	279(±1.83)
4	20	1.00	1205(±2.51)	374(±1.00)	1254(±2.11)	281(±1.35)
5	30	1.00	1024(±1.66)	289(±0.98)	940(±1.81)	252(±1.32)

Initial biomass : Fresh weight : 360(±3.25) mg
Dry weight : 72(±1.47) mg

Data collected after 60 days of incubation

Each value is the mean ±SEM of 20 independent determinations
All values compared with those of Sl.No.1. In all cases $p < 0.05$

't' values of Table 3.8
(Comparison of dry weight)

't' values between	't' values	't' values between	't' values
2A and 3A	55.216	3B and 4B	1.243*
2A and 4A	10.673	4B and 5B	21.718

* not significant at 5% level

whereas among the different concentrations of NAA, 20 µM along with 1 µM BAP has given the highest biomass accumulation.

Generally the callus cultures of *C. fenestratum* were slow growing and they were deep yellow or yellowish brown in colour. The solid agar medium on which the calli were grown changed to deep yellow due to the secretion of an yellow substance into the medium. On continuous growth, the calli became more brown and this situation was prevented by frequent transfer to fresh medium.

Higher concentration of BAP increased the brown colour of the calli whereas higher concentration of auxins particularly 2,4-D reduced the brown colour and increased the yellow colour along with fragility of the callus tissue.

3.3.1.6. *Rauwolfia tetraphylla*

In *R. tetraphylla (R. cannescens)* all the explants induced calli after 20 to 35 days of incubation. Young stem internodes and young leaves were the most suitable explant tissues for callus induction.

The effect of various hormone combinations on callus initiation were attempted using the stem explant tissue. Callus initiation occurred in all the cases after 20 to 25 days of incubation to varying proportions. The

results are presented in Table 3.1. Higher concentrations of 2,4-D (10 µM) with BAP at concentrations of 1 µM produced calli in 90 to 95 percent of explants. The percentages were slightly reduced when 2,4-D was replaced with NAA. Complete elimination of auxins significantly lowered the percentage of callus initiation but the absence of BAP does not appear to have much effect on callus induction. However BAP at higher concentrations brought down the extent of callus induction.

The callus tissue formed were separated from the explant and cultured in the fresh media of the same composition and were maintained by subculturing every 25 days. The results of the effect of hormone combinations used for callus initiation and biomass accumulations are given in Table 3.9. Maximum biomass formation was observed in the media with 10 µM 2,4-D and 0.5 µM BAP. Supplementation of BAP at a concentration of 1.0 and 5.0 µM were also found to be favourable for biomass formation. In media supplemented with NAA the highest biomass accumulation was observed at a concentration of 10 µM in combination with 0.5 µM BAP.

The calli were yellowish white and had a fragile as well as compact nature. High concentration of BAP increased the compactness and green colour of the callus

Table 3.9 : *Rauwolfia tetraphylla* callus cultures - Effect of different hormone combinations on biomass accumulation

Sl. No.	2,4-D/NAA (µM)	BAP (µM)	2,4-D		NAA	
			Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)
				A		B
1	10	0.00	4566.80(±1.63)	861.50(±1.08)	3826.30(±5.23)	735.76(±0.92)
2	10	0.25	4832.68(±2.32)	945.39(±1.25)	4288.92(±2.55)	818.87(±2.20)
3	10	0.50	5210.50(±2.53)	972.04(±1.80)	4293.36(±3.11)	911.49(±2.65)
4	10	1.00	4985.86(±3.89)	908.17(±3.42)	4530.76(±1.92)	877.24(±4.82)
5	10	5.00	4328.88(±1.83)	844.50(±1.52)	3766.85(±4.80)	788.20(±1.30)
6	5	5.00	2592.38(±1.99)	515.08(±1.92)	2995.00(±3.06)	574.45(±1.30)
7	5	1.00	3990.75(±2.52)	716.40(±2.11)	3581.80(±3.91)	739.11(±1.04)
8	5	0.50	3422.85(±4.55)	648.57(±3.56)	3640.92(±3.80)	685.91(±1.04)
9	5	0.25	3214.80(±1.36)	606.64(±1.63)	3675.96(±1.89)	690.65(±2.59)
10	0	1.00	1065.69(±1.87)	217.48(±1.30)	1058.10(±2.32)	217.81(±1.24)

Initial biomass : Fresh weight : 365(±2.41) mg
Dry weight : 76(±1.33) mg

Data collected after 40 days of incubation

Each value is the mean ±SEM of 20 independent determinations
All values compared with those of Sl.No.1. In all cases $p < 0.05$

't' values of Table 3.9
(Comparison of dry weight)

't' value between	't' values	't' value between	't' values
1A and 2A	71.806	5A and 6A	16.415
2A and 3A	17.195	8A and 9A	15.192
3A and 4A	23.368	3B and 4B	8.804
4A and 5A	24.055	8B and 9B	2.401

tissue. They were more fragile in media containing 2,4-D than NAA. The callus cultures did not show any organogenetic response in any of the hormone combination used.

3.3.2. Kinetics of callus growth

The callus cultures of each plant varied greatly in the growth and biomass accumulation. Therefore experiments were conducted to study the growth kinetics of callus cultures. The culture media in which the maximum biomass obtained in each case was selected and used for this experiment. The results obtained are presented in Table 3.10 to 3.15. The dry weights of the callus cultures were plotted against time and growth curves were developed for each callus culture. Each curve was a typical one, with a lag phase, logarithmic phase and a stationary phase. Each of these phases varied greatly from plant to plant. Generally, the lag phase extended upto 21 to 35 days. It was more prolonged in the case of *P. nigrum*, *C. fenestratum* and *T. cordifolia*. In plants like *L. nicotianifolia* a lag period of 14 days was observed. The logarithmic phase in this case started between 20 and 30 days and extended upto 50 to 60 days. In others which had prolonged lag phase, the logarithmic phase started between 25 and 35 days and it prolonged upto 65 to 70 days. The results are presented in Figures 3.4 to 3.9. Specific growth rate (μ) and doubling time (tg) were calculated in each case from the logarithmic

Table 3.10 : *Sida cordifolia* callus cultures - Time course studies on growth, DNA and Protein content

Time (days)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Protein (mg/100mg)	DNA (mg/100 mg)
0	612.55(±1.73)	118.54(±1.25)	6.11(±0.93)	3.52(±0.34)
7	630.60(±1.04)	121.08(±1.03)	6.31(±0.08)	3.55(±0.08)
14	795.93(±2.11)	125.40(±1.44)	6.56(±0.12)	3.87(±0.15)
21	987.54(±1.81)	186.94(±0.81)	7.18(±0.53)	4.60(±0.27)
28	1319.63(±1.65)	342.32(±1.93)	7.79(±0.44)	4.93(±0.33)
35	2407.30(±2.53)	482.80(±1.36)	10.45(±1.03)	5.67(±0.25)
42	4511.88(±1.93)	891.22(±1.20)	13.81(±0.66)	5.98(±0.18)
49	5567.00(±2.10)	1220.79(±1.28)	15.34(±0.81)	6.25(±0.34)
56	5883.65(±2.55)	1123.41(±1.77)	15.71(±1.11)	6.59(±0.81)
63	5840.92(±1.83)	1105.38(±1.22)	14.24(±0.38)	6.90(±0.75)
70	5781.75(±2.89)	948.30(±1.49)	13.52(±0.25)	7.06(±0.51)

Each value is the mean ±SEM of 10 independent determinations

Table 3.11 : *Lobelia nicotianifolia* callus cultures - Time course studies on growth, DNA and protein content

Time (days)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Protein (mg/100mg)	DNA (mg/100mg)
0	378.08(±6.00	117.90(±0.88)	4.53(±0.18)	2.56(±0.06)
7	718.56(±1.02)	128.50(±1.10)	5.12(±0.07)	2.72(±0.04)
14	866.92(±1.38)	155.43(±0.52)	6.33(±0.14)	3.11(±0.06)
21	1163.73(±1.54)	211.45(±1.33)	7.18(±0.13)	3.63(±0.09)
28	1895.00(±0.88)	356.20(±1.15)	8.83(±0.17)	4.43(±0.10)
35	3715.78(±1.20)	681.65(±1.21)	12.51(±0.12)	4.88(±0.06)
42	4120.35(±1.62)	968.26(±0.76)	15.80(±0.24)	5.84(±0.11)
49	4680.65(±1.34)	1371.06(±2.10)	15.63(±0.20)	5.72(±0.07)
56	4721.28(±1.66)	1455.77(±1.56)	14.38(±0.43)	5.86(±0.05)
63	4760.55(±1.72)	1486.26(±1.31)	12.91(±0.29)	6.05(±0.08)
70	4741.83(±1.24)	1405.28(±1.68)	12.53(±0.18)	6.12(±0.10)

Each value is the mean ±SEM of 10 Independent determinations

Table 3.12 : *Tinospora cordifolia* callus cultures - Time course studies on growth, DNA and protein content

Time (day)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Protein (mg/100mg)	DNA (mg/100mg)
0	472.88(±1.26)	92.59(±0.66)	4.81(±0.02)	3.25(±0.37)
7	487.53(±1.73)	93.24(±1.34)	4.53(±0.34)	3.41(±0.05)
14	489.26(±1.94)	98.70(±1.92)	5.22(±0.24)	3.43(±0.05)
21	518.56(±1.14)	101.52(±0.85)	7.34(±0.15)	3.75(±0.11)
28	834.63(±1.37)	167.28(±0.59)	8.82(±0.03)	4.14(±0.06)
35	1167.61(±1.26)	235.04(±0.25)	10.44(±0.09)	4.52(±0.11)
42	1728.52(±1.37)	365.40(±1.30)	12.59(±0.12)	4.89(±0.05)
49	2536.48(±1.85)	496.79(±1.55)	14.86(±0.09)	5.57(±0.02)
56	3861.93(±2.11)	641.00(±0.51)	14.24(±0.11)	6.28(±0.10)
63	3359.70(±1.53)	671.81(±1.08)	11.96(±0.07)	6.94(±0.03)
70	3140.18(±1.96)	654.16(±1.75)	10.47(±0.17)	7.11(±0.03)

Each value is the mean ±SEM of ten independent determinations

Table 3.13 : *Piper nigrum* callus cultures - Time course studies on growth, DNA and protein content

Time (days)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Protein (mg/100mg)	DNA (mg/100mg)
0	636.18(±2.11)	132.92(±1.26)	4.80(±0.12)	2.18(±0.03)
7	641.25(±1.56)	132.41(±1.08)	4.76(±0.08)	2.21(±0.05)
14	640.81(±1.24)	140.12(±0.81)	4.88(±0.13)	2.35(±0.21)
21	643.65(±1.66)	138.86(±1.51)	4.96(±0.26)	2.40(±0.11)
28	658.73(±1.33)	148.77(±0.39)	5.36(±0.52)	2.63(±0.53)
35	924.38(±1.19)	198.81(±0.72)	9.26(±0.73)	3.51(±0.13)
42	1255.51(±1.24)	262.76(±1.99)	13.81(±0.81)	4.36(±0.26)
49	2423.87(±1.55)	475.09(±0.35)	12.24(±0.45)	5.11(±0.18)
56	3598.15(±1.38)	763.42(±1.85)	11.53(±0.38)	5.93(±0.09)
63	3723.56(±1.91)	767.15(±0.72)	11.13(±0.14)	6.00(±0.02)
70	4110.70(±1.68)	775.87(±0.89)	11.24(±0.11)	6.11(±0.14)

Each value is the mean ±SEM of ten independent determinations

Table 3.14 : *Coscinium fenestratum* callus cultures - Time course studies on growth, DNA and protein content

Time (days)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Protein (mg/100mg)	DNA (mg/100mg)
0	396.31(±2.26)	78.08(±0.71)	4.53(±0.14)	2.81(±0.07)
7	398.35(±1.35)	83.47(±1.03)	4.72(±0.85)	2.84(±0.26)
14	423.50(±1.86)	85.53(±1.25)	4.96(±0.80)	2.96(±0.33)
21	481.03(±1.22)	97.84(±0.92)	5.25(±0.51)	3.24(±0.51)
28	610.40(±0.99)	126.53(±1.74)	5.72(±0.27)	3.55(±0.30)
35	665.41(±1.53)	135.48(±1.05)	7.46(±0.06)	4.67(±0.55)
42	835.85(±1.77)	117.33(±1.71)	9.15(±0.12)	5.16(±0.73)
49	918.13(±1.31)	180.62(±0.83)	11.92(±0.10)	5.83(±0.21)
56	1020.11(±1.76)	184.19(±1.11)	12.34(±0.31)	6.50(±0.34)
63	984.39(±1.50)	173.58(±1.59)	11.85(±0.66)	6.58(±0.08)
70	920.56(±0.89)	178.55(±1.68)	11.33(±0.71)	6.68(±0.12)

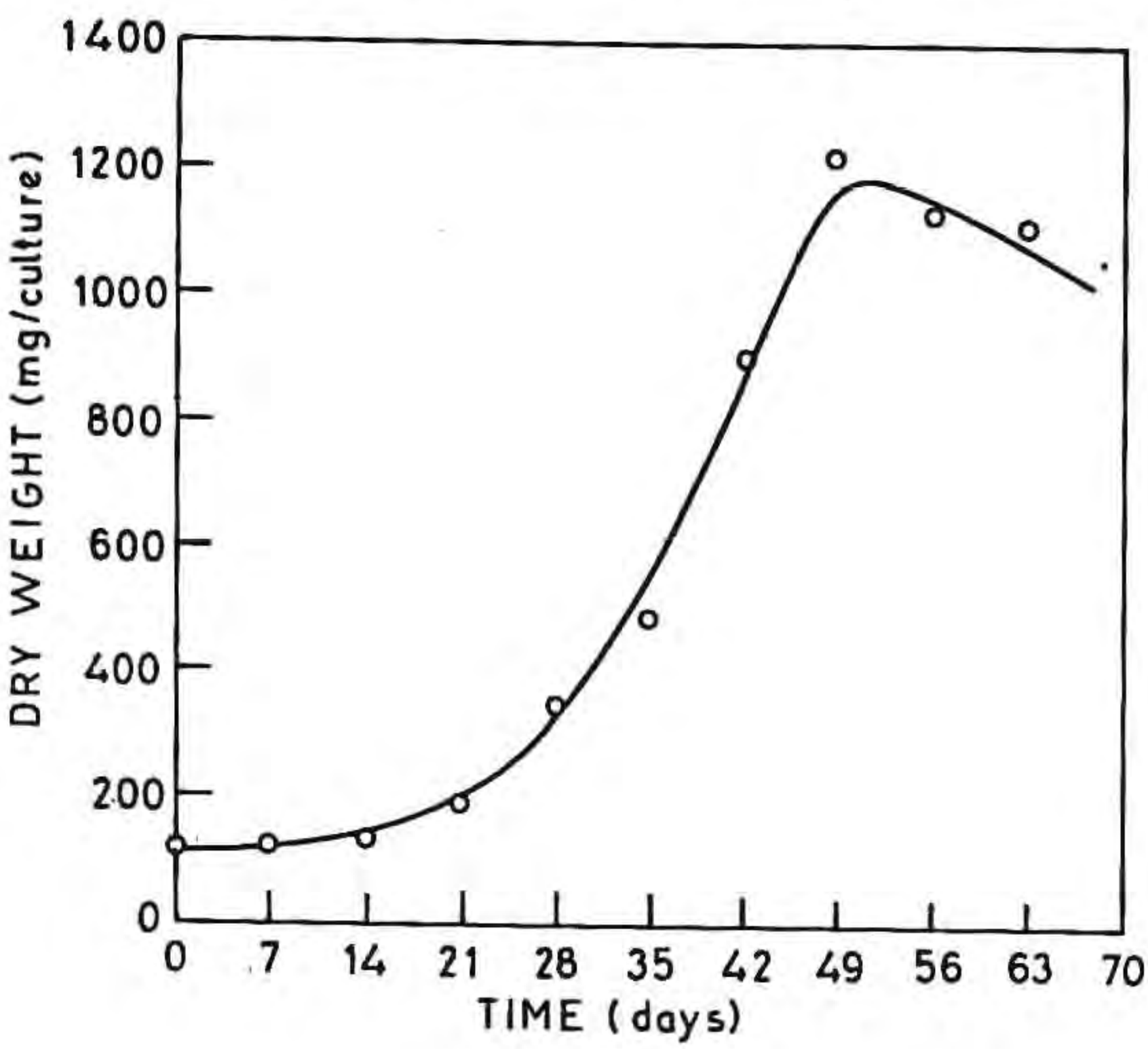
Each value is the mean ±SEM of ten independent determinations

Table 3.15 : *Rauwolfia tetraphylla* callus cultures - Time course studies on growth, DNA and protein content

Sl. No.	Time (days)	Fresh wt (mg/culture)	Dry wt. (mg/culture)	Protein (mg/100mg)	DNA (mg/100mg)
1	0	480.53(±1.05)	113.23(±0.27)	5.26(±0.14)	3.1(±0.21)
2	7	487.00(±0.83)	113.81(±1.11)	5.32(±0.11)	3.03(±0.03)
3	14	672.85(±1.13)	143.91(±0.50)	5.92(±0.16)	3.18(±0.34)
4	21	788.56(±0.55)	168.45(±1.85)	6.71(±0.31)	3.42(±0.26)
5	28	939.66(±0.93)	192.72(±1.35)	7.42(±0.22)	3.93(±0.13)
6	35	1183.25(±1.56)	241.42(±1.17)	10.47(±0.15)	4.25(±0.26)
7	42	1846.95(±2.11)	377.58(±0.91)	12.05(±0.33)	4.74(±0.32)
8	49	2617.72(±1.31)	545.33(±1.26)	13.21(±0.08)	4.96(±0.80)
9	56	3544.57(±1.22)	693.46(±1.55)	11.26(±0.17)	5.28(±0.13)
10	63	3748.83(±1.49)	764.89(±2.14)	10.80(±0.13)	5.73(±0.28)
11	70	3623.24(±1.25)	658.13(±1.79)	10.88(±0.32)	5.81(±0.33)

Each value is the mean ±SEM of ten independent determinations

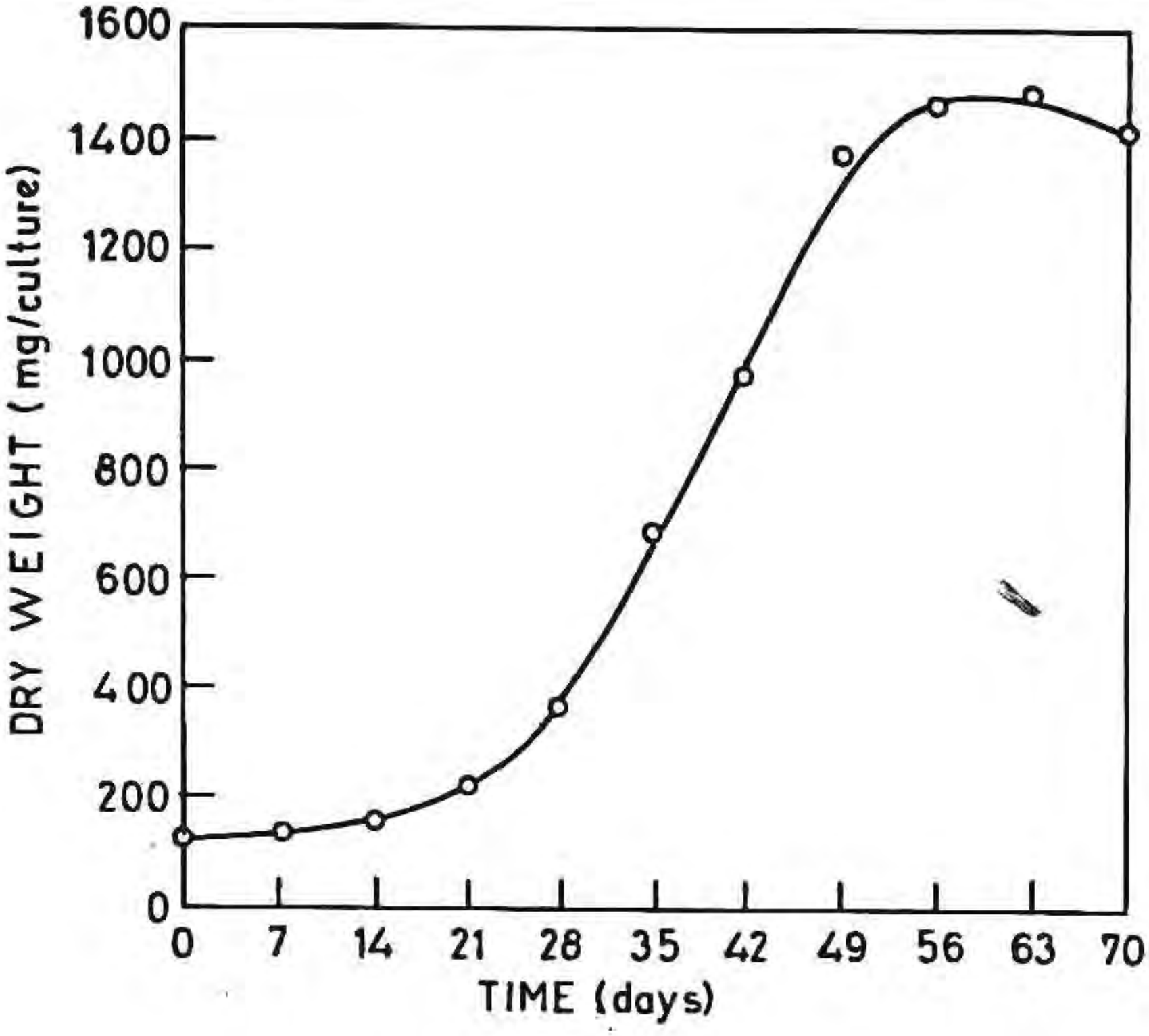
Fig.3.4 : Growth curve of *Sida cordifolia* callus cultures



The cultures were grown in MS basal media supplemented with 10 µM 2,4-D and 5 µM BAP.

The values plotted are the mean of twenty independent determinations.

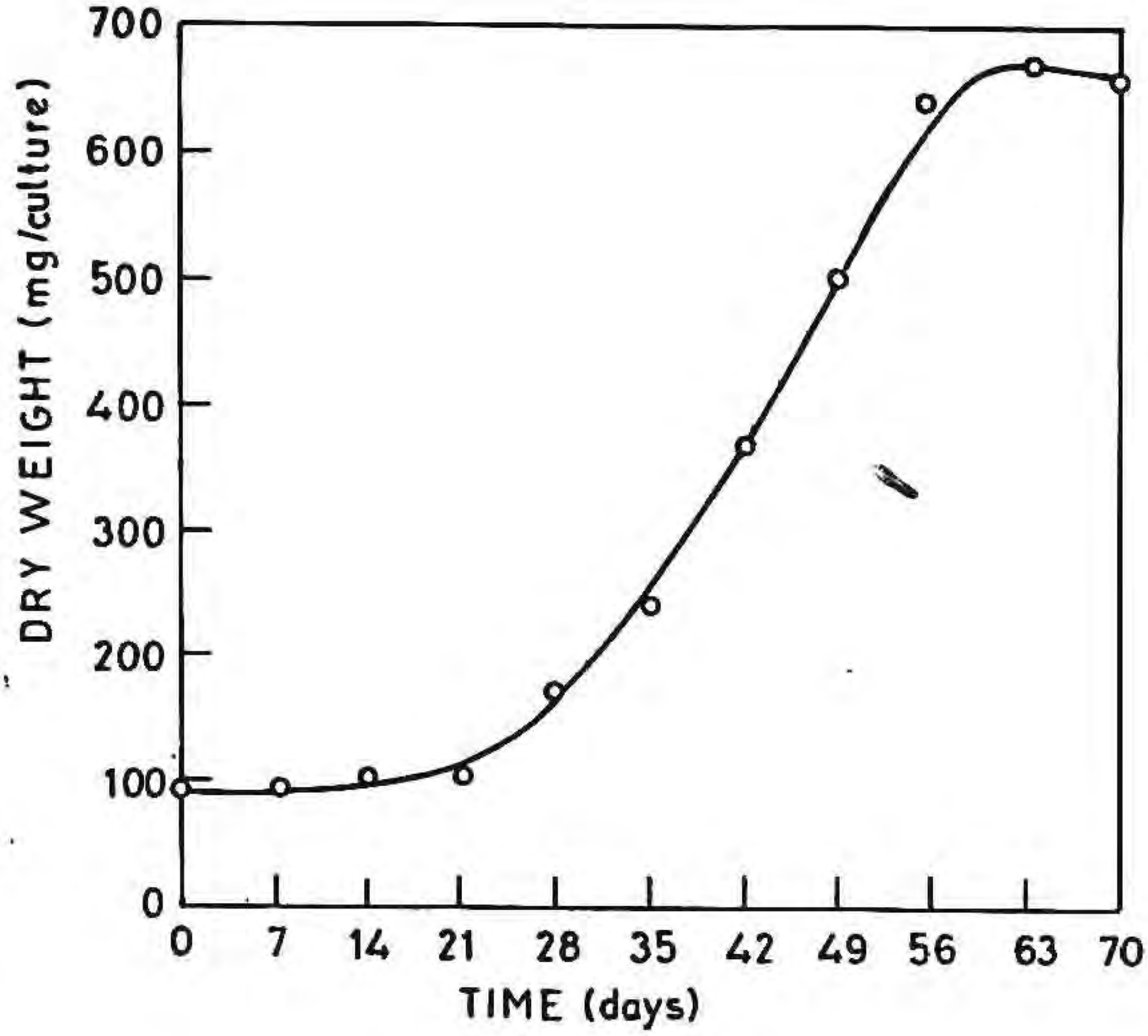
Fig.3.5 : Growth curve of *Lobelia nicotianifolia* callus cultures



The cultures were grown in MS basal media supplemented with 10 µM 2,4-D and 5 µM BAP.

The values plotted are the mean of twenty independent determinations.

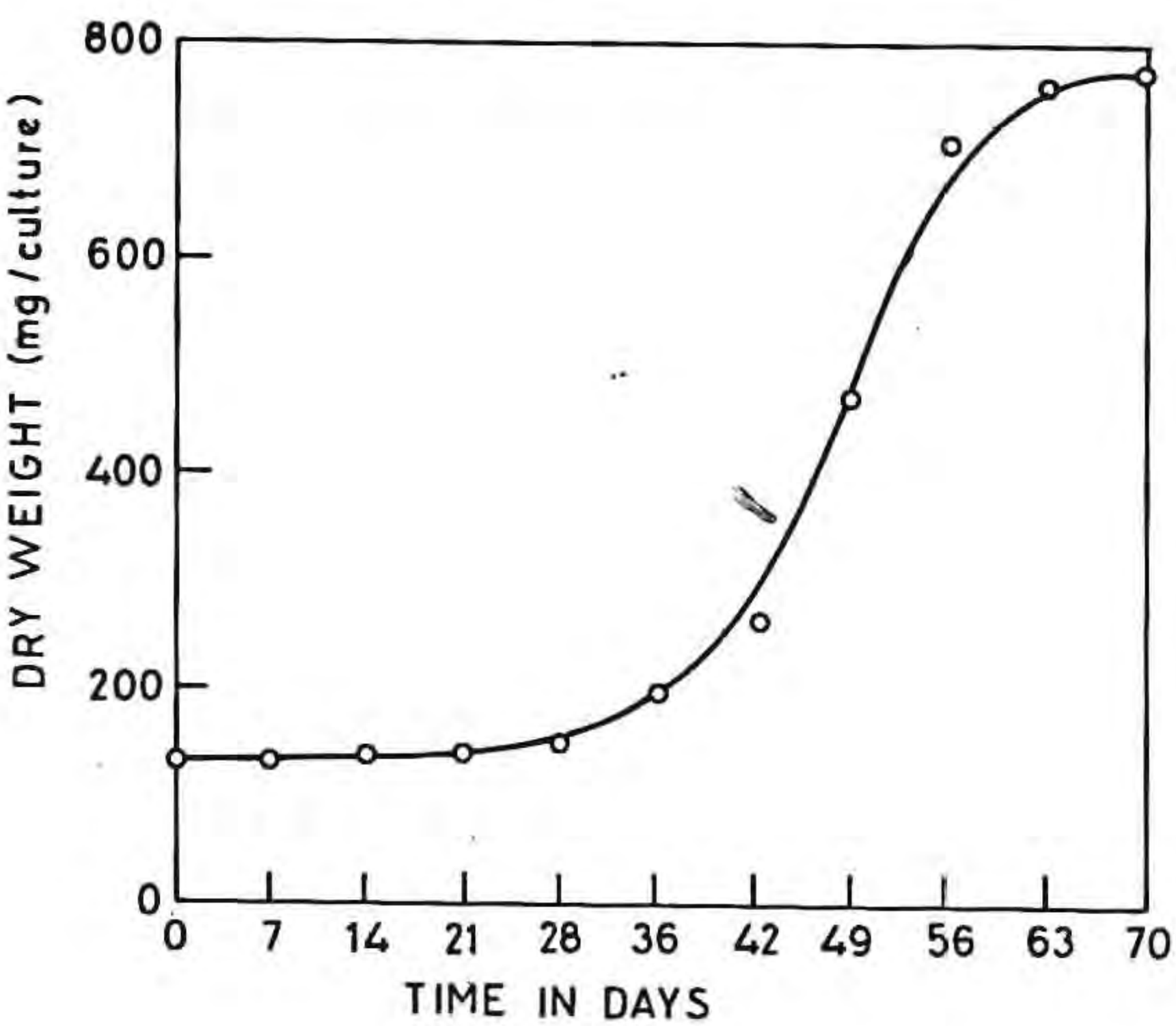
Fig.3.6 : Growth curve of *Tinospora cordifolia* callus cultures



The cultures were grown in MS basal media supplemented with 10 µM 2,4-D and 3 µM BAP.

The values plotted are the mean of twenty independent determinations.

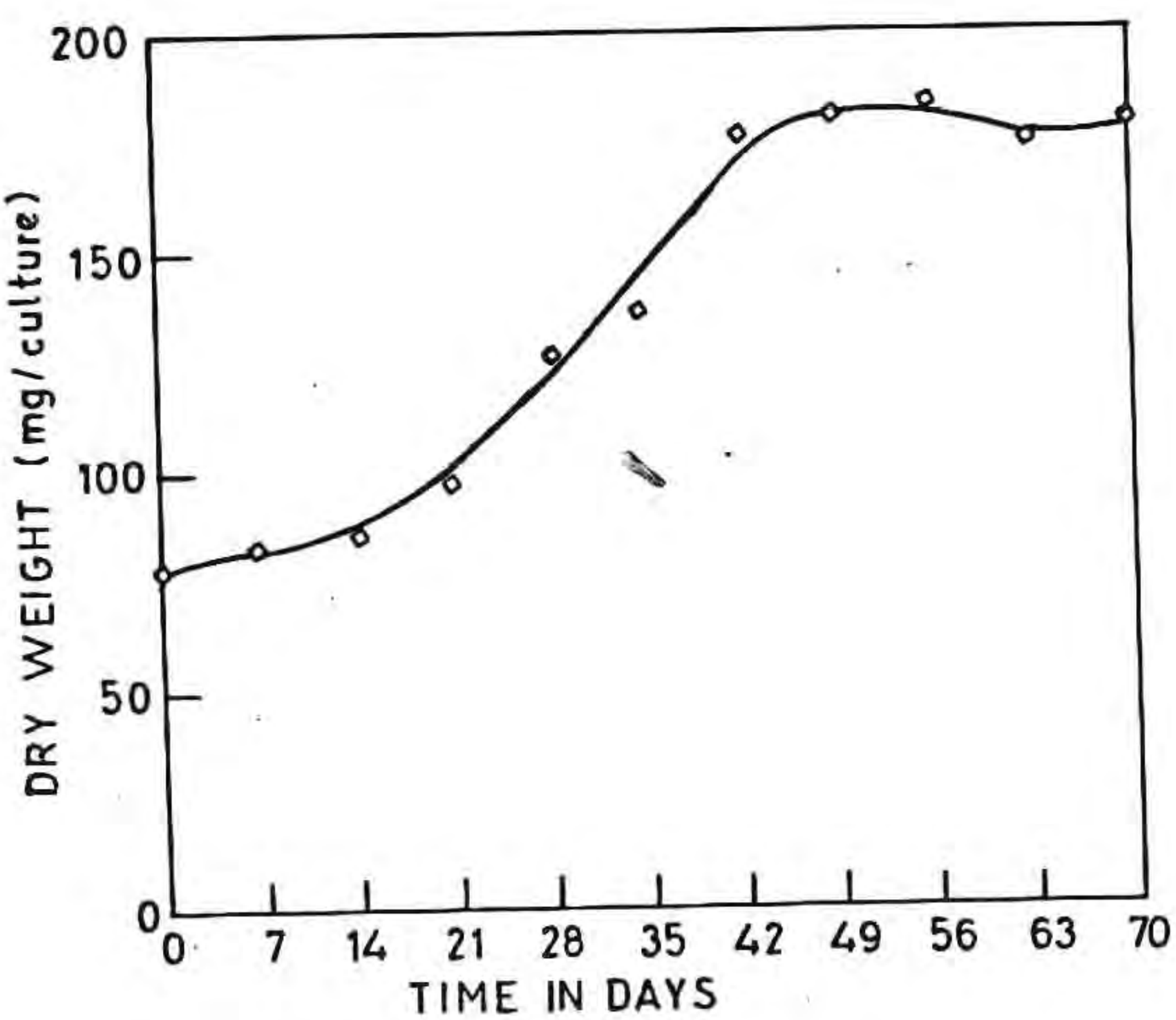
Fig.3.7 : Growth curve of *Piper nigrum* callus cultures



The cultures were grown in MS basal media supplemented with 10 µM 2,4-D and 0.25 µM BAP.

The values plotted are the mean of twenty independent determinations.

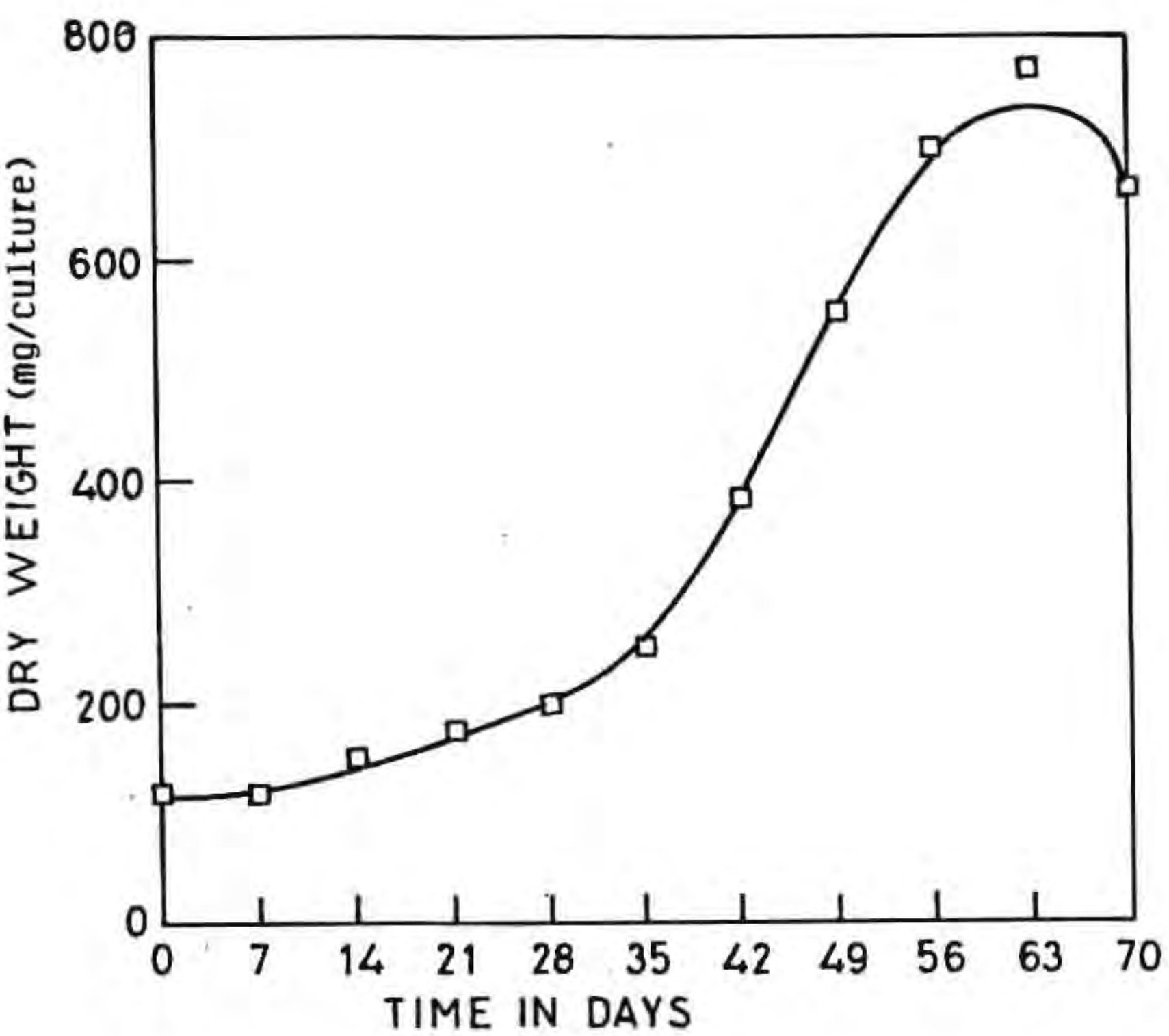
Fig.3.8 : Growth curve of *Coscinium fenestratum* Callus cultures



The cultures were grown in MS basal media supplemented with 10 µM 2,4-D and 2 µM BAP.

The values plotted are the mean ±SEM of twenty independent determinations

Fig.3.9 : Growth curve of *Rauwolfia tetraphylla* callus cultures



The callus cultures were grown in MS media supplemented with 10 µM 2,4-D and 0.5 µM BAP.

The values plotted are the mean of twenty independent determinations

phase of the growth curve and the results are presented in Table 3.16. Among the callus cultures of the six plants, specific growth rate (μ) was very low in *C. fenestratum* and was highest in *L. nicotianifolia* and *T. cordifolia*. The doubling time (tg) was lowest in *L. nicotianifolia* (11.26 days) and highest in *C. fenestratum* (23.89 days).

The DNA and protein content of the callus tissues were also determined in each case and are presented in Figures 3.10 and 3.11 respectively. The total DNA content gradually increased corresponding to the biomass increase particularly the dry biomass in all the callus cultures. There was no significant change in the DNA content observed during the stationary phase of the growth. But protein content varied greatly with respect to the growth phase. In all the cases, protein content gradually increased reaching a maximum between 30 and 50 days of the incubation period which was the logarithmic phase of growth in most of the callus cultures and decreased thereafter during the stationary phase.

3.3.3. Isolation and Identification of secondary metabolites

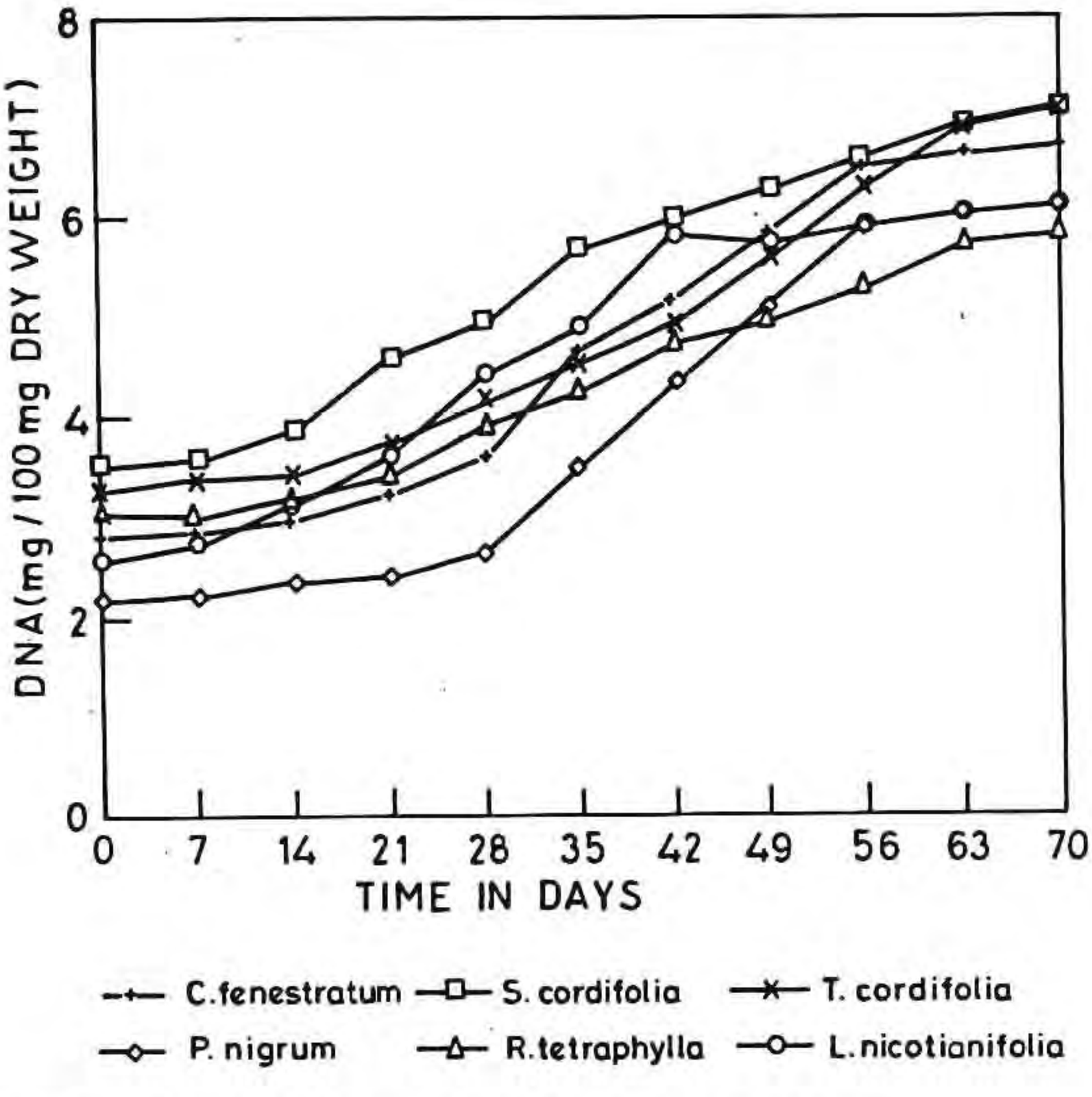
Seventy days old callus cultures were dried under reduced pressure and extracted as described in Chapter II. Thin layer chromatographic analysis showed that only four of the six plants studied were able to synthesize their active metabolites in the *in vitro* conditions. The callus

Table 3.16 : Kinetic parameters of callus growth of different plants

Callus cultures	*Sida cordifolia*	*Lobelia nicotianifolia*	*Tinospora cordifolia*	*Piper nigrum*	*Coscinium fenestratum*	*Rauwolfia tetraphylla*
Specific growth rate (μ /day)	0.060	0.062	0.048	0.0514	0.029	0.059
Doubling time (tg) (days)	11.630	11.260	14.560	13.480	23.890	11.740

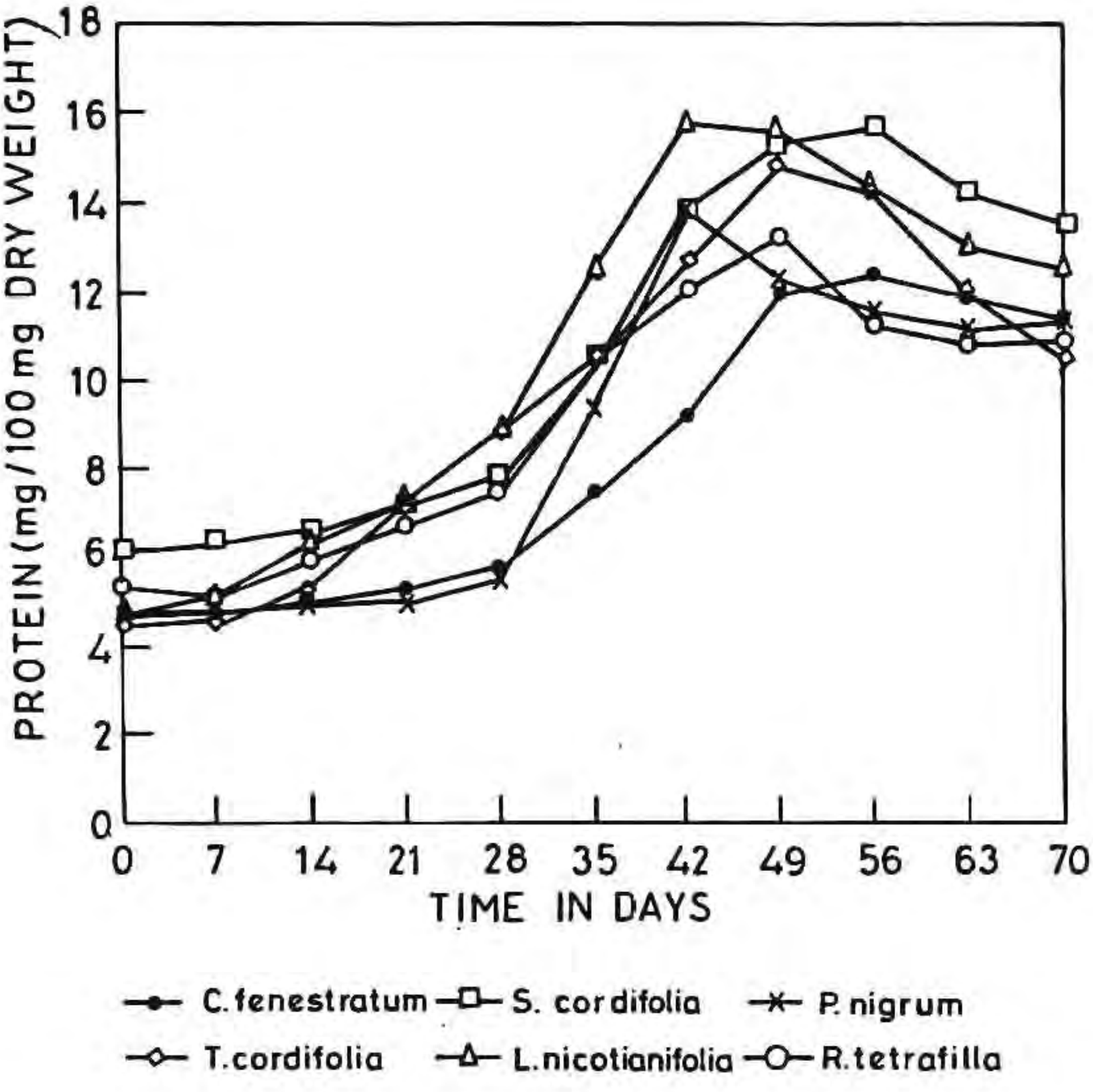
Values were calculated from the growth curve of each callus culture

Fig.3.10 : DNA content in the callus cultures during different stages of growth



The values plotted are the mean ±SEM of ten independent determinations

Fig.3.11 : Protein content in the callus cultures during different stages of growth



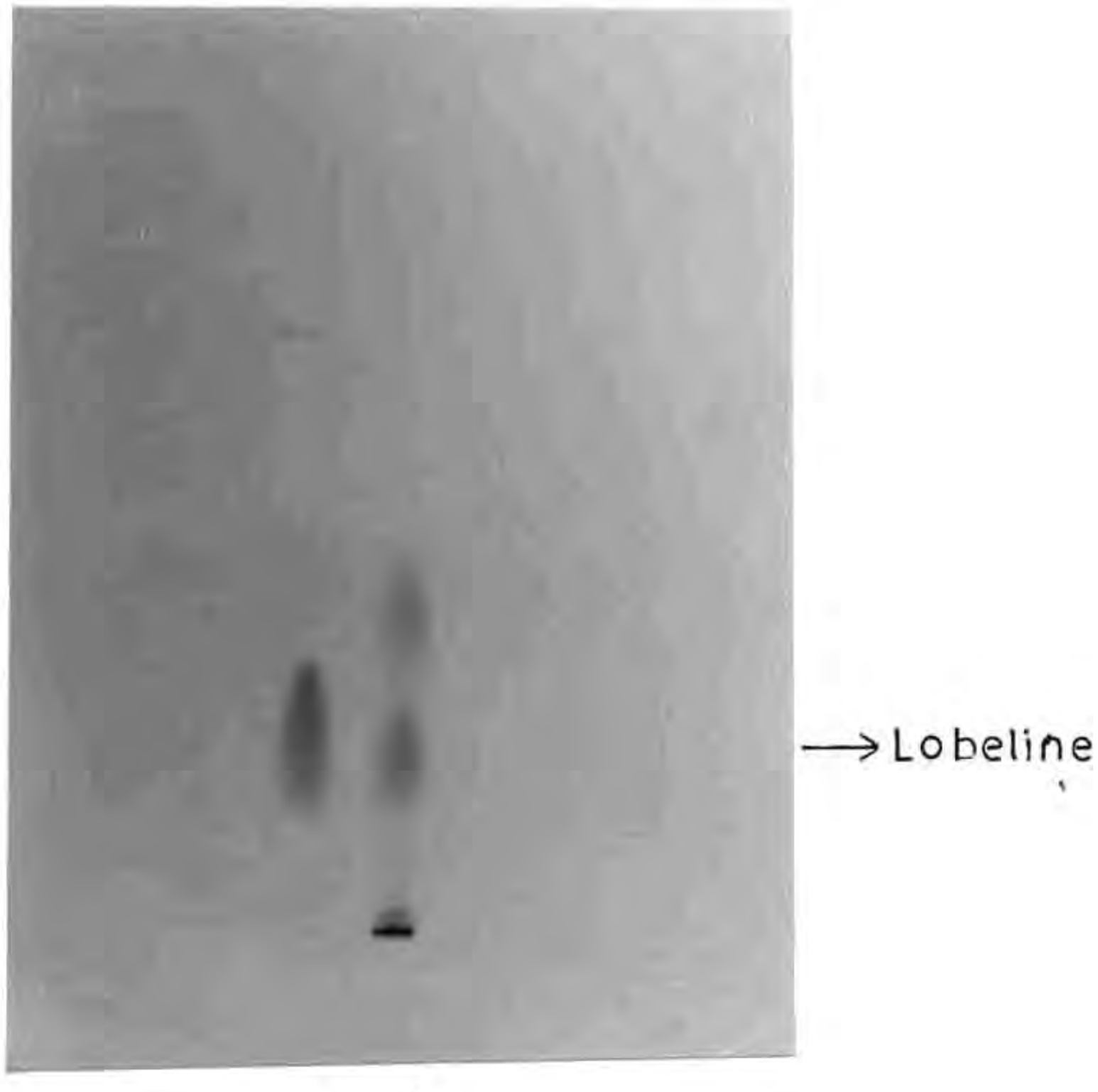
The values plotted are the mean of ten independent determinations

cultures of *P. nigrum* and *S. cordifolia* were not able to synthesize their active metabolites piperine and ephedrine respectively. The TLC plates after development were sprayed with 10 percent methanolic sulphuric acid or kept in iodine chamber and observed. No characteristic spots having the same R_f as that of the standard samples were seen on the chromatoplate.

Dichloromethane extracts of the *L. nicotianifolia* was applied on TLC and developed as described in the procedure. It was sprayed with Dragendorff reagent. The alkaloid spots developed a light brown colour. One of the spots was observed to have same R_f as that of the authentic sample of Lobeline. This spot was also detected by keeping the chromatographic plates in an iodine chamber (Fig.3.12). Analysis was carried out using HPLC as described in Chapter II. The HPLC chromatogram showed a peak having the identical retention time 7 minutes to that of the standard (Fig.3.13). The Lobeline content of the sample was estimated using HPLC and found to be 73 µg/g dry weight of the callus tissue.

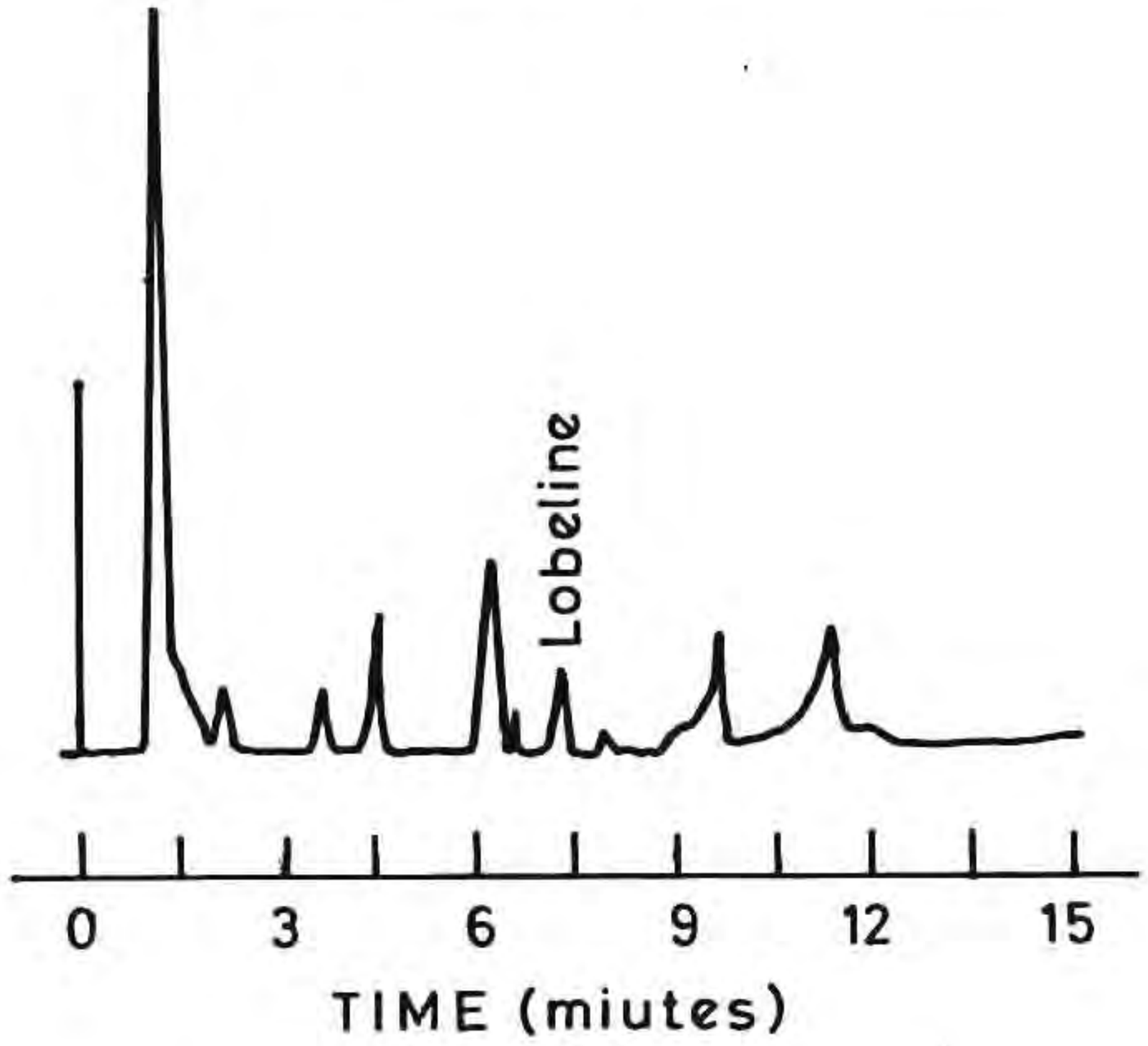
The methylene chloride extract of *R. tetraphylla* was analysed by TLC along with the authentic standard. The chromatographic plates after developing with the solvent system were viewed under UV light at 320 nm. Reserpine appeared as blue bright fluorescent spot corresponding

Fig.3.12 : TLC pattern of alkaloid extract of the callus cultures of *Lobelia nicotianifolia*



The chromatogram after development was treated with Iodine vapour: (a) Authentic sample (b) Alkaloid extract

Fig. 3.13 : HPLC elution pattern of alkaloid extract of the callus cultures of *Lobelia nicotianifolia*



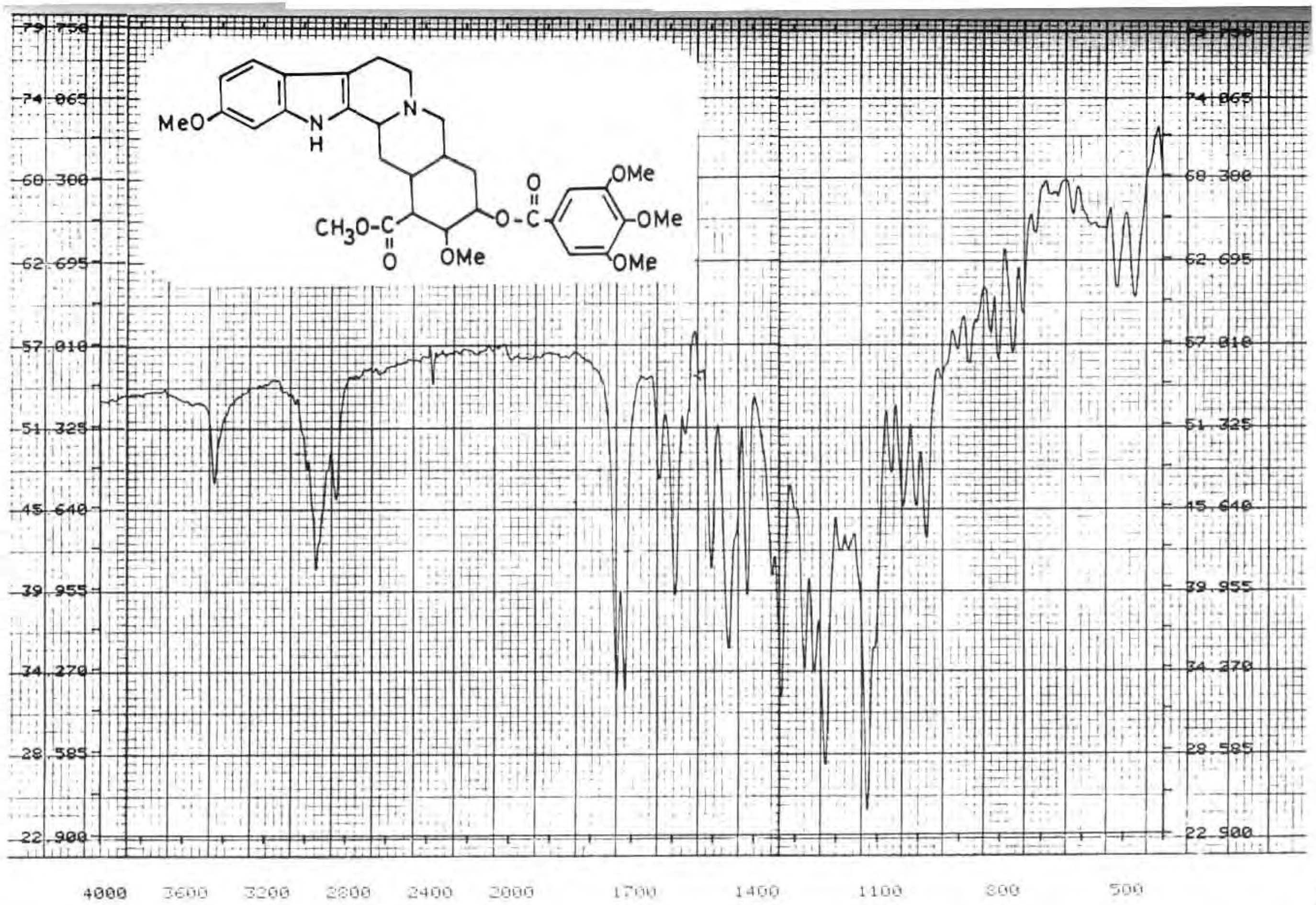
Alkaloid extract (20 µl) was injected into the column and the chromatogram obtained after 15 min.

to the R_f of the authentic sample. The reserpine spots were also detected by keeping the chromatographic plates in Iodine Chamber (Fig.3.14). A number of unidentified blue spots were also seen. Reserpine was isolated and purified by column chromatography followed by preparative TLC. The blue fluorescent area corresponding to the spot of authentic standard was scraped out and eluted with methylene chloride. The solvent was removed under vacuum and the weight of the sample was determined. The reserpine content of the callus tissue was estimated to be 12 mg/100g of dry weight. The structural confirmation of the pure sample isolated was carried out by studying the IR and ^1H NMR spectra (Fig.3.15 and 3.16), In addition to reserpine, some other light blue fluorescent spots were also observed under UV light.

The callus cultures of *T. cordifolia* and *C. fenestratum* were also extracted and analysed by TLC and HPLC. The yellow substance extracted from the cultured tissues was observed to be fluorescent under the UV. The above substance separated on silica gel G using different solvent systems described in Chapter II, gave two spots in both the cases. The R_f of one of the spots was identical with authentic berberine. The alkaloid spots were visible as lemon yellow spots which have the same R_f as that of the standard and gave a positive test for berberine with Dragendorff reagent. These alkaloid spots were also detected under UV light of 320 nm as flourescent yellow

Fig.3.14 : TLC pattern of alkaloid extract of the callus cultures of *Rauwolfia tetraphylla*

The chromatogram after development was treated with Iodine vapour (a) Authentic sample (b) Alkaloid extract



Fig· 3·15 IR Spectrum of Reserpine

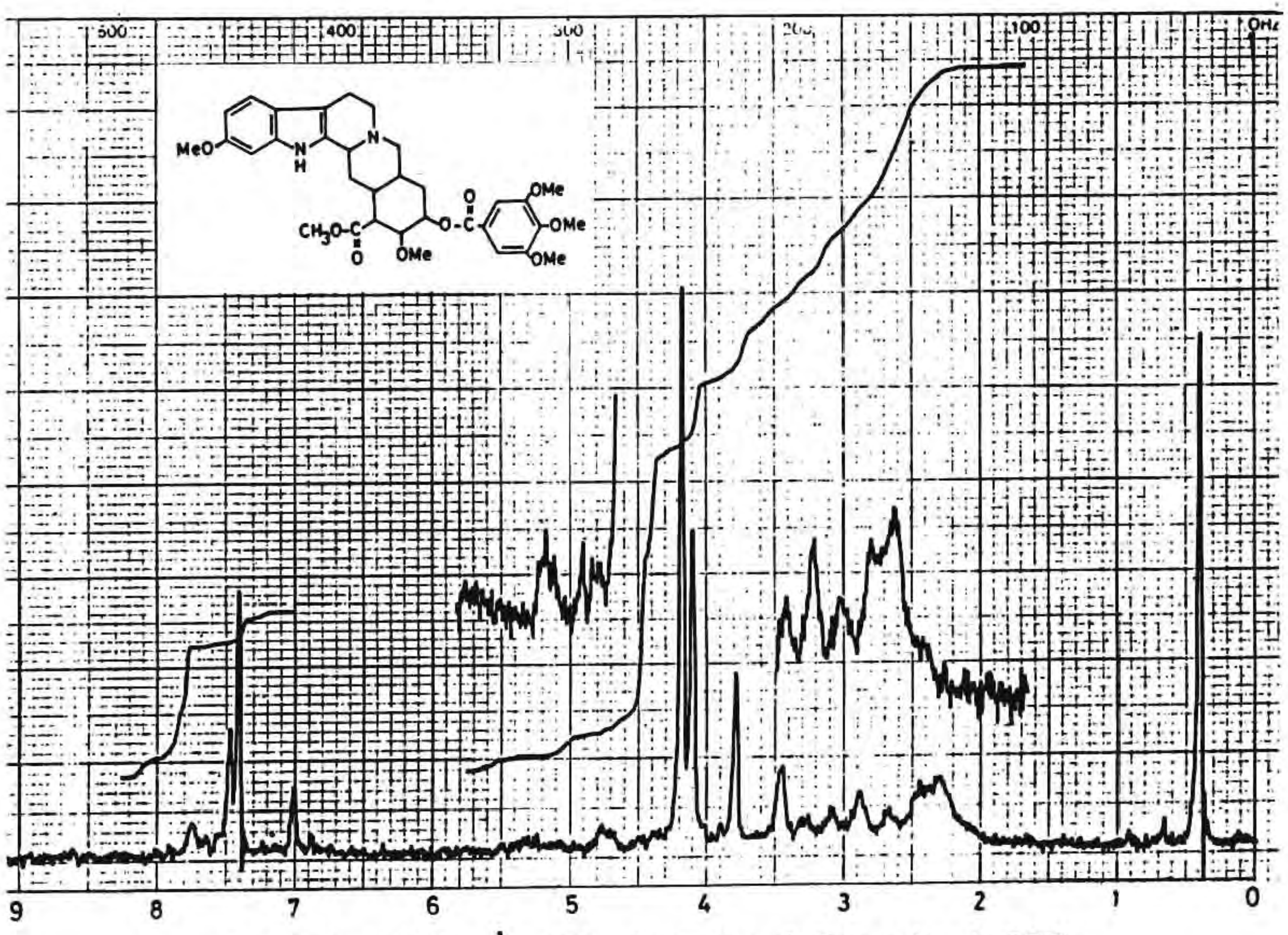


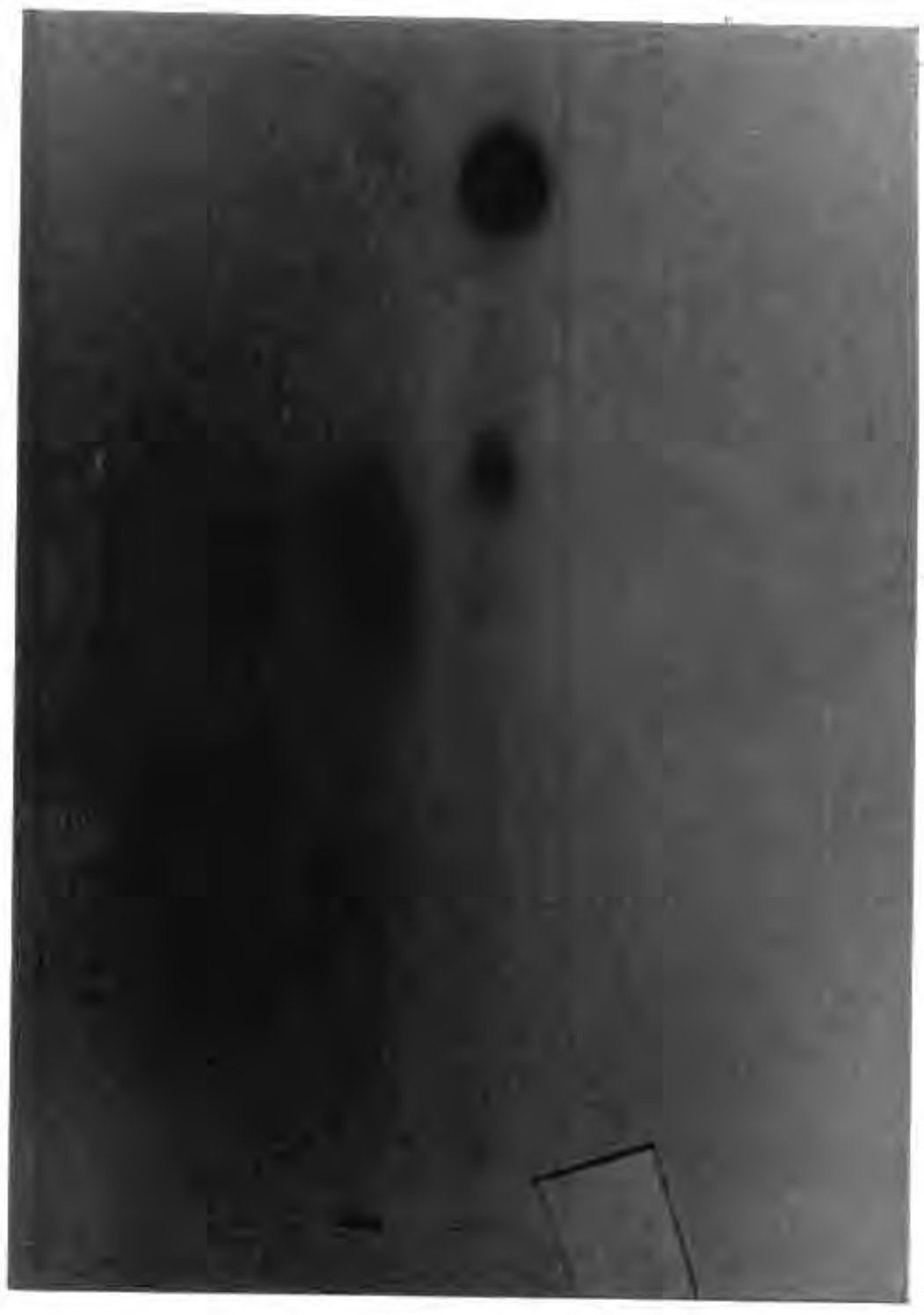
Fig. 3.16 60MHz ^{1}H NMR Spectrum of Reserpine in $CDCl_3$

spots (Fig.3.17). Another fluorescent yellow spot having an R_f higher than the standard berberine has been detected in both *T. cordifolia* and *C. fenestratum* which was not identified.

Berberine content of the samples was also analysed qualitatively and quantitatively using HPLC as described in Chapter II. Berberine was detected as a peak having a retention time of five minutes (Fig.3.18) which was similar to that of standard in both the samples. But another unidentified peak was also observed in both cultures, having a retention time of 15.30 minutes. Quantitative analysis by HPLC showed that the berberine content of *C. fenestratum* callus tissues was approximately seven percent of the dry weight while it was only 0.42 percent in *T. cordifolia*.

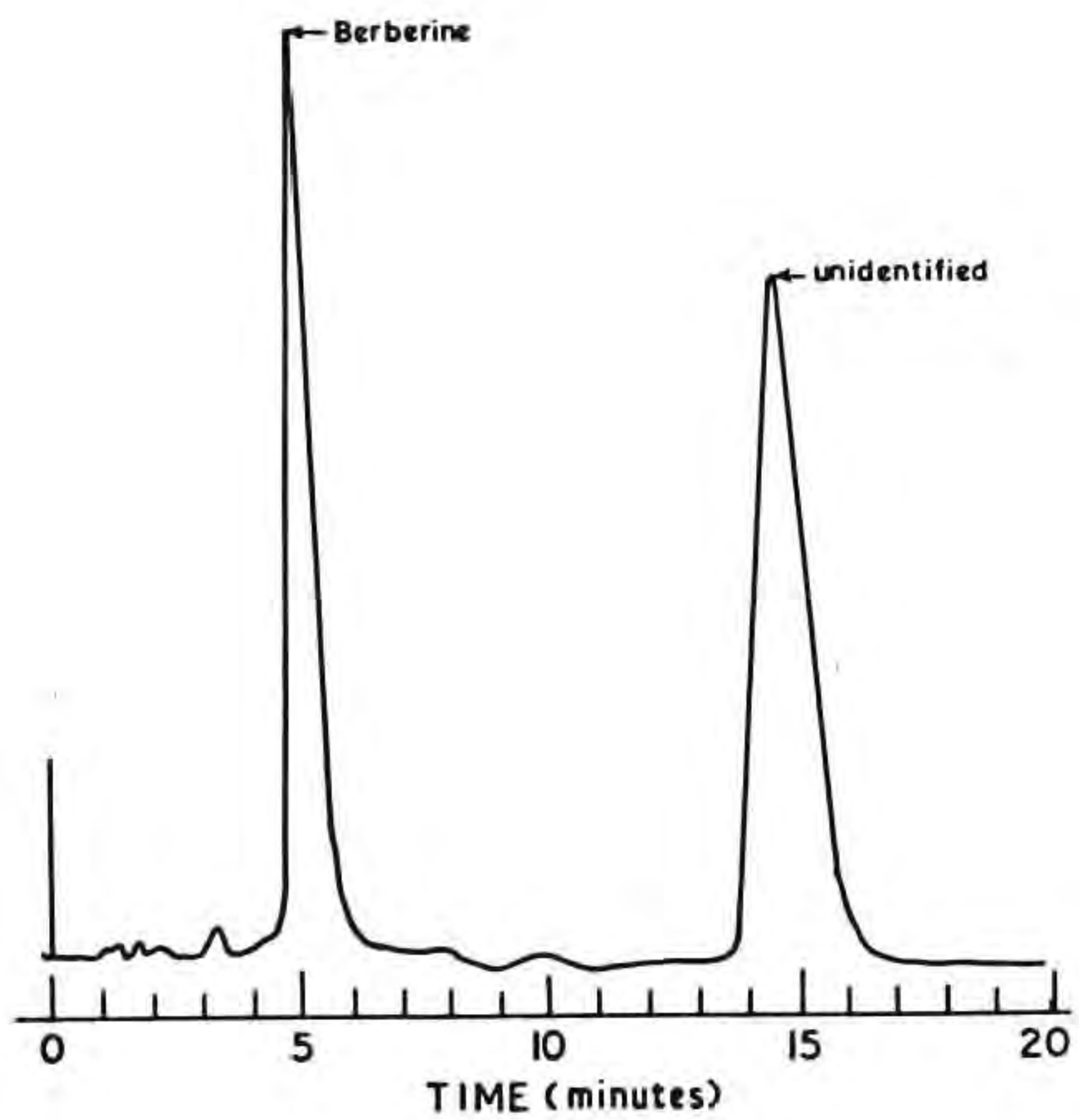
Berberine was isolated in pure form from both the samples by quantitative TLC. The fluorescent yellow zone corresponding to the authentic sample of berberine was scraped out and eluted with 90 percent methanol acidified with 2N HCl. Berberine hydrochloride was recrystallized from water and was further identified by co-TLC and HPLC along with the standard. Structural confirmation was made by comparing UV, IR and ^1H NMR spectra (Figs.3.19 to 3.21) and melting point (192°C) of the sample with that of the standard.

Fig.3.17 : TLC pattern of alkaloid extract of *C. fenestratum* and *T. cordifolia* Callus cultures



A. Standard, B. *C. fenestratum* C. *T. cordifolia*
The berberine content in *C. fenestratum* is higher than that of *T. cordifolia*

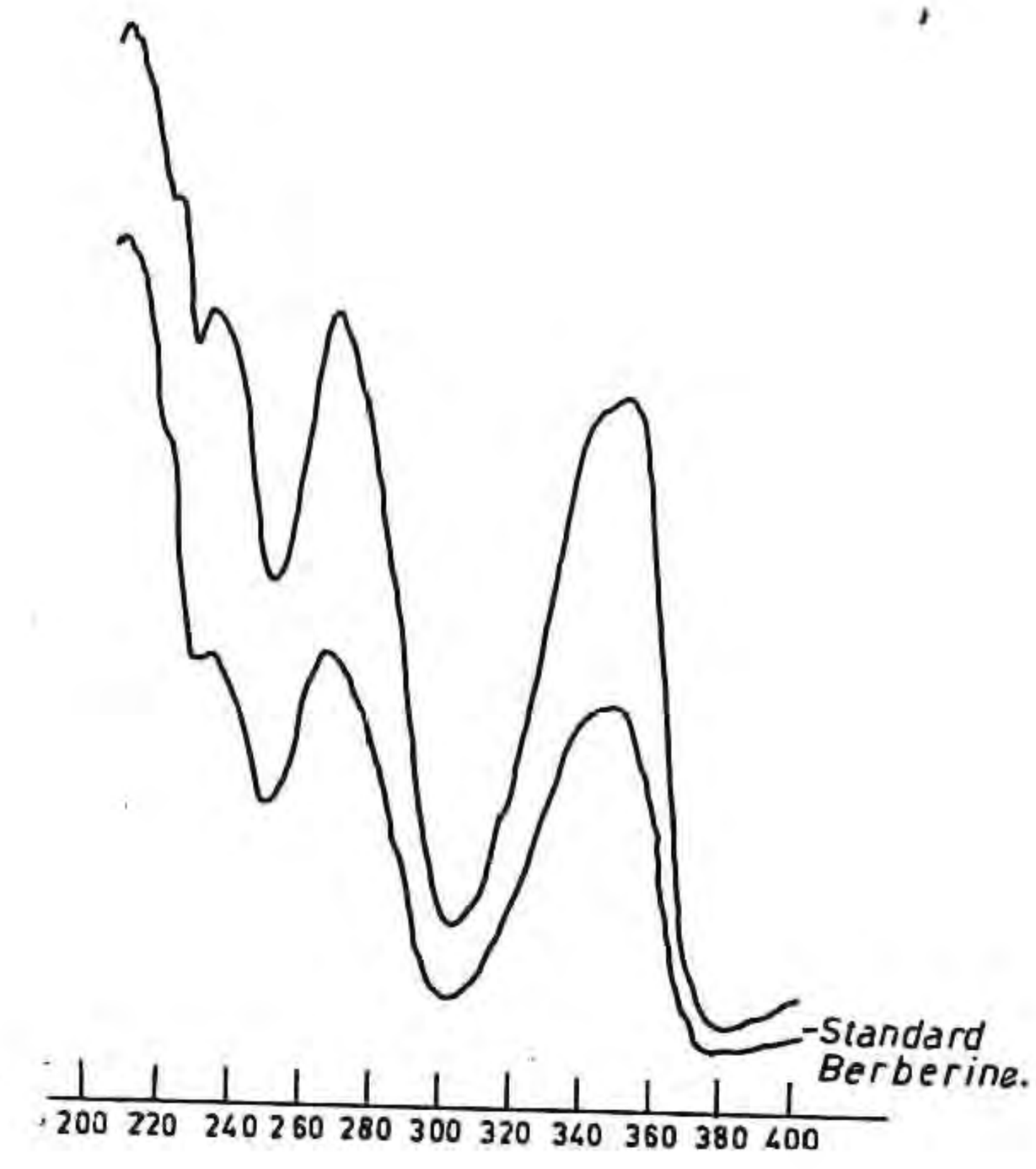
Fig.3.18 : HPLC elution pattern of berberine extract of *Coscinium fenestratum/Tinospora cordifolia* callus cultures



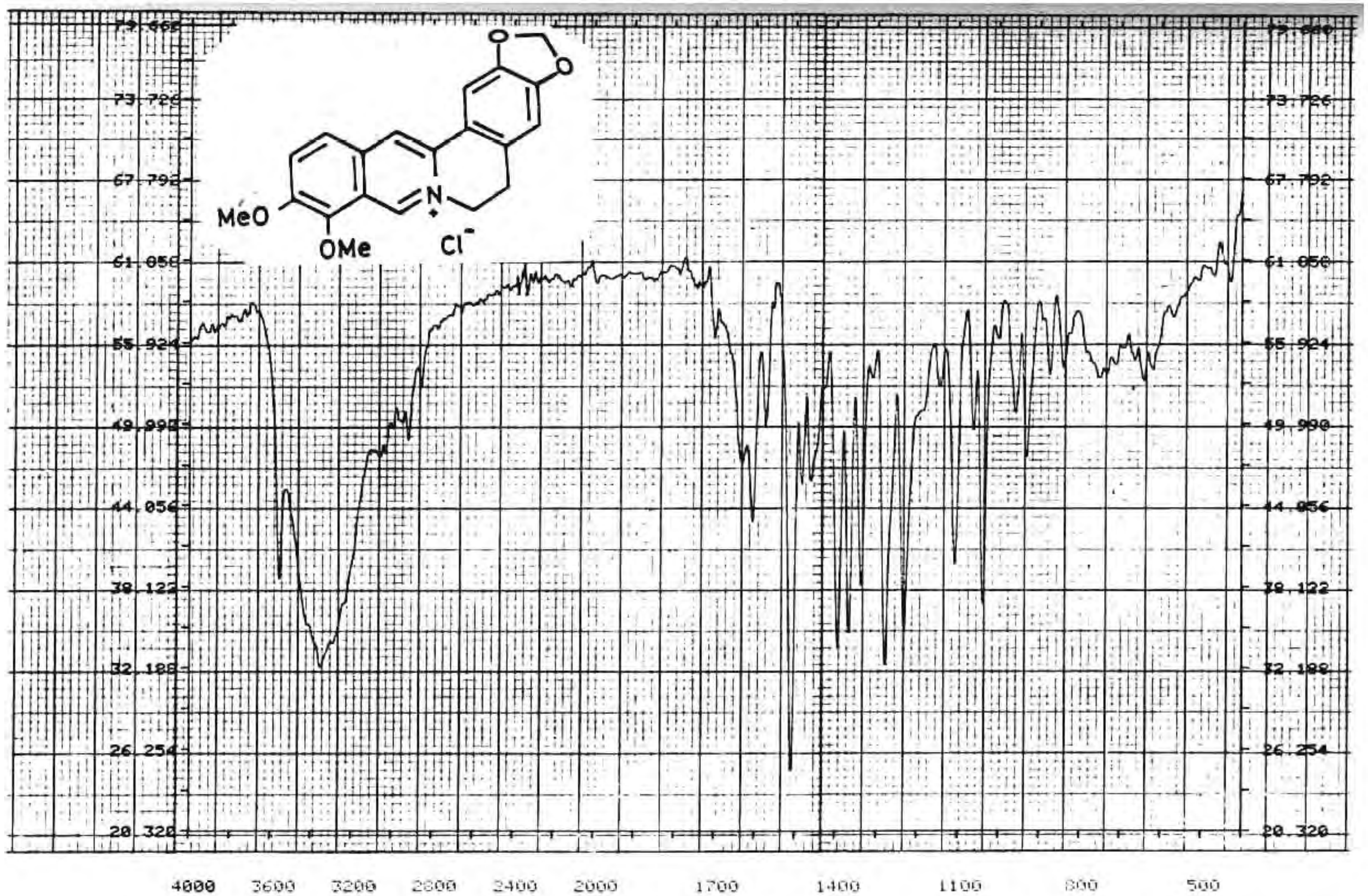
Alkaloid extract (20 µl) was injected into the column and the chromatogram obtained after 20 min.

Alkaloid extract from both callus cultures showed similar pattern

Fig.3.19 : UV spectrum of berberine



UV spectrum was taken in the medium - methanol



Fig·3·20 IR Spectrum of Berberine Hydrochloride

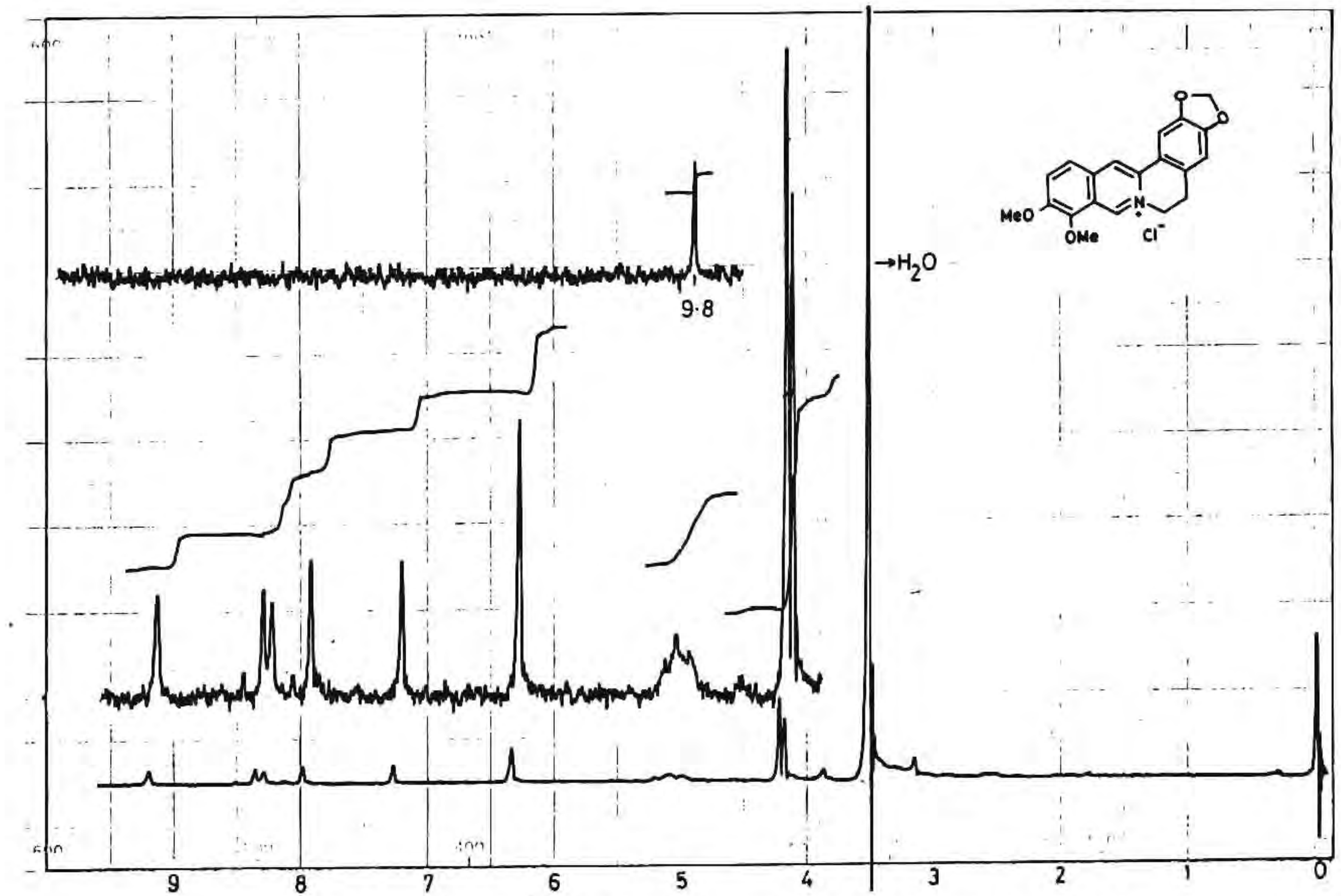


Fig. 3·21 60MHz ^{1}H NMR Spectrum of Berberine Hydrochloride in DMSO - d_6

3.4. DISCUSSION

This study was undertaken (1) to standardize the conditions for rapid production of callus cultures (2) to optimize the hormone combination for obtaining the highest biomass formation within a short culture period (3) to compare the growth pattern, kinetic parameters of growth and variations in protein and DNA content during growth cycle in the callus cultures of each plant under ideal nutritional conditions and (4) to investigate the ability of callus cultures to synthesize the respective secondary metabolite under *in vitro* conditions.

Identification of suitable explant and appropriate nutrient media conditions are very important for the rapid induction of callus cultures and its maintenance for further experiments. The phytohormones used for these studies include two synthetic auxins, 2,4-D, NAA and a synthetic cytokinin BAP. These hormones were used in different combinations to find out the suitable one, giving very good response. Different parts of each plant were tried for callus initiation to identify the suitable explant. It was found 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 that is prevalent in the explant tissue[214].

In *Lobelia nicotianifolia*, *Sida cordifolia*, *Rauwolfia tetraphylla* and *Tinospora cordifolia* young parts were the most suitable explant source for callus initiation. But it was not true in the case of *Piper nigrum* and *Coscinium fenestratum*. The young explant tissues of *Piper nigrum* became blackened due to polyphenol oxidase activity and did not induce callus. Blackening was comparatively less when mature stem internodal tissues were used as explant source. They produced calli in media containing higher concentration of 2,4-D. This may be due to reduced level of polyphenol oxidase activity in the mature parts compared to young parts. In *Coscinium fenestratum* only the pulvinate region of the petiole was able to produce callus, after 40 to 60 days of incubation in media containing 2,4-D at concentrations of 20 to 30 µM. All other parts including mature and young parts become blackened and died due to the formation and exudation of polyphenols. The problem of blackening was more severe in the case of *Coscinium* than of *Piper nigrum*. Administration of antioxidants like activated charcoal, ascorbic acid, polyvinyl pyruvate and phloroglucinol were not effective in preventing the blackening and promoting callus formation. The polyphenol oxidase activity of *Coscinium fenestratum* requires special attention as it is more active than other plants and caused blackening of all tissue parts immediately after its excision from the plant.

Among the auxins tried for callus initiation 2,4-D is the most effective one. NAA is also found equally good for callus initiation in *L. nicotianifolia* and *S. cordifolia*. Eventhough high concentration of 2,4-D favoured callus induction, the results indicate that there should be an optimum auxin-cytokinin ratio for callus induction because the complete elimination of BAP or auxins reduced the percentage of callus induction. It can differ from plant to plant and explant to explant.

Results obtained from the studies on the effect of different hormone combinations on biomass accumulation in callus cultures of different plants indicated that the hormone combination which has given the highest percentage of callus induction was not suitable for obtaining the highest biomass. The hormone combination suitable for callus initiation and callus growth are not the same in most cases. The results obtained further indicate that the auxin-cytokinin ratio required for maximum biomass yield is different from that for callus initiation. Since the callus inducing system and the biomass generating conditions were not found to be identical, the strategy adopted for maximum biomass production was to initiate callus in the medium which favours callus induction and then transfer into medium which favours biomass production.

The variations in the hormone combination also influenced the appearance and nature of the callus tissue which is the reflection of its metabolic condition. The fragility of callus tissue was more with 2,4-D than with NAA. The auxin-cytokinin ratio influenced the general morphology of callus tissue and also the physiological condition as it was understood from the colour of the callus tissue in all cases. For example, a hormone combination consisting of higher concentration of BAP and lower concentratins of auxins caused the formation of chlorophyll in the callus cultures of *S. cordifolia*, *L. nicotianifolia* and *R. tetraphylla*. In the callus cultures of *T. cordifolia* and *C. fenestratum* the auxin-cytokinin ratio greatly influenced the yellow colour of the tissue, in which high concentration of BAP caused its browning. The browning and blackening of callus and other plant tissue is due to the secretion and subsequent oxidation of phenolic substances into the medium by the tissue[215]. The intensity of browning was less in media containing high concentration of auxins. This may be due to the ability of the auxins particularly that of 2,4-D to inhibit production of polyphenols[216]. The preliminary experiment indicated that the hormonal condition of the culture system has got a greater role in regulating the metabolism under *in vitro* conditions.

The kinetic parameters of growth of the callus cultures of the different plants indicated that growth rate is lowest in the callus cultures of *C. fenestratum* and it is highest in *L. nicotianifolia*.

In *L. nicotianifolia* of the different auxin-cytokinin ratio used for the callus induction, the combination caused the regeneration of shoots in the callus cultures, which was induced in the same medium. Therefore, plant regeneration from the callus cultures of this plant was studied in detail and that led to the standardization of a rapid method for the successful regeneration of *L. nicotianifolia* plantlets from its callus cultures. An appropriate combination of NAA and BAP was found to be a critical factor for rapid regeneration of shoot buds. Similarly the whole plant regeneration depends mainly on root initiation in the regenerated shoots. We have succeeded in initiating root formation in the shoots without the formation of callus. The plantlets were transferred to soil.

The increase in fresh weight may not be the actual biomass increase, as it contains varied quantity of water in different experiments. Therefore the estimation of total DNA and protein of the cells cultured for different periods is necessary. The total DNA content increased gradually and came to a steady state during the stationary phase of growth. This indicates that there is no new

cell formation during this phase. The variation in protein content of callus cultures indicate the metabolically active stages of the growth cycle. Protein content gradually increased from the exponential phase and became maximum during the logarithmic phase. It declined during the stationary phase, probably suggesting a retardation in the overall metabolic activity of cells.

Since plant cells are totipotent[217] all the necessary genetic and physiological potential for natural product formation should be present in an isolated cell. According to this theory, cultured cells obtained from any part of the plant might be expected to yield secondary compounds similar to those of plant grown *in vitro*.

Production of secondary metabolites by the callus cultures has been reported in a large number of plants. But there are cases of non-production of secondary metabolite in the callus cultures as exhibited in the callus cultures of *Mentha spicata* which failed to accumulate the essential oils[218]. In some cases, the rate of production of metabolite remains very low in callus or cell cultures as in the case of production of tropane alkaloids such as Scopolamine by cell culture[219]. This aspect has been reviewed in the introduction. In our study, chemical analysis of the callus cultures of six plants under investigation revealed that two of them were not able to accumulate

their active components under *in vitro* conditions. *Sida cordifolia* callus cultures did not produce ephedrine, the alkaloid present in the leaves and roots of the plant. Similarly the callus cultures of *Piper nigrum* did not accumulate piperine, the main alkaloid present in the pepper berries. The secondary metabolites of plant kingdom are characteristic of a group, genus or sometimes of a species. Some compounds are organ or tissue specific and are closely related to cell and tissue differentiation. The callus cultures of *Mentha spicata* failed to accumulate the essential oils *in vitro* eventhough the regenerated plantlets accumulated the compounds. The ability to synthesize secondary metabolites in the cultured cells is dependent on the degree of differentiation of these cells. A number of studies have indicated that highly differentiated cells have the greater ability for accumulation of secondary metabolites[220-224]. In *Piper nigrum*, piperine is reported to be present in highly differentiated tissue viz. pepper berries and was not detected in other parts of the plant. Therefore, piperine formation may be associated with the development and differentiation of pepper fruits. In the case of *Sida cordifolia* ephedrine is reported to be present in the roots and leaves but their callus cultures failed to accumulate ephedrine. These observations are against the totipotency principle of plant cell. The inability of these callus cultures to accumulate the

specific compound can be attributed to the metabolic blockage in the biosynthetic pathway. This blockage may be due to absence of one or more enzymes of the pathway. The expression of the genes of these enzymes are sometimes regulated by the outcome of tissue differentiation. But in the other four cases, callus cultures accumulated the respective desired compounds present in the intact plants. *Lobelia nicotianifolia* callus cultures accumulated lobeline, the main alkaloid present in the leaves and roots of the plant. Even then quantitatively the product was much lower (0.0073 percent) compared to that of the whole plant, which is around 1.8 percent of the dry weight of the tissue.

Eventhough no reports are available regarding the tissue culture, micropropagation and *in vitro* production of lobeline in *L. nicotianifolia*, production of lobelia alkaloids by callus culture and hairy root cultures of *L.inflata* are available[177,224]. The lobeline content 18-24 µg/g) of hairy root cultures were found to be equivalent to that of callus cultures as reported by Krajewska et al[224] and was also equivalent to that of the whole plant of *L. inflata*. Eventhough the callus cultures of *L. nicotianifolia* showed an increased lobeline content in our studies (73 µ g/g or 0.007 percent) compared to the above reports, it was still very low compared to that of the whole plant.

In *Rauwolfia tetraphylla*,the callus cultures accumulated the desired indole alkaloid reserpine. Here also the quantity of the products was much lower compared to that of the whole plant. Callus and cell suspension cultures of *R. serpentina* produced a number of indole alkaloids including reserpine and ajmaline[225-231]. *Catharanthus roseus* a related plant also produced a range of indole alkaloids which include the rauwolfia alkaloids such as serpentine and ajmalicine[88]. In addition to reserpine *R. tetraphylla* callus cultures also produced a number of unidentified alkaloids. They appeared as blue fluorescent spots on TLC when visualized under UV light.

The callus cultures of *Tinospora cordifolia* and *Coscinium fenestratum* produced berberine with some other unidentified fluorescent protoberberine alkaloids. The TLC and HPLC analysis clearly indicated that berberine is the main alkaloid produced by the callus cultures of both the plants. In *Coscinium fenestratum*, berberine was the prominant spot detected by TLC. Berberine was isolated and its structural confirmation was made by spectroscopic studies. The quantitative analysis of berberine by HPLC showed that berberine content of the callus cultures of *C. fenestratum* was much higher, coming upto seven percent of the dry weight of the callus tissue as against 3.5 percent in the whole plant. But berberine

content of callus cultures of *T. cordifolia* was very low when compared to its content in the whole plant and that of the callus cultures of *C. fenestratum*. Berberine production by the callus cultures of *Coptis japonica*[232] and *Thalictrum minus* has been reported earlier[114]. Furuya et al[20] induced the callus cultures of *Coptis japonica* and observed that the cells grew very slowly and berberine content was low. Yamada and Sato[112] could produce a fragile cell line of *Coptis japonica* from root derived callus cultures, which showed higher growth rate and higher berberine content (two to four percent on dry weight basis). The callus cultures of *C. fenestratum* also grew very slowly but contained higher percent of berberine. Compared to this, *Tinospora cordifolia* produced more biomass but contained very low percent of berberine.

Since the callus cultures of *C. fenestratum* and *T. cordifolia* accumulated the desired compound in higher quantities than that present in the intact plants and also the product content is much higher when compared to the cases of other plants studied, the callus cultures of *C. fenestratum* and *T. cordifolia* were selected for further detailed investigations so as to improve the rate of biomass formation and product synthesis.

CHAPTER IV

CHAPTER IV

BIOSYNTHESIS AND SECRETION OF BERBERINE BY CELL SUSPENSION CULTURES OF *TINOSPORA CORDIFOLIA* AND *COSCINIUM FENESTRATUM*

4.1 INTRODUCTION

Berberine is a bright lemon yellow isoquinoline alkaloid (Protoberberine type) (Fig.4.1) found in a large number of genera belonging to families such as Beriberidaceae Menispermaceae, Ranunculaceae, Papaveraceae, Rutaceae, Rubiaceae, Rhamnaceae, Magnoliaceae and Annonaceae.

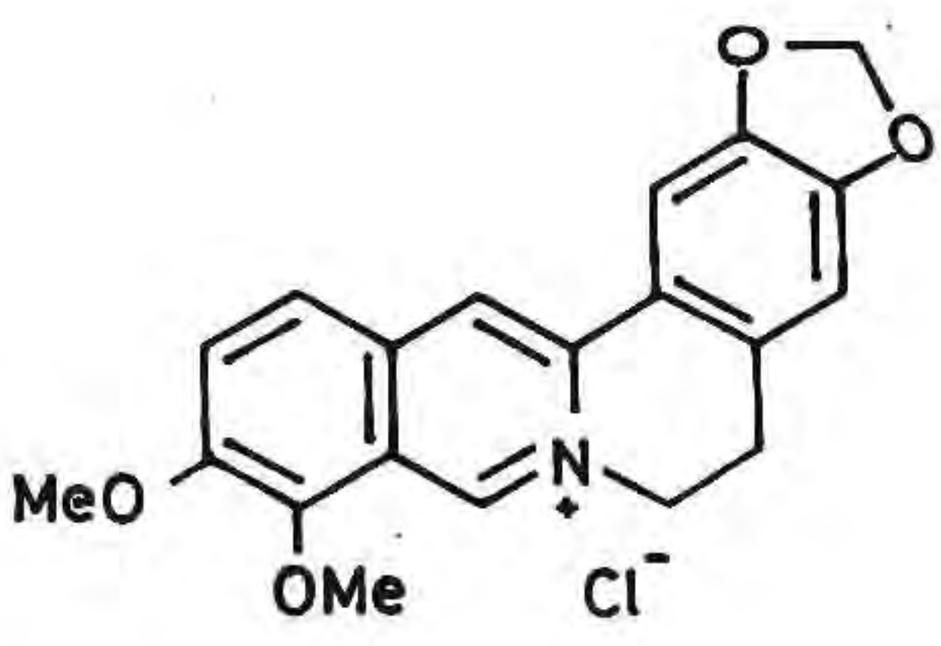


Fig. 4.1

Berberine has got various therapeutic and pharmacological properties. It was found toxic to various types of protozoans including *Leishmania tropica*. Hence it

is found useful in the treatment of malaria and amoebic dysentry. It also has antimicrobial activity against various types of pathogenic bacteria. It acts as a provocative agent in the diagnosis of latent malaria and as an intestinal antiseptic compound. It was shown that berberine sulphate is very effective in curing oriental sore and ulcers which is caused by *Leishmania tropica*[233-236]. This compound in combination with some other components or the extract of plants containing berberine is used against eye diseases like conjunctivitis and chronic opthalmia. Since berberine has got a permanent bright yellow colour it has also been used as a dye for the last hundred years or so in the tanning and colouring of leather and textile industry. Berberine is employed in India as a bitter tonic alternative astringent, stomachic, diapharetic, gentle aperient and as curative of piles[237].

In India, *Coscinium fenestratum* and berberis sp. are the major sources of berberine and among these the former is the richest source amounting to 3.5 percent. Bark and roots are the major raw material used for berberine extraction. In all cases particularly in *C. fenestratum* it takes considerably long period of about seven to ten years from the time of seedling to obtain the raw materials for the extraction of berberine. These plants are on the verge of extinction due to the over exploitation. Under

these circumstances it is very important and highly advantageous to employ the cell culture system as an alternate source for berberine production.

Experiments described in the previous chapter showed that the callus cultures of *Coscinium fenestratum* and *Tinospora cordifolia* were able to synthesize berberine as the major component. The quantity of berberine in these callus cultures was found to be higher when compared to the products obtained from the callus cultures of other plants studied. In addition to these, the berberine content in the callus cultures of *C. fenestratum* was found to be much more than in the intact plant. But callus cultures cannot be used for continuous production of berberine and the extraction of the product from the solid medium is less efficient. Hence a detailed study has been undertaken to optimise berberine production by cell suspension cultures of *T. cordifolia*.or *C. fenestratum*.

The cell suspension cultures are induced to generate rapidly large amounts of cell material with a homogenous cell population. This can be used to examine the growth and metabolism of secondary metabolites as well as for their continuous production.

This chapter presents the results of investigations on the induction and maintenance of cell suspension cultures

of both *C. fenestratum* and *T. cordifolia*. The kinetics of cell growth and berberine production by suspension cultures of both plants were calculated and compared to select the better cell culture system with respect to rate of berberine production. The cell culture system with higher berberine production rate was chosen for further experiments with a view to standardize the media and hormonal conditions which can produce maximum amount of berberine and cell biomass.

4.2. MATERIALS AND METHOD

4.2.1. Induction of cell suspension cultures

Callus tissues grown in MS basal media supplemented with 10 µM 2,4-D and 1 µM BAP were used as the source of inoculum for the induction of cell suspension cultures. Fragile fragments of callus tissues of both *C. fenestratum* and *T. cordifolia* were inoculated aseptically into MS liquid media containing 2,4-D and BAP as above and agitated continuously on a rotary shaker at 140±5 rpm at 25±2°C under continuous light or dark. After two weeks, the cells were filtered and transferred to fresh media. Subculturing of the cells were carried out on every 20 days by separating the cells by filtration and transferring the cells to fresh media. An inoculum of 350 mg fresh weight of cells were inoculated in each case. The cells in the culture were periodically examined for its cytological and morphological changes.

4.2.2. Kinetic comparison of growth and berberine production

Kinetics of cell growth was studied in the cell cultures of *T. cordifolia* and *C. fenestratum* in MS liquid medium containing 10 µM 2,4-D and 1 µM BAP by measuring the increase in fresh weight and dry weight every four days upto 32 days. The total berberine content of the cultures also was calculated by extracting and estimating the alkaloid content of the cells and medium of the suspension cultures. The values were plotted against time and using this growth curve and berberine production curve, specific growth rate (µ), doubling time (tg), product formation rate, yield, productivity and specific product formation rate were calculated as described in Chapter II.

4.2.3. Quantitative estimation of berberine

The cells were separated from the medium by filtration and both medium and cells were extracted with methanol. Berberine content was estimated separately and the total berberine content of the cultures was expressed as percentage of dry weight of the cell biomass.

4.2.4. Effect of different basal media on cell growth and berberine production

Stock cultures grown in MS medium were filtered aseptically and a known amount of cells were transferred to flasks containing 50 ml fresh medium containing either

of MS, B_5, E1 or LS basal media supplemented with plant growth hormones as given above. An initial inoculum of 300 to 350 mg fresh weight of cells per flask was used and the cultures were maintained under identical conditions. Cultures were harvested on every fourth day and fresh weight, dry weight and berberine content were determined as explained earlier upto 32 days of inoculation. The values were plotted against time and the kinetic parameters of cell growth and berberine production were calculated from the growth curve and product formation curve as described under general materials and methods.

4.3. RESULTS

4.3.1. Induction of cell suspension cultures

Cell suspension cultures of *T. cordifolia* and *C. fenestratum* were initiated from the fragile yellowish callus cultures capable of synthesizing and secreting berberine into the solid agar medium. The callus fragments inoculated into the liquid culture medium dispersed well and grown as a suspension on continuous agitation on a rotary shaker. The cells in the suspension culture were examined periodically under light microscope. By the second transfer, the large cell aggregates disappeared and the suspension consisted of both single cells and small groups or clumps consisting of 5 to 20 cells and also small number of large cell aggregates having numerous

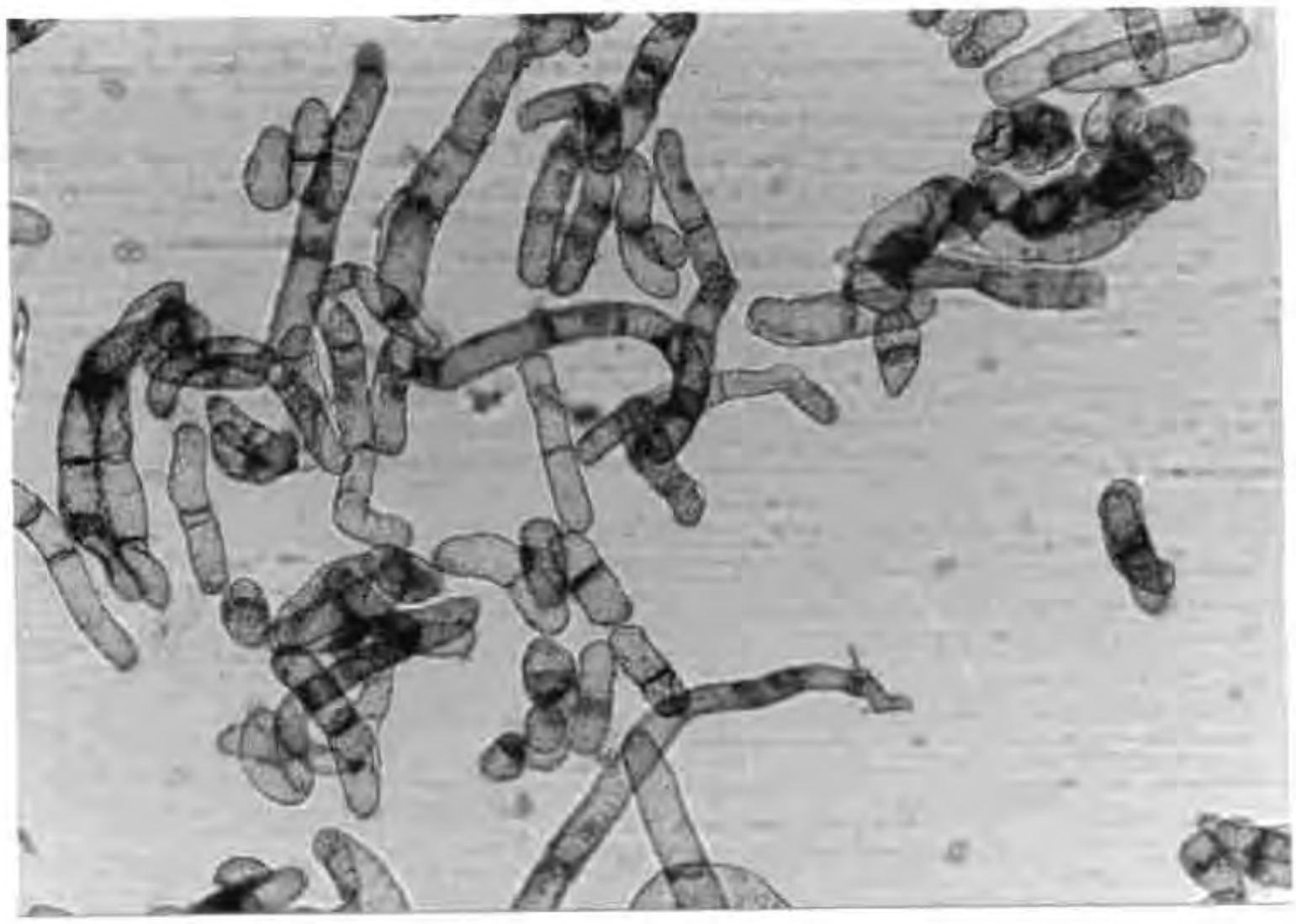
cells. There was not much difference observed in the degree of cell dispersion in the suspension culture of *T. cordifolia* and *C. fenestratum*. There was no cell differentiation such as the formation of liquefied cells or tracheids observed in both cell cultures but consisted of oval or elongated parenchymatous cells. Most of the cells and cell aggregates were observed to have vacuoles and unidentified granular materials of varying sizes particularly at the later stages of growth cycle in both cell suspension cultures (Figs.4.2 and 4.3).

The cell cultures of *T. cordifolia* and *C. fenestratum* synthesized and secreted the yellow alkaloid berberine into the medium. The liquid medium gradually turned yellow after four to five days of inoculation. The colour of the media in *C. fenestratum* cell cultures became deep yellow after 10 to 19 days and the colour was less in the media of *T. cordifolia* cell cultures.

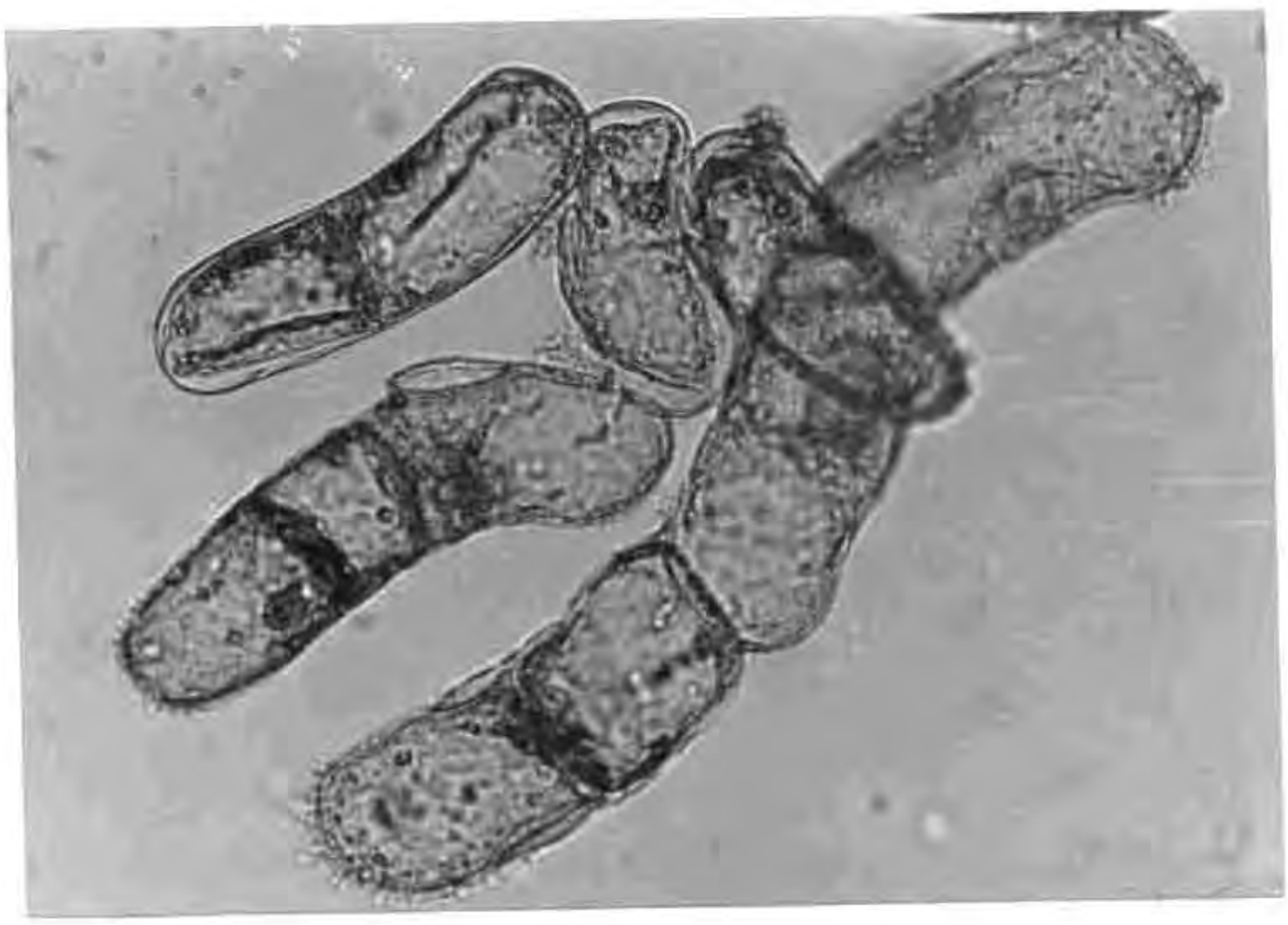
Berberine was isolated both from the media and cells separately. Its identity was confirmed by TLC, HPLC, melting point determination, UV, IR and ^{1}H NMR spectra as shown in the case of callus cultures (Chapter III). In the suspension cultures of *C. fenestratum*, the yellow alkaloid synthesized and secreted into the medium by the cells was found to consist of berberine alone by HPLC analysis (Fig.4.4). But in suspension cultures of *T. cordifolia* the media and cells contained an unidentified

Fig.4.2 : Cells in suspension cultures of *Coscinium fenestratum* at stationary phase of growth

A



B



A. Magnification 80 X

B. Magnification 320 X

Fig.4.3 : Cells in suspension cultures of *T. cordifolia*, at stationary phase

A

B

A. Magnification 80 X

B. Magnification 320 X

Fig.4.4 : HPLC elution pattern of berberine extracted from the medium of cell suspension culture of *C. fenestratum*

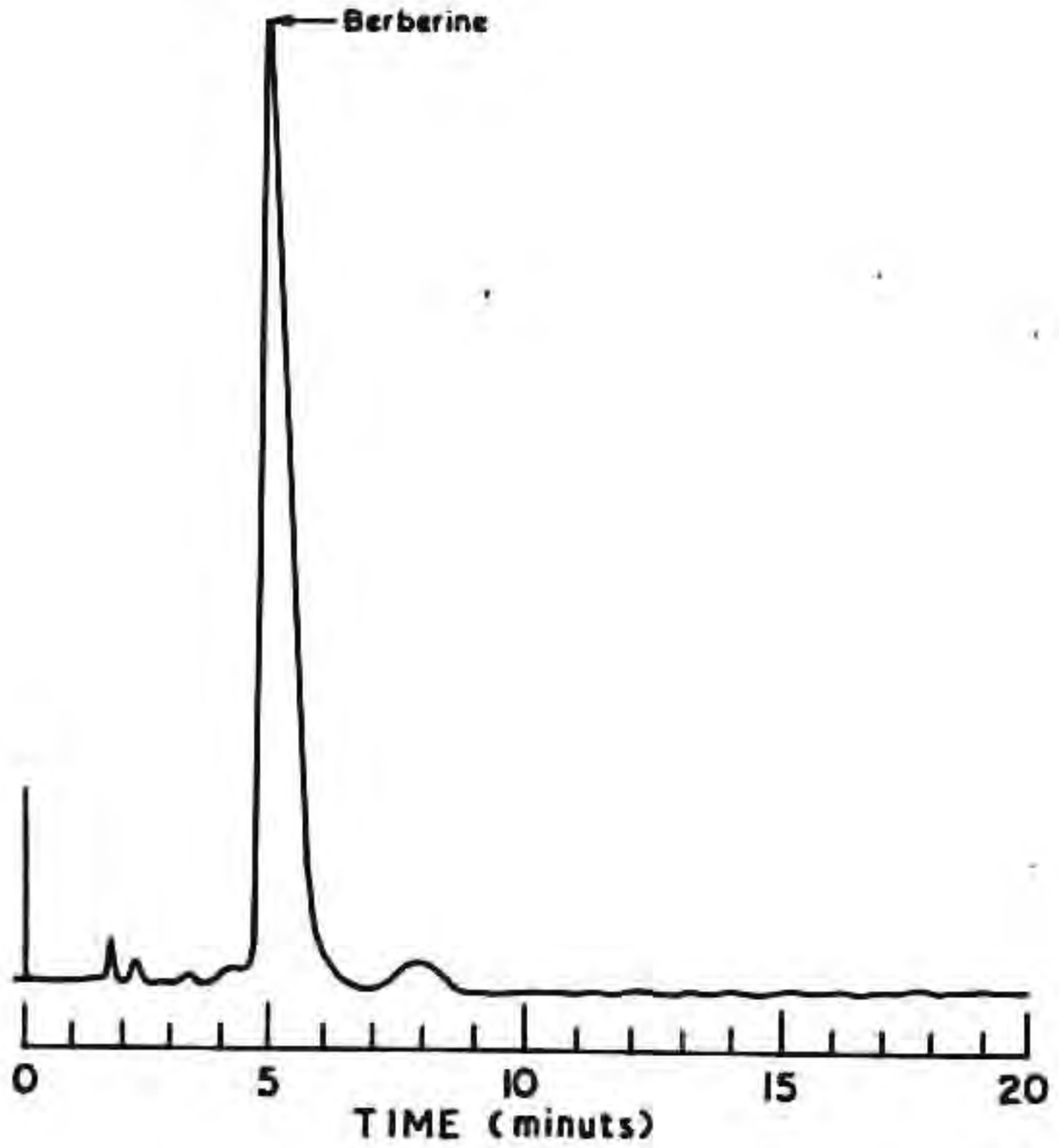
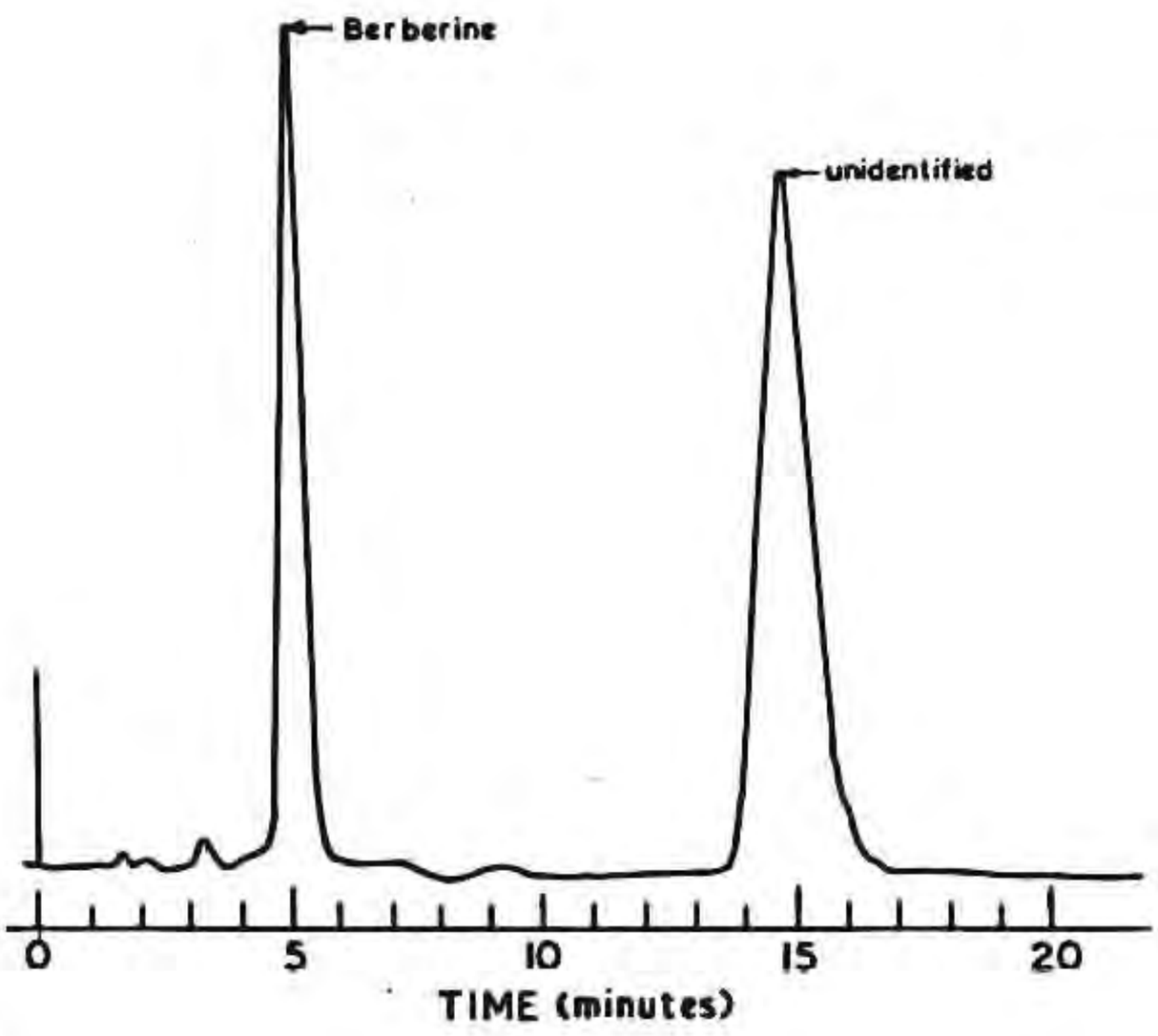


Fig.4.5 : HPLC elution pattern of berberine extract from the medium of cell suspension culture of *T. cordifolia*



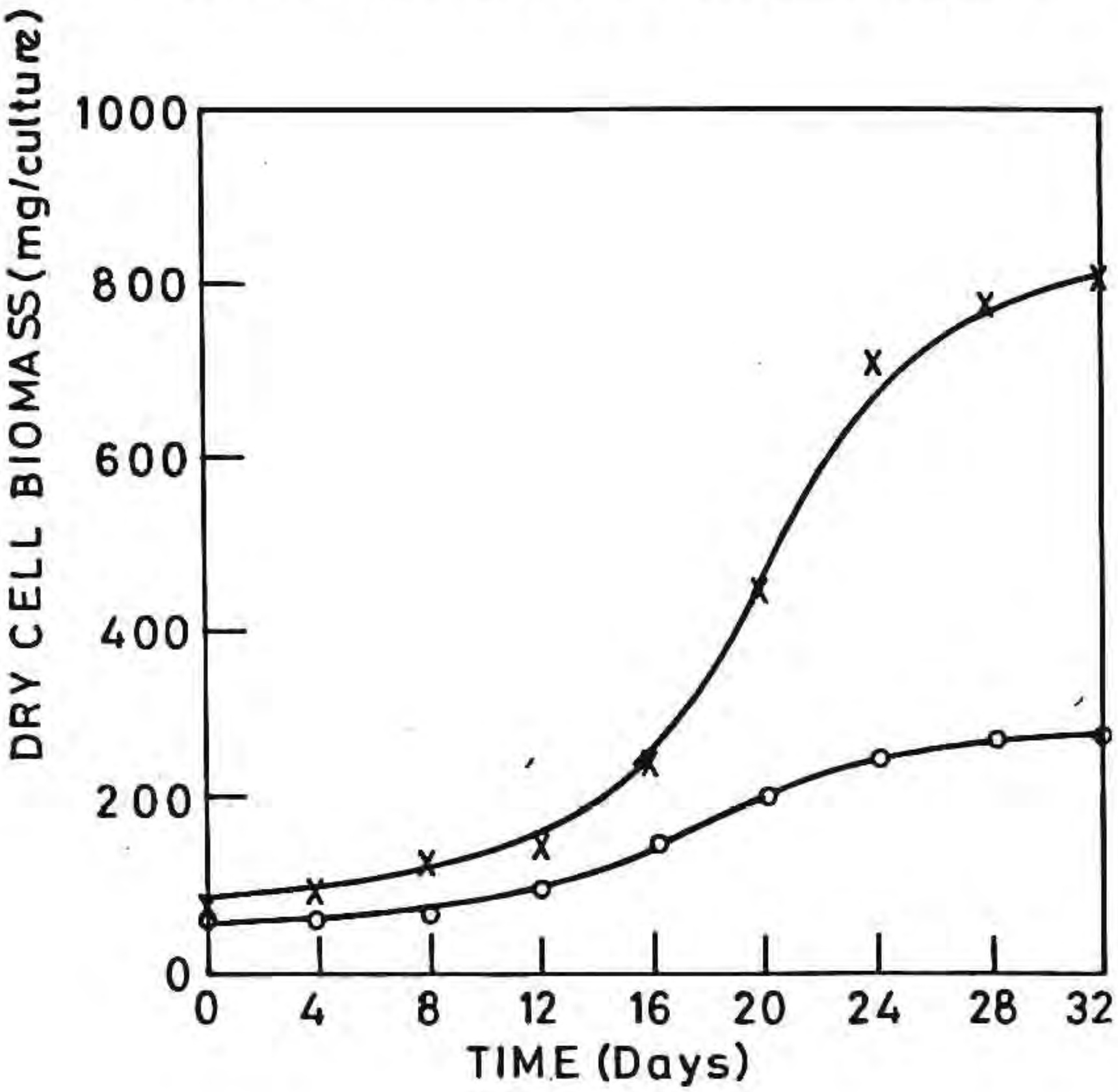
compound in addition to berberine (Fig.4.5) as observed in the previous chapter.

4.3.2. Kinetics of growth and berberine - production in *C. fenestratum* and *T. cordifolia*

The time course of cell growth and berberine production in both cell suspension cultures was studied and the results are shown in Figs.4.6 and 4.7 . The growth cycle is characterized by a relatively long lag phase upto 12 to 15 days and a short exponential growth phase of approximately 4 to 6 days. The logarithmic growth phase or the linear growth phase lasted for 8 to 12 days before the cells enter into the stationary phase. Thus in general, the pattern of cell growth was almost similar in *C.fenestratum* and *T. cordifolia* cell cultures but for the net increase in cell biomass at the stationary phase (Fig.4.6). During the growth cycle, the dry weight of the cells increased four to five fold in *C. fenestratum* (Table 4.1) and around eight fold in *T. cordifolia* cell cultures(Table 4.2) over the initial inoculum.

The pattern of berberine production in the cell cultures of both *C. fenestratum* and *T. cordifolia* is shown in Fig.4.7. Berberine production started within a few days after inoculation and increased almost in parallel with cell growth. The total amount of berberine obtained at the end of the culture period was more in *C. fenestratum*

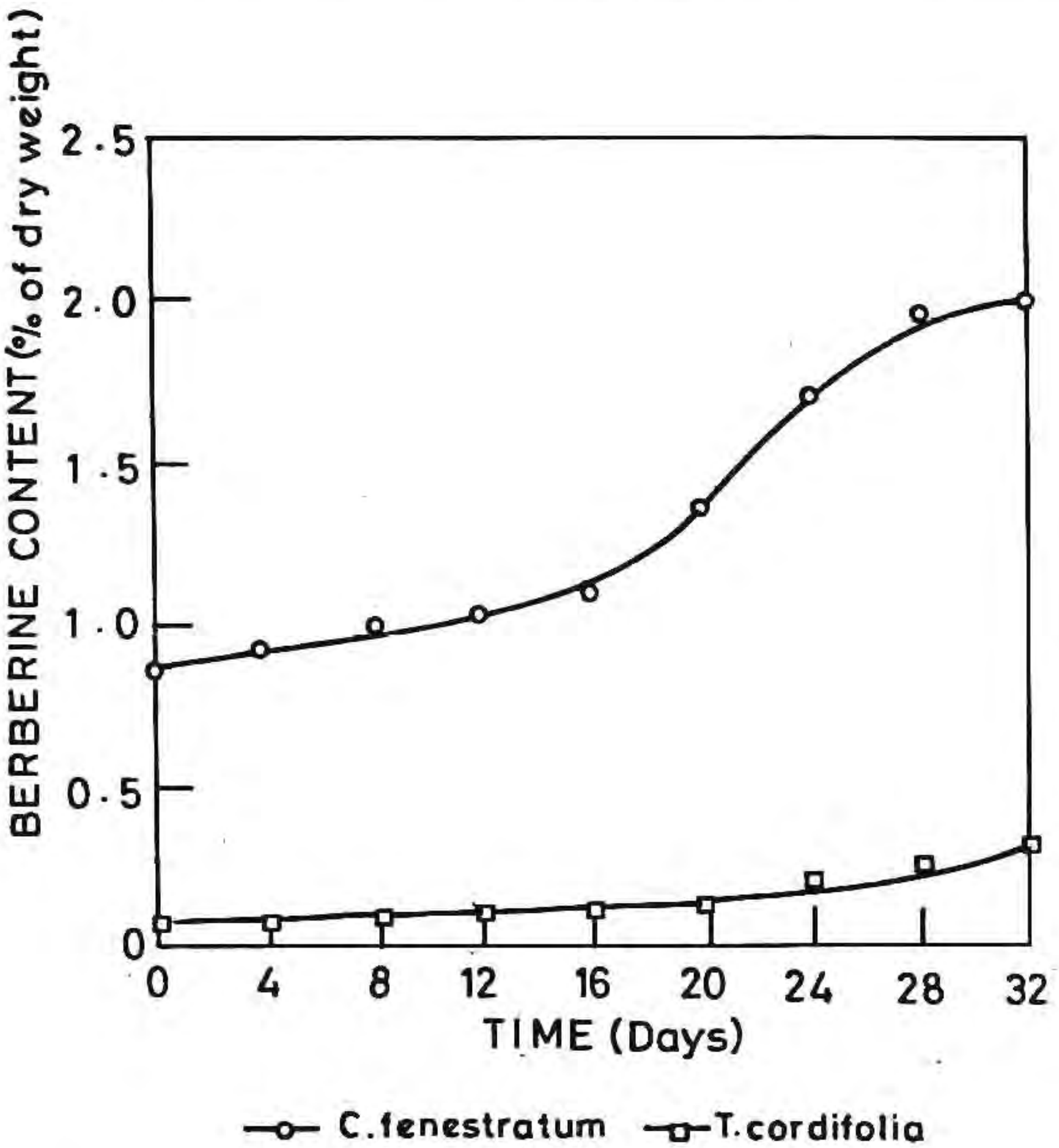
Fig.4.6 : Growth curve of cell suspension cultures of *Coscinium fenestratum* and *Tinospora cordifolia*



The cells were grown in MS basal medium supplemented with 10 µM 2,4-D and 1 µM BAP.

The values plotted are the mean of five independent determinations.

Fig.4.7 : Production of berberine in the cell suspension cultures of *Coscinium fenestratum* and *Tinospora cordifolia* at different stages of growth



Cells were grown in MS basal media supplemented with 10 µM 2,4-D and 1 µM BAP.

The values plotted are the mean of five independent determinations

Table 4.1 : Growth and berberine content in cell suspension cultures of *Coscinium fenestratum* (Medium: MS basal + 10 µM 2,4-D + 1 µM BAP)

Time (days)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
0	337.67(±3.66)	63.30(±0.55)	0.0000	0.540(±0.060)	0.86
4	323.11(±2.81)	62.58(±1.33)	0.118(±0.062)	0.563(±0.052)	0.90
8	330.06(±2.25)	67.21(±0.83)	0.189(±0.041)	0.651(±0.110)	0.97
12	458.33(±4.73)	93.80(±1.52)	0.310(±0.062)	0.937(±0.131)	1.02
16	730.99(±3.65)	143.59(±1.82)	0.515(±0.033)	1.550(±0.091)	1.08
20	1055.12(±3.72)	202.40(±3.11)	1.132(±0.036)	2.670(±0.136)	1.32
24	1334.27(±4.66)	250.81(±2.56)	1.812(±0.088)	4.189(±0.260)	1.67
28	1415.49(±5.75)	271.00(±3.26)	2.810(±0.160)	5.200(±0.121)	1.92
32	1362.29(±7.15)	275.66(±3.72)	2.680(±0.201)	5.403(±0.188)	1.96

Each value is the mean ±SEM of five independent determinations

Table 4.2 : Growth and berberine content in cell suspension cultures of *Tinospora cordifolia* (Medium : MS basal + 10 µM 2,4-D + 2 µM BAP)

Time (days)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
0	370.00(±6.30)	76.52(±2.30)	0.000	0.04(±0.01)	0.05
4	415.56(±2.24)	91.33(±1.18)	0.02(±0.00)	0.06(±0.01)	0.06
8	528.12(±1.63)	128.57(±1.73)	0.03(±0.01)	0.09(±0.02)	0.07
12	573.93(±2.70)	146.50(±2.55)	0.05(±0.01)	0.13(±0.02)	0.09
16	843.72(±5.26)	237.80(±1.92)	0.08(±0.01)	0.23(±0.01)	0.10
20	1628.00(±3.75)	443.25(±3.20)	0.16(±0.05)	0.51(±0.06)	0.11
24	2534.70(±5.20)	710.85(±3.62)	0.48(±0.12)	1.26(±0.15)	0.18
28	2912.67(±3.58)	774.25(±6.26)	0.76(±0.15)	1.68(±0.17)	0.22
32	2980.81(±7.11)	806.63(±4.55)	0.89(±0.13)	1.94(±0.14)	0.24

Each value is the mean ±SEM of five independent determinations

(Table 4.1) than in *T. cordifolia* (Table 4.2). At the stationary phase of growth about 50 percent of the total berberine was recovered from the medium. Berberine content in the medium never increased beyond this. Similarly there was no change in the berberine content in the medium in the stationary phase.

Kinetic parameters of growth and berberine production were calculated from the growth curve and product formation curve of both cell cultures. The results are given in Table 4.3. The cell suspension cultures of *T. cordifolia* showed higher specific growth rate with lower generation time (tg) compared to the cell cultures of *C. fenestratum*. But the specific product formation rate was higher in *C. fenestratum* compared to *T. cordifolia*. The other kinetic parameters such as productivity, yield and product formation rate were also higher in *C. fenestratum* compared to *T. cordifolia*. From all these results, it was clear that the cell culture of *C. fenestratum* was suitable for berberine production and hence it was selected for further optimization of media and hormonal conditions with a view to increase the berberine production and growth rate.

The cell cultures were also grown in light and dark and the biomass production and berberine formation was observed. There was no significant change between cultures grown in light or dark on biomass formation and berberine production. But a slight decrease in biomass was noted

Table 4.3 : Kinetic parameters of growth and berberine production in the cell cultures of *Coscinium fenestratum* and *Tinospora cordifolia*

Cell culture	cell growth			berberine production		
	Specific growth rate (μ /day)	Generation time (tg) (days)	Specific product formation rate (μg/mg dry cell biomass/ day)	Yield (μg/mg dry cell biomass)	Productivity (μg/day)	Product * formation rate (μg/day)
C. fenestratum	0.815	8.56	0.614	19.60	169	304
T. cordifolia	0.137	5.06	0.075	2.40	60	119

*Calculated from the product formation curve

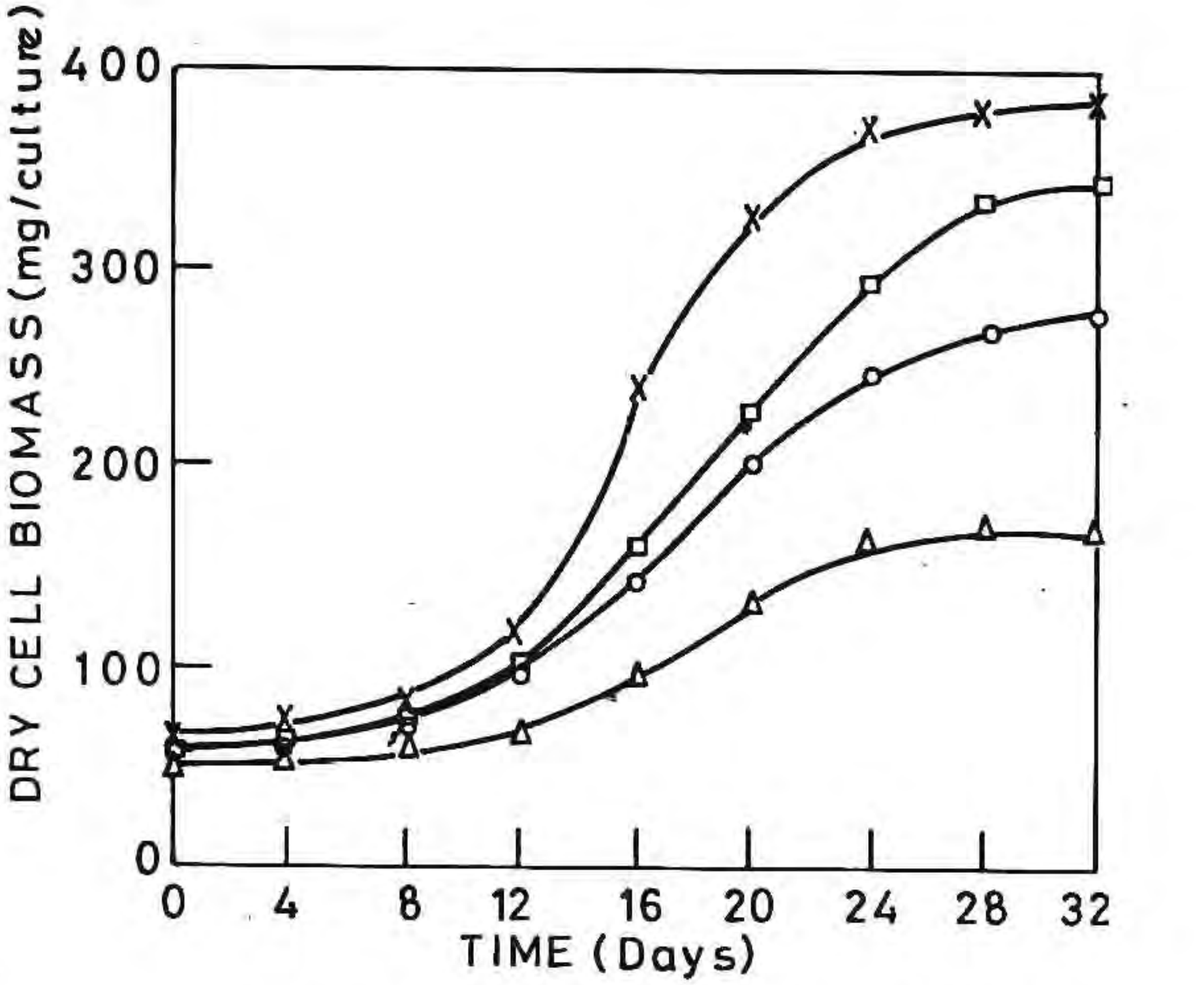
in the case of *C. fenestratum* when grown in continuous light.

4.3.3. Effect of basal media on growth and berberine production by cell cultures of *C. fenestratum*

Time dependent studies were conducted to compare the growth and berberine production by the cell suspension cultures of *C. fenestratum* in four commonly used basal media.

The results in Fig.4.8 show the growth pattern of the cells in the four liquid media - MS, B_5, LS and E1 which are found to be similar with some differences in the duration of logarithmic phase and lag phase. In all media except LS medium, the growth curves consist of a lag phase of eight to ten days and logarithmic phase of different durations with varying growth rate. The cells entered into the stationary phase after 28 or 32 days of inoculation in all media. In LS medium, the growth curve is characterized by 12 to 14 days of lag phase and approximately 8 days of logarithmic phase before coming to the stationary phase after 26th or 28th day of inoculation. The length of logarithmic phase in MS medium was almost equivalent to that of LS but the total biomass formation was higher in the former accounting to five fold of initial inoculum. In LS medium there was only about three fold increase. In B_5 and E1 medium the

Fig.4.8 : Growth of cells in suspension cultures of *Coscinium fenestratum* - Effect of different basal media



All the basal media were supplemented with 10 µM 2,4-D and 1 µM BAP.

The values plotted are the mean of five independent determinations

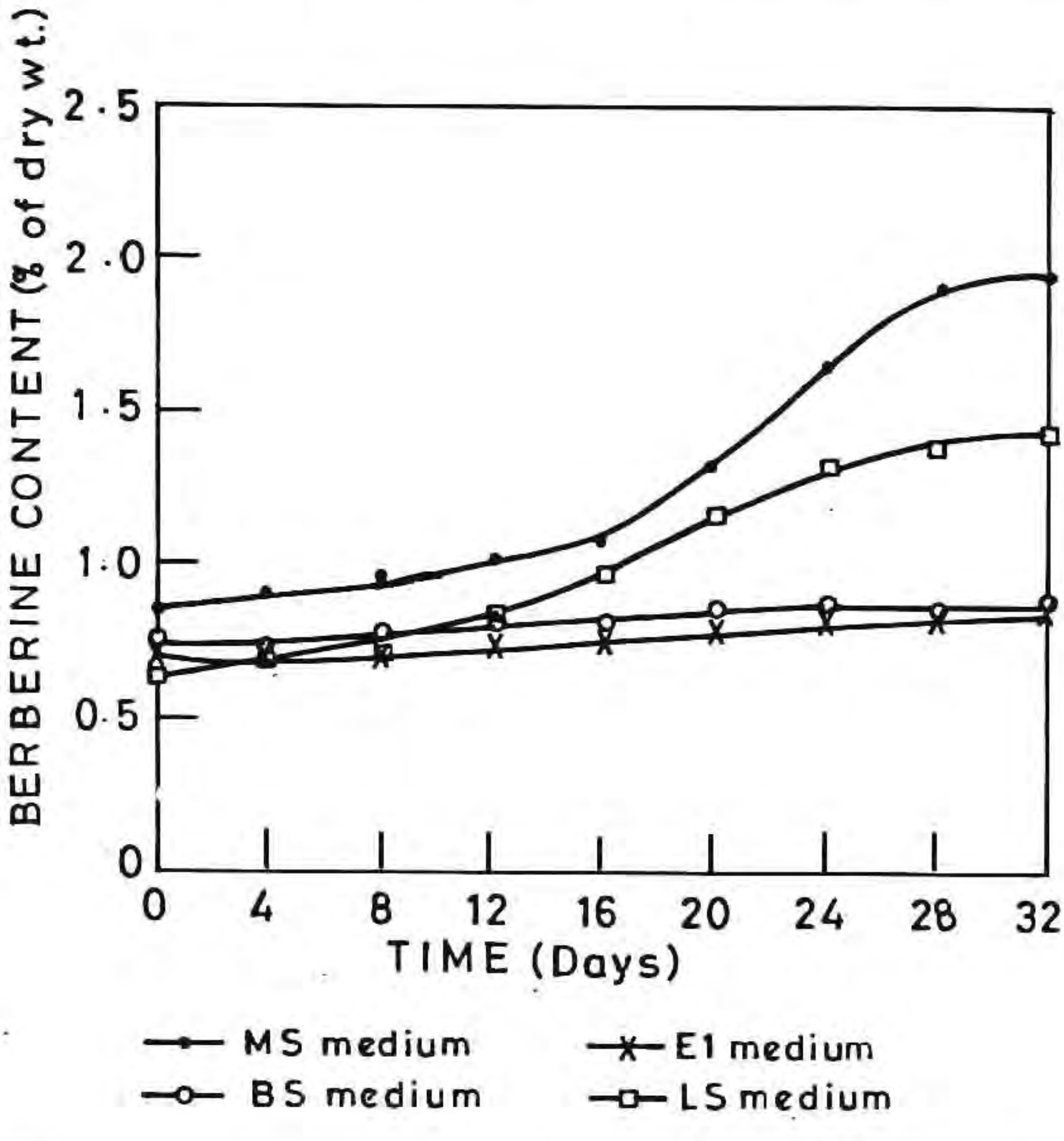
lag phase was comparatively shorter. Logarithmic phase in B_5 medium lasted for about 10 days with a six fold increase in biomass and it lasted for 12 to 14 days with almost same increase in biomass in E1 medium. Thus the growth pattern of cells in the suspension cultures grown in B_5 and E1 medium were almost similar under identical conditions.

The kinetic parameters of growth have been calculated from the growth curves for each media (Table 4.7). The results indicate that the specific growth rate was highest in B_5 medium with shortest doubling time and was lowest in LS medium. Thus among the four basal media studied for cell growth, B_5 is the most appropriate one for obtaining high growth rate and biomass formation.

Berberine produced by the cell cultures at different time intervals were determined and were expressed as percentage of dry weight of cell biomass. These values were plotted against time (Fig.4.9).

The pattern of product formation was almost identical (Tables 4.1,4.4 to 4.6). However the net quantity of berberine at the end of growth cycle or at the stationary phase was not so. Berberine production started after three to four days of inoculation and increased almost in parallel with cell growth. The percentage of berberine

Fig.4.9 : Berberine production in the cell suspension cultures of *Coscinium fenestratum* - Effect of different basal media



All basal media were supplemented with 10 µM 2,4-D and 1 µM BAP

The values plotted are the mean of five independent determinations

Table 4.4 : Growth and berberine content in cell suspension cultures of *Coscinium fenestratum* (Medium: E1 basal + 10 µM 2,4-D + 1 µM BAP)

Time (days)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
0	297.35(±.6.92)	58.87(±2.67)	0.000	0.38(±0.01)	0.71
4	266.95(±7.11)	51.92(±1.56)	0.11(±0.06)	0.36(±0.11)	0.69
8	364.07(±5.52)	69.34(±1.34)	0.17(±0.05)	0.50(±0.12)	0.72
12	403.05(±4.24)	97.18(±2.55)	0.26(±0.01)	0.79(±0.09)	0.74
16	953.49(±8.50)	159.25(±2.38)	0.46(±0.01)	1.20(±0.21)	0.75
20	1146.77(±6.22)	227.39(±3.18)	0.76(±0.07)	1.80(±0.34)	0.79
24	1442.63(±5.43)	292.81(±1.88)	0.99(±0.12)	2.40(±2.83)	0.82
28	1746.62(±5.11)	335.51(±2.56)	1.38(±0.15)	2.83(±0.13)	0.84
32	1899.82(±7.60)	342.76(±3.92)	1.33(±0.31)	2.97(±0.36)	0.87

Each value is the mean ±SEM of five independent determinations

Table 4.5 : Growth and berberine content in cell suspension cultures of *Coscinium fenestratum* (Medium: B_5 basal + 10 µM 2,4-D + 1 µM BAP)

Time (days)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
0	373.38(±2.38)	62.00(±3.63)	0.000	0.53(±0.08)	0.76
4	365.89(±1.81)	73.16(±1.22)	0.19(±0.03)	0.51(±0.06)	0.70
8	392.54(±3.83)	79.25(±1.58)	0.26(±0.07)	0.61(±0.03)	0.77
12	548.82(±2.93)	116.86(±3.73)	0.34(±0.11)	0.95(±0.11)	0.81
16	1261.23(±5.11)	241.56(±2.88)	0.91(±0.10)	2.01(±0.33)	0.83
20	1471.45(±6.28)	327.79(±5.23)	1.21(±0.1)	2.86(±0.26)	0.87
24	1856.21(±5.83)	371.50(±3.44)	1.39(±0.15)	3.29(±0.29)	0.89
28	1773.41(±4.77)	380.00(±4.21)	1.63(±0.06)	3.31(±0.33)	0.87
32	1912.36(±7.22)	384.35(±5.73)	1.73(±0.14)	3.41(±0.24)	0.89

Each value is the mean ±SEM of five independent determinations

Table 4.6 : Growth and berberine content in cell suspension cultures of *Coscinium fenestratum* (Medium: LS basal + 10 µM 2,4-D + 1 µM BAP)

Time (days)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
0	242.37(±3.70)	48.50(±0.68)	0.00	0.30(±0.03)	0.61
4	254.91(±2.16)	42.00(±1.33)	0.15(±0.08)	0.35(±0.02)	0.67
8	308.30(±2.93)	59.80(±0.93)	0.25(±0.08)	0.43(±0.03)	0.71
12	323.14(±4.55)	63.53(±0.85)	0.28(±0.07)	0.53(±0.03)	0.83
16	514.36(±1.99)	96.58(±1.55)	0.37(±0.09)	0.95(±0.05)	0.98
20	694.19(±3.65)	130.80(±1.32)	0.61(±0.05)	2.15(±0.09)	1.33
24	832.65(±3.65)	162.00(±0.98)	1.27(±0.09)	2.15(±0.21)	1.30
28	856.46(±4.51)	171.80(±1.83)	1.25(±0.01)	2.39(±0.28)	1.39
32	813.20(±3.14)	166.24(±1.77)	1.36(±0.13)	2.39(±0.18)	1.44

Each value is the mean ±SEM of five independent determinations

content was higher in the cell suspension cultures grown in MS liquid medium (2 percent) and lower in B_5 medium (0.89 percent). There was no change in the total quantity of berberine during the stationary phase in any of the culture media studied. The production of berberine was highest during the logarithmic phase of cell growth and the product formation rate declined during the stationary phase in all cell suspension cultures.

The kinetic parameters of berberine synthesis is given in Table 4.7. The specific product formation rate of berberine is highest in MS medium and lowest in B_5 medium. There was no significant difference in yield of berberine in B_5 and E1 medium. Same is the case with productivity which represents product formation per time. Yield and productivity were low in B_5 when compared with MS and LS media and were highest in MS medium coming to 196 µg/mg dry weight. The product formation rate at the steady state expressed as mg of berberine/day also was highest in M S. medium and was least in E1 medium.

Thus cells of *C. fenestratum* cultured in MS basal liquid medium showed highest rate of berberine production and that grown in B_5 medium showed highest rate of cell growth.

Table 4.7 : Kinetic parameters of cell growth and berberine production in different basal media by *Coscinium fenestratum* cultures

Medium	Specific growth rate (μ/day)	Generation time (tg) (days)	Specific product formation rate (μg/mg dry wt./day)	Yield (μg/mg dry weight)	Productivity (μg/day)	Product formation rate $(\frac{dp}{dt})$ (μg/day)*
MS medium	0.081	8.56	0.614	196.00	169	304
B_5 medium	0.096	7.22	0.277	8.85	106	168
E1 medium	0.090	7.70	0.271	8.68	93	141
LS medium	0.077	9.00	0.450	14.00	75	135

* Calculated from the product formation curve during logarithmic phase

4.4. DISCUSSION

This part of the thesis mainly discusses:

(1) The induction of cell suspension cultures in *T. cordifolia* and *C. fenestratum*, isolation of berberine from the media and cells of the suspension cultures.

(2) Kinetic comparison of growth and berberine production between the cell cultures of *C. fenestratum* and *T. cordifolia* to select the cell culture system having comparatively high potential for berberine synthesis.

(3) Effect of different basal media on cell growth and berberine production by the cell cultures of *C. fenestratum* which has been found to be the suitable cell culture system with higher berberine production.

The induction of cell suspension cultures was successfully achieved using the fragile callus stock cultures in both *C. fenestratum* and *T. cordifolia*. By the second transfer itself a fine suspension culture consisting of single cells, small groups and small number of large cell aggregates were obtained in both cases. The cells in suspension cultures also showed the ability to synthesize and secrete berberine into the media.

Eventhough cell suspension cultures are initiated easily in both *T. cordifolia* and *C. fenestratum* the behaviour of the cells under identical nutritional and

hormonal conditions were not similar with respect to the product formation and cell growth. The cell culture system of *T. cordifolia* which has given higher biomass is not efficient in product synthesis. Cell cultures of *C. fenestratum* are capable of synthesizing and secreting high quantities of berberine eventhough the cell biomass formation is much less in MS medium supplemented with 10 µM 2,4-D and 1 µM BAP. The growth curves and product formation curves of both cell culture systems indicate that the sequence of cell growth and product formation are identical eventhough the net result is quantitatively different. It reveals that the synthesis of berberine occurs parallel to the cell growth and follows the same sequence in both cultures. Berberine synthesis was maximum during the logarithmic phases of cell growth and the rate of production declines during the stationary phase. This may not mean that berberine synthesis is a growth dependent process, as the cell culture system with higher growth rate and biomass formation is poor in product synthesis eventhough the biosynthetic pathway operates along with growth cycle. The process of berberine synthesis may be somehow linked to the primary metabolism or metabolic events which operates the cell cycle and cell multiplication. There are a number of reports where the accumulation of secondary metabolites occurs during the stationary

phase of growth cycle. In such cases the biosynthesis of the compound is independent of cell growth but operates only when the cells enter into the stationary phase. The biosynthetic pathway of the compound might be blocked and becomes operational only during the stationary phase. This behaviour is common for many of the plant cell cultures[238-240]. This helps the system to make optimum use of the available substrate for growth and major house keeping activities and when the cells enter into stationary phase these substrates are spared for secondary metabolite production.

Production of berberine by the callus and cell cultures of *Coptis japonica*, different species of Thalictrum such as *T. minus*, *T. rugosum* and *T. deptrocarpum* and berberis sps. has been reported[81,113-115,241-243]. Among these the cell cultures of *C. japonica* and *T. minus* are capable of producing high quantity of berberine compared to the whole plant. Berberine is secreted into the medium in large quantities and the excess berberine crystallized in the medium by a high berberine producing cell line of *T. minus*[115] in LS liquid medium containing 100 µM NAA and 10 µM BAP. In the case of *C. japonica*, eventhough the cell cultures contained high amount of berberine only a small amount is secreted into the medium[113,115].

The kinetic parameters of growth such as specific growth rate (μ) and generation time (tg) and kinetic parameters of product formation such as specific product formation rate, yield, productivity and product formation rate were calculated for the cell cultures of *Tinospora cordifolia* and *C. fenestratum*. These parameters were very much helpful in identifying the cell culture system which can synthesize more product in terms of biomass and time. The plant cell suspension and callus cultures very much resemble a microbial population in culture which goes through a growth cycle with a lag phase, logarithmic phase and attaining a stationary phase. It again starts when the population at the stationary phase is diluted by transfering to a fresh medium. Hence the kinetics of growth and product formation can be applied to the plant cell culture systems.

Kinetic analysis of biomass production and berberine formation by cell suspension cultures of *T. cordifolia* and *C. fenestratum* indicated that the former produced more biomass with less product formation while the latter produced less biomass and significantly higher amounts of berberine.

Based on the kinetic data of product formation, *C. fenestratum* cell cultures have been selected for further detailed studies and media optimization for increasing

the berberine production and rate of growth. Among the different basal media tried, B_5 medium was the one which gave highest growth rate and biomass production and was least in MS and LS media. The activity of E1 medium with respect to growth and alkaloid production was similar to B_5 and that of LS was almost identical with MS. Here also, it was observed that the production and secretion of berberine was lower in those media which has given higher biomass.

The cell growth and alkaloid production were found to be different in different basal media used. The exact reason for this difference is not clear. The formulation of one medium differs from the other in the composition of macro and micronutrients and even organic supplements like myo-inositol, vitamins and sugar. A major difference between B_5 and MS medium is that in B_5 medium, concentration of NO_3^- is higher than NH_4^+ ions whereas in MS, NH_4^+ ions are in higher concentration than B_5. Further there is a significant difference in the pH of the media particularly between MS and B_5 medium. E1 medium is a modified form of B_5 and similarly LS is a modified form of MS medium.

In *T. cordifolia* and *C. fenestratum* cell cultures, berberine was secreted into the medium from the time of lag phase and exponential growth phase itself. But there

was not much increase in the berberine content of the medium as the rate of production was retarded towards the stationary phase of growth cycle. Therefore these results can be attributed to the passive secretion of berberine into the medium because of pH gradient or concentration gradient. The pH of the medium was on the acidic side in the beginning (5.5 to 5.6) and it goes to the basic side during the later stages of the culture period. Since berberine is a base, this may be one of the driving forces behind its secretion, at least in the beginning of the culture period. Another observation for supporting the possibility of the passive secretion of berberine is that its content in the medium in almost all cultures both in *C. fenestratum* and *T. cordifolia* was found to be approximately 50 percent (50±5) of the total berberine content of the cultures at the stationary phase. Thus berberine secretion by the cells may be linked to the biosynthesis and accumulation of berberine in the cells. These results indicated that secretion of berberine into the culture medium by the cells was operated passively towards the concentration gradient. Berberine secretion stops at the stationary phase when the system reaches an equilibrium with respect to the concentration of berberine in the medium and cell. There was no increase or decrease in the concentration of berberine in the medium which probably indicates the absence of an energy dependent

mechanism for berberine secretion against the concentration gradient and also indicates that there is no degradation of the product within the cells or in the medium. Reabsorption of berberine by the cells similar to that reported earlier[85,184] in the cell cultures of *Thalictrum flavum* and *Coptis japonica* cannot be ruled out because there may be a cyclic process of reabsorption and secretion simultaneously without any change in the net quantity at equilibrium. Thus it can be concluded that the driving forces behind the secretion of berberine into the culture medium by the cell suspension cultures of *C. fenestratum* and *T. cordifolia* could be pH gradient (electrostatic gradient) and concentration gradient of berberine across the cell and medium of the suspension cultures unlike the cell cultures of *Thalictrum minus* where it is energy dependent[86]. However the possibility of secretory processes such as ATP/energy dependent secretion, secretion through reverse pinocytosis could not be completely excluded.

There are a number of reports where the primary metabolites such as citric acid[244] and proteins[245-247] were released from the cultured plant cells into the medium Most of the plant secondary metabolites are reported to be accumulated within the cultured cells particularly in vacuoles or specialized cells. However there are a

few reports regarding the release of secondary metabolites from the cultured cells. Franke and Bohom[120] reported the release of protopine by the cell cultures of *Macleaya microcarpa*. Tsukada and Tabata[248] reported the release of shikonin derivatives in *Lithospermum erythrorhyzon* Release of monoterpenoids in *Thuja occidentalis*[249] and capsaicin in *Capsicum fruitescens*[250] were also reported. In all these cases, the mechanism behind this phenomenon is not elucidated or understood. But it was demonstrated in the cell cultures of *Thalictrum minus* that berberine was secreted into the medium through an energy requiring process[85]. But this cannot be generalized in all berberine producing or secreting cell cultures as there are different secretory mechanisms for different cell culture systems[251]. The secretory activity of berberine in different cell cultures systems were found to be distinctly different from each other. Cell cultures of *Thalictrum depterocarpum* released berberine into the medium only during a period of exponential growth phase. This activity was significantly different from *Thalictrum minus* cell cultures[252]. The cell cultures of *Thalictrum minus* secrete most of the berberine produced into the medium during the whole period of cell growth[115,116]. Excess berberine secreted into the medium crystallized out. In this case, the secretory activity appears to be energy dependent

since it occurs against the concentration gradient. But it was noted in this species that berberine is not released into the medium by every culture strain of *T. minus*. A low alkaloid producing cell line formed crystals of berberine only within the vacuole and only very small amount of berberine was secreted out. The same result was observed in the cell cultures of *Coptis japonica*[241], *Berberis sp.*[81] and *Thalictrum rugosum* In contrast to the *Thalictrum minus*, Coptis cells in culture accumulate most of their berberine alkaloids in the vacuole releasing only a small part of the alkaloid into the medium probably during the autolysis of senescent cells[115]. In berberis cell cultures, the alkaloids consisting mainly of jeteorhizine were usually retained in the cells but were released into the medium when partial autolysis occurred. In *Thalictrum rugosum* usually the cells release very little berberine into the medium but it was shown that berberine was released into the medium when the cells were grown in a fresh medium deficient in phosphate[243]. Similarly when the cultures were grown in dark, the extracellular berberine content increased[193] whereas illuminated culture produced less than five percent of the total berberine as extracellular berberine. Thus light and also ions such as calcium and phosphate are found to be important in regulating the permeability of the cell membrane for the release of

secondary metabolites. According to Drapeau et al[253] the transport of alkaloid into the medium might occur by an active or passive mechanism driven by light sensitive pH difference between the vacuoles and the medium. In the case of *Tinospora cordifolia* and *C. fenestratum* cell cultures, the secretory mechanism of berberine appeared to be passive and dependent on the intracellular berberine content and also the difference in the pH between the medium and intracellular sites. The effect of light on cell growth and berberine production, particularly in the secretion of berberine was not considerable. Even then the presence of light increased the brown colour of the cell cultures as may be due to the light induced secretion of polyphenols and polyphenol oxidase activity as described by Berlin et al[254] in Rose cell suspension cultures.

From these studies it was concluded that the cell cultures of *T. cordifolia* and *C. fenestratum* are capable of synthesizing and secreting berberine into the media. The total berberine content of the cell suspension cultures were found to be less when compared to the corresponding callus cultures. This may be due to the effect of cell dispersion and loss of cell to cell contact in suspension cultures compared to callus cultures. The secretion of berberine into the medium occurred in both cell cultures.

This is advantageous in the industrial application of the cell cultures for continuous or semicontinuous production of berberine and brings down the cost of production of the compound. The cell cultures of *C. fenestratum* was selected as it was shown to be more efficient in berberine synthesis and its secretion, for further detailed studies and optimization of conditions. Among the four basal media, B_5 was selected for obtaining high cell biomass and MS media was selected for berberine synthesis.

CHAPTER V

CHAPTER V

SECTION A

INFLUENCE OF GROWTH REGULATORS ON BERBERINE PRODUCTION BY CELL SUSPENSION CULTURES OF *COSCINIUM FENESTRATUM*

5A.1. INTRODUCTION

Plant growth substances play an important role in cell differentiation, growth and development of higher plants. These substances, referred to as plant growth hormones, are divided into several classes, each of which may be represented by one or more structural analogues, which functions essentially like parent compound. Plant hormones which include auxins, characterized by an indole ring; Gibberellins, first isolated from a fungus - *Gibberella fujikuroi;* Cytokinins, closely related to kinetine, a nucleic acid degradation product, first described in fish sperms; abscissic acid; brassins; several classes of short and long chain alcohols, and ethylene. Plant growth hormones are present in almost all plant parts. Their site of action in the plant system is remote to their site of synthesis. Each of these hormones is characterized by a multiplicity of observed effects on plant growth, cell division and differentiation. Numerous studies have revealed an interaction between plant hormones with regard to the effect of a given concentration of one hormone with regard to the effect of equilibrium concentration of other hormones in the same tissue; that is the addition

of a given concentration of exogenous hormone to one tissue may elicit a completely different response than the equivalent amount of the same hormone added to a different tissue; which possess a distinctly different balance of other plant hormones[214]. A slight change in the hormone balance of the living system is likely to have a profound influence on the resultant product, be it a whole plant or a single secondary metabolite of interest.

Hormones are of paramount importance in the *in vitro* culture and growth of plant cells, and are effective triggers of secondary metabolism[255]. Both quality and quantity of auxins initially present in media or administered during the course of culture development have been shown to have a marked effect on primary[256,257] and secondary metabolism[258]. With the exception of habituated and oncogenic cultures, i.e. auxins and cytokinins to media for consistent growth by cell division. On the other hand, growth by differentiation or morphogenesis can usually be induced by lowering the auxin concentration or by supplying less active plant growth hormones. Since the production of secondary metabolites in plant cell cultures is a function of both cell multiplication and differentiation, growth regulators have a potential role in determining the productivity.

There are numerous examples in literature about the types of growth regulator effects on secondary metabolism *in vitro*[259,260]. The effect of auxin quality on secondary product synthesis has been investigated in cultures of numerous species. Indole-3-acetic acid (IAA) was found to be better than α-Naphthaleneacetic acid (NAA) or 2,4-Dichlorophenoxyacetic acid(2,4-D)for stimulating the production of thebaine by the suspension cultures of *Papaver bracteatum*[261,263]. NAA stimulated the synthesis of anthraquinones whereas 2,4-D totally suppressed its production in suspension cultures of *M. citrifolia*. A wide range of concentrations of these auxins were also tried and confirmed that the difference in the biosynthetic ability was due to the auxin type and its activity[217,262].

Effects of different auxins on the production of other alkaloids such as nicotine are well known. When *Nicotiana tabacum* cv Bright Yellow was cultured in the presence of 2,4-D for five years, no alkaloids were detected, whereas alkaloids such as nicotine, anatabine and anabasine were readily found in callus growing in media supplemented with IAA[263]. By transferring callus from 2,4-D to IAA supplemented media and vice versa it was demonstrated that nicotine biosynthesis was activated by IAA and suppressed by 2,4-D. The same effect of 2,4-D was also reported in the production of shikonin derivatives by the cell

cultures of *Lithespermum erythrorhyzon*[266]. But Buchner and Staba[265] reported that media without any auxin produced more amount of cardenolides by Digitalis Cultures. Similar effects were also reported in alkaloid production by callus cultures of Peganum[266]. and *Catharanthus roseus*[267]. In all these cases omission of auxin resulted in culture differentiation and morphogenesis.

Thus it is evident that the appropriate plant growth substances are required to be present in optimum amounts to promote cell growth and production of secondary metabolites. An understanding of the role of these plant growth substances on the biosynthesis of berberine by cells in culture is necessary to establish suitable conditions for optimum utilization of the substrates for the biosynthesis of berberine. Experiments to this effect have been carried out to find out an optimum concentration and combination of growth regulators - mainly auxins and cytokinin for increased cell growth and berberine biosynthesis.

Different types of synthetic auxins viz. 2,4-Dichlorophenoxy acetic acid (2,4-D),α-Naphthaleneacetic acid (NAA), Indole-3-acetic acid(IAA), Indole-3-butyric acid (IBA) and 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) and cytokinins-Benzylaminopurine (BAP) and kinetin (6-furfurylamino-

purine) were tried at different concentrations and combinations in these experiments.

5A.2. MATERIALS AND METHOD

The cell suspension cultures were grown and maintained in Gamborg's B_5 basal medium containing 10 µM 2,4-D and 1 µM BAP. The stock suspension cultures were continuously agitated on an orbital shaker at a speed of 140±5 rpm at 25±2°C in the dark. The cells were subcultured at two week intervals till the experiment was over. To study the effect of plant growth regulators on berberine production and growth, about 350 mg of cells were collected by filtration from the stock cultures and inoculated into 50 ml B_5 medium containing various concentrations of different auxins (2,4-D, NAA, IAA, IBA and 2,4,5-T) and cytokinins (BAP or kinetin) in Erlenmeyer flasks and cultured for 28 days under the culture conditions as described above. On 28th day the cultures were harvested, cells were separated and biomass determined by recording the dry weight and fresh weight. Berberine content of the spent media and cells was determined separately and total berberine content was calculated.

5A.2.1. Quantitative analysis of berberine

The amount of berberine in the medium and cultured cells was determined as described in Chapter II.

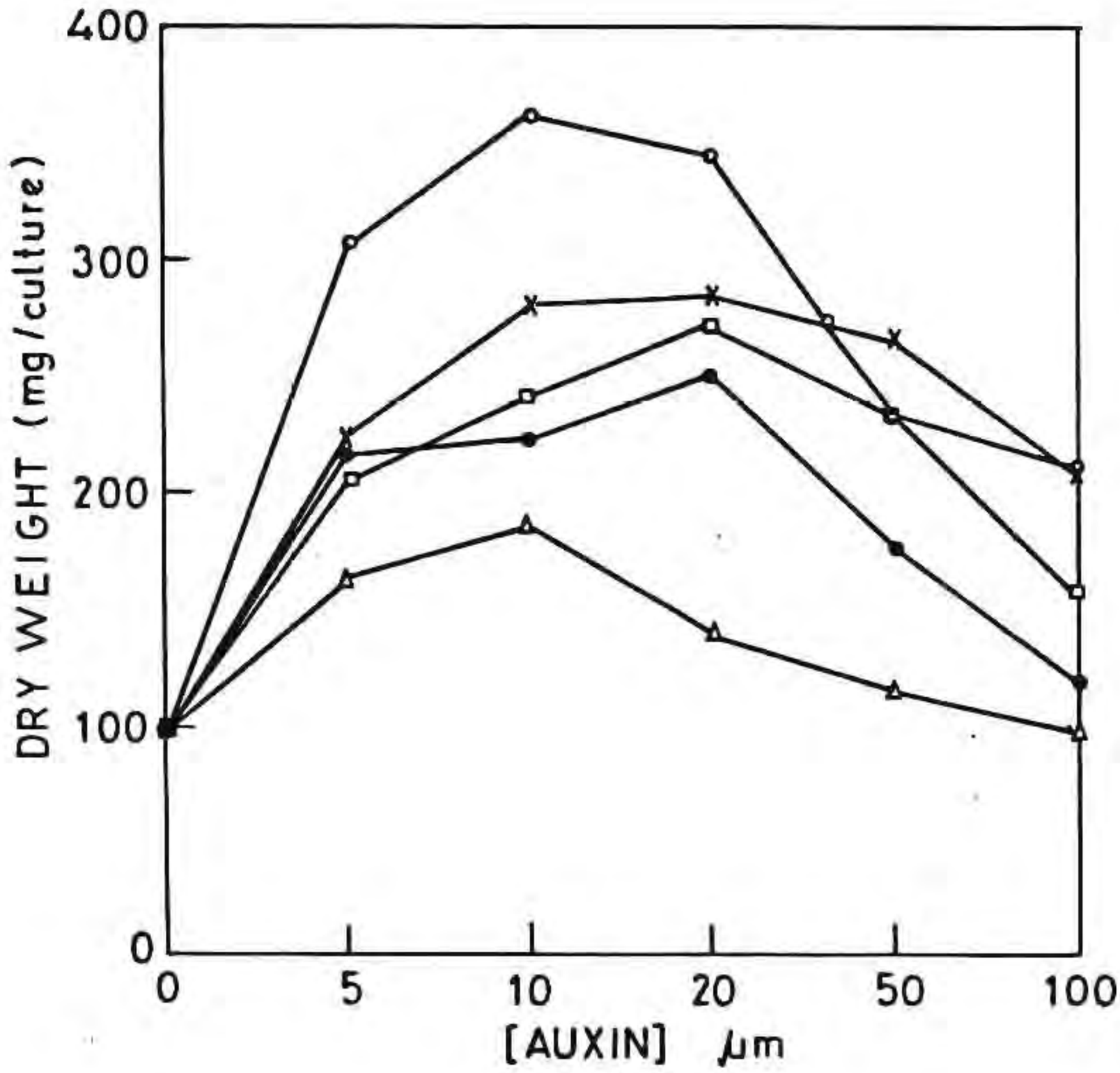
5A.3. RESULTS

5A.3.1. Effect of auxins on growth and berberine production

Fig.5A.1 shows the effect of the five different types of auxins - 2,4-D, NAA, IAA, IBA and 2,4,5-T on biomass formation. Among the different auxins tried 2,4-D was found to be suitable and maximum biomass was obtained at a concentration of 10 µM followed by IBA, IAA and NAA. Biomass formation was least in the case of 2,4,5-T. For all the types of auxins, the maximum biomass formation occurred at concentrations between 5 µM and 50 µM. On further increase in the concentrations, the biomass content declined irrespective of the nature of the auxin.

The effect of auxins on berberine production in the cell cultures is given in Fig.5A.2. Of the various types of auxins, NAA gave the maximum berberine production when compared to the other auxins. Comparatively large amount of berberine was produced at concentrations of 10, 20 and 50 µM. Highest product formation occurred at 20 µM concentration both for NAA and 2,4-D and was significantly higher in the case of NAA. The total berberine content increased with increase in the concentration and attained maximum at 20 µM and then decreased gradually. Auxins such as IAA, IBA and 2,4,5-T were comparatively

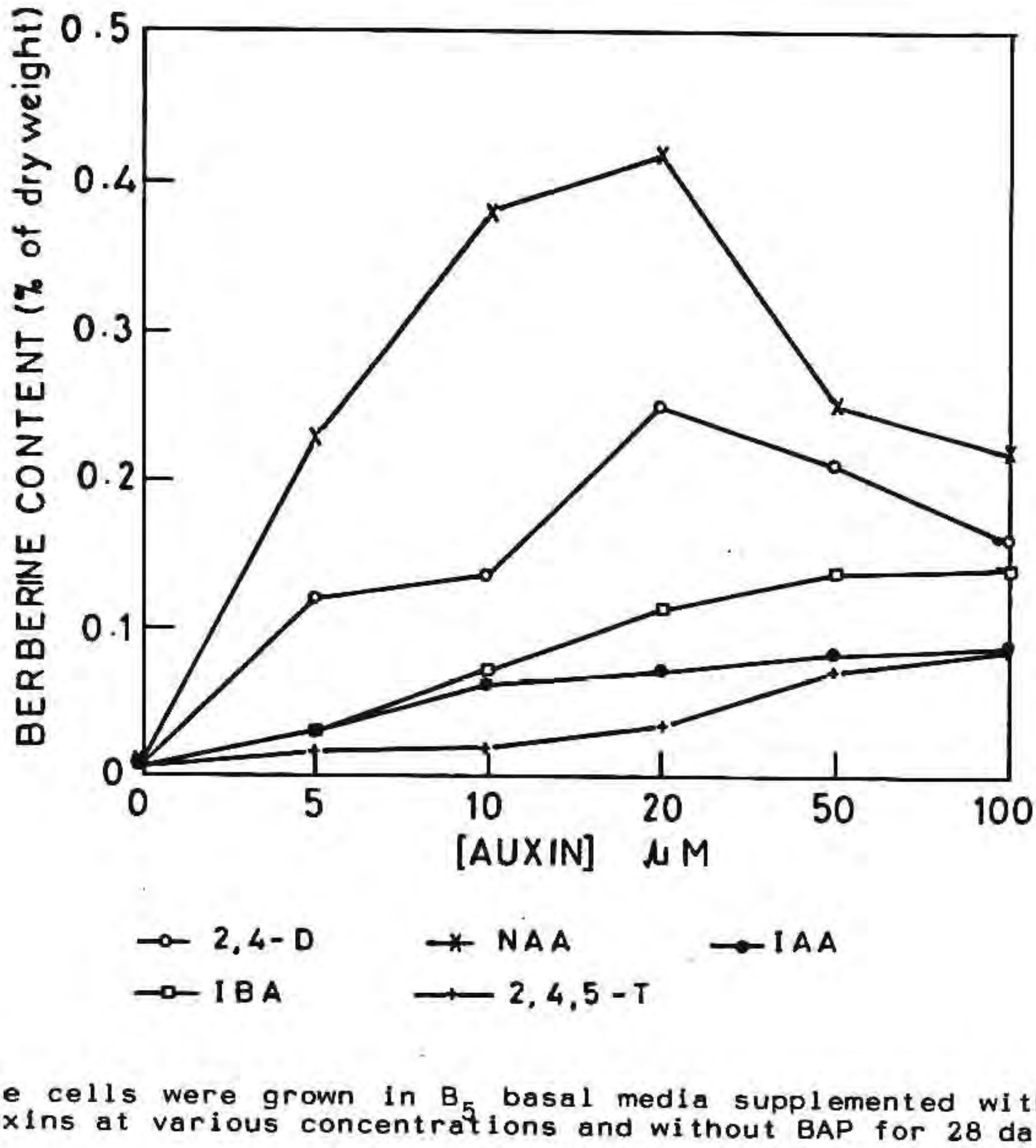
Fig.5A.1 : Effect of different auxins on growth of cell suspension cultures of *Coscinium fenestratum*



The cells were grown in B_5 basal medium supplemented with different concentrations of auxins and without cytokinin for 28 days

The values plotted are the mean of five independent determinations

Fig.5A.2 : Effect of different auxins on berberine production in the cell suspension cultures of *Coscinium fenestratum*



The cells were grown in B_5 basal media supplemented with auxins at various concentrations and without BAP for 28 days

The values plotted are the mean of five independent determinations

less effective. In these cases, berberine content of the cultures increased with increase in the concentration of hormones eventhough the quantity was not comparable to that of NAA. The auxin 2,4,5-T was not effective both for biomass formation and berberine production. The values are also presented in the Table 5A.1 to 5A.5 for each type of auxins. In all the experiments approximately 50 percent of the total berberine was recovered from the medium of the cell cultures.

5A.3.2. Effect of auxins on growth and berberine production in combination with Cytokinin (BAP)

The above experiments were repeated with the supplementation of 1 µM BAP along with the auxins. The results are presented in the Tables 5A.6 to 5A.10. It was observed that supplementation of BAP along with auxins enhanced the berberine biosynthesis. This BAP induced enhancement was observed in all the different concentrations of auxins tried. Figs.5A.3 to 5A.7 gives a comparative picture of berberine content and biomass formation with BAP (1 µM) supplementation. Berberine biosynthesis was maximum when 1 µM BAP and 20 µM NAA was added to the medium. But the biomass formation was reduced by the addition of BAP along with all the concentrations of auxins studied when compared to that without BAP. The media with 2,4-D, supplementation of 1 µM BAP enhanced both biomass and

Table 5A.1 : Effect of different concentrations of 2,4-D on cell biomass and berberine production in cell suspension cultures of *Coscinium fenestratum* (Medium: B_5 basal + 2,4-D)

Sl. No.	Concentration (µM)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	0	461.95(±1.88)	93.85(±1.13)	0.005(±0.001)	0.008(±0.001)	0.009
2	5	982.41(±8.40)	306.58(±3.25)	0.135(±0.021)	0.367(±0.051)	0.120
3	10	1215.00(±4.56)	362.34(±5.90)	0.215(±0.027)	0.400(±0.035)	0.135
4	20	1312.70(±7.53)	344.75(±3.82)	0.448(±0.031)	0.865(±0.071)	0.251
5	50	837.20(±5.90)	233.67(±3.55)	0.236(±0.025)	0.489(±0.044)	0.210
6	100	813.77(±6.29)	212.60 (±3.85)	0.146(±0.033)	0.339(±0.050)	0.160

Initial biomass : Fresh weight : 357.66(±2.18) mg
Dry weight : 69.32(±0.92) mg

Data collected on 28th day of inoculation
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Sl.No.1. In all cases $p < 0.05$

't' values of Table 5A.1

't' values between	't' values of group A	't' values of group B
1 and 2	78.203	8.902
3 and 4	3.165	7.430
4 and 5	26.943	5.693
5 and 6	5.089	2.848

Table 5A.2 : Effect of different concentrations of NAA on cell biomass and berberine production in cell suspension cultures of *Coscinium fenestratum* (Medium: B_5 basal + NAA)

Sl. No.	Concentration (μM)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	0	461.95(±1.88)	93.85(±1.13)	0.005(±0.001)	0.008(±0.001)	0.009
2	5	834.72(±6.80)	217.82(±3.55)	0.212(±0.026)	0.500(±0.049)	0.230
3	10	875.65(±4.38)	223.41(±5.13)	0.413(±0.038)	0.851(±0.035)	0.380
4	20	912.25(±7.11)	250.85(±3.37)	0.483(±0.041)	1.054(±0.067)	0.420
5	50	723.70(±4.95)	176.69(±5.25)	0.260(±0.063)	0.513(±0.046)	0.250
6	100	466.82(±5.93)	118.73(±4.23)	0.117(±0.021)	0.268(±0.040)	0.220

Initial biomass : Fresh weight : 357.66(±2.18) mg
Dry weight : 69.32(±0.92) mg

Data collected on 28th day of inoculation
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Sl.No.1. In all cases $p < 0.05$.

't' values of Table 5A.2

't' values between	't' values to group A	't' values to group B
1 and 2	42.091	12.698
3 and 4	5.654	3.396
4 and 5	15.036	8.420
5 and 6	10.874	5.083

Table 5A.3 : Effect of different concentrations of IAA on cell biomass and berberine production in cell suspension cultures of *Coscinium fenestratum* (Medium: B_5 basal + IAA)

Sl. No.	Concentration (µM)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	0	461.95(±1.88)	93.85(±1.13)	0.005(±0.001)	0.008(±0.010)	0.009
2	5	765.52(±8.13)	206.23(±0.42)	0.026(±0.011)	0.066(±0.013)	0.032
3	10	883.14(±3.77)	240.35(±2.93)	0.063(±0.014)	0.154(±0.012)	0.064
4	20	882.59(±7.11)	270.81(±3.79)	0.091(±0.018)	0.193(±0.017)	0.072
5	50	705.28(±5.33)	232.50(±3.53)	0.093(±0.013)	0.198(±0.015)	0.085
6	100	582.59(±3.99)	154.65(±4.11)	0.057(±0.020)	0.132(±0.011)	0.086

Initial biomass : Fresh weight : 375.66(±2.18) mg
Dry weight : 69.32(±0.92) mg

Data collected on 28th day of inoculation
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Sl.No.1. In all cases $p < 0.05$.

't' values of Table 5A.3

't' values between	't' values of group A	't' values of group B
1 and 2	117.983	5.626
1 and 3	56.598	3.342
5 and 6	18.175	4.488

Table 5A.4 : Effect of different concentrations of IBA on cell biomass and berberine production in cell suspension cultures of *Coscinium fenestratum* (Medium: B_5 basal + IBA)

Sl. No.	Concentration (µM)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	0	461.95(±1.88)	93.85(±1.13)	0.005(±0.001)	0.008(±0.001)	0.009
2	5	720.78(±5.31)	225.44(±3.96)	0.024(±0.080)	0.067(±0.010)	0.030
3	10	887.55(±3.82)	279.83(±4.45)	0.084(±0.010)	0.195(±0.031)	0.070
4	20	896.32(±5.19)	286.38(±3.56)	0.148(±0.013)	0.320(±0.018)	0.112
5	50	925.50(±6.95)	265.49(±7.11)	0.172(±0.117)	0.368(±0.023)	0.139
6	100	624.77(±6.25)	208.36(±4.95)	0.169(±0.012)	0.290(±0.021)	0.142

Initial biomass : Fresh weight : 357.66(±2.18) mg
Dry weight : 69.37(±0.92) mg

Data collected on 28th day of inoculation
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Sl.No.1. In all cases $p < 0.05$.

't' values of Table 5A.4

't' values between	't' values of group A	't' values of group B
1 and 2	40.419	7.425
1 and 4	65.202	21.891
1 and 6	28.527	16.966

Table 5A.5 : Effect of different concentrations of 2,4,5-T on cell biomass and berberine production in cell suspension cultures of *Coscinium fenestratum* (Medium: B_5 basal + 2,4,5-T)

Sl. No.	Concentration (μM)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	0	461.95(±1.88)	93.85(±1.13)	0.005(±0.001)	0.008(±0.001)	0.009
2	5	637.62(±6.18)	163.20(±2.58)	0.011(±0.003)	0.026(±0.007)	0.016
3	10	628.00(±5.29)	185.34(±4.19)	0.014(±0.004)	0.033(±0.010)	0.018
4	20	531.78(±6.50)	137.92(±4.53)	0.021(±0.007)	0.046(±0.013)	0.034
5	50	471.59(±3.28)	113.78(±5.83)	0.039(±0.010)	0.081(±0.011)	0.072
6	100	403.93(±5.88)	96.33(±4.25)*	0.042(±0.011)	0.087(±0.009)	0.090

Intial biomass : Fresh weight : 357.66(±2.18) mg
Dry weight : 69.32(±0.92) mg

Data collected on 28th day of inoculation
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Sl.No.1. In all cases $p < 0.05$. *$p > 0.05$

't' values of Table 5A.5

't' values between	't' values of group A	't' values of group B
1 and 2	31.144	3.219
1 and 4	11.939	3.686
1 and 6	0.713*	11.035

* not significant at 5% level

Table 5A.6 : Effect of different concentrations of 2,4-D with 1 µM BAP on cell biomass and berberine production in cell suspension cultures of *Coscinium fenestratum* (Medium: B_5 basal + 2,4-D + 1 µM BAP)

Sl. No.	Concentration (µM)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	0	362.73(±2.16)	72.87(±0.90)	0.009(±0.001)	0.019(±0.004)	0.026
2	5	1273.52(±3.99)	368.50(±6.22)	0.310(±0.071)	0.650(±0.081)	0.180
3	10	1384.43(±7.28)	396.81(±8.55)	0.483(±0.088)	1.026(±0.100)	0.260
4	20	1512.00(±7.93)	406.67(±6.29)	0.610(±0.093)	1.258(±0.132)	0.310
5	50	1120.75(±8.48)	312.93(±5.77)	0.421(±0.018)	0.925(±0.001)	0.290
6	100	842.60(±6.33)	234.70(±6.21)	0.211(±0.016)	0.468(±0.083)	0.200

Initial biomass : Fresh weight : 316.92(±1.72) mg
Dry weight : 67.88(±0.81) mg

Data collected on 28th day of inoculation
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Sl.No.1. In all cases $p < 0.05$.

't' values of Table 5A.6

't' values between	't' values of group A	't' values of group B
1 and 2	59.387	9.841
3 and 4	1.175*	1.772*
5 and 6	11.673	6.964

* not significant at 5% level

Table 5A.7 : Effect of different concentrations of NAA with 1 µM BAP on cell biomass and berberine production in cell suspension cultures of *Coscinium fenestratum* (Medium: B_5 basal + NAA + 1 µM BAP)

Sl. No.	Concentration (M)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	0	342.73(±2.16)	72.87(±0.98)	0.009(±0.001)	0.019(±0.004)	0.031
2	5	867.80(±5.93)	225.79(±3.52)	0.278(±0.016)	0.630(±0.021)	0.280
3	10	918.55(±3.91)	236.48(±2.83)	0.585(±0.034)	1.210(±0.037)	0.510
4	20	966.25(±4.72)	260.75(±3.41)	0.881(±0.051)	1.682(±0.110)	0.650
5	50	920.00(±5.21)	146.67(±3.11)	0.389(±0.037)	0.810(±0.072)	0.360
6	100	634.38(±4.98)	130.85(±4.49)	0.173(±0.018)	0.394(±0.013)	0.300

Initial biomass : Fresh weight : 316.92(±8.73) mg
Dry weight : 67.88(±0.81) mg

Data collected on 28th day of inoculation
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Sl.No.1. In all cases $p < 0.05$.

't' values of Table 5A.7

't' values between	't' values of group A	't' values of group B
1 and 2	52.938	36.192
4 and 5	31.266	8.389
5 and 6	3.663	7.192

Table 5A.8 : Effect of different concentrations of IAA with 1 µM BAP on cell biomass and berberine production in cell suspension cultures of *Coscinium fenestratum* (Medium: B_5 basal + IAA + 1 µM BAP)

Sl. No.	Concentration (µM)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	0	362.73(±2.60)	72.87(±0.98)	0.009(±0.001)	0.019(±0.004)	0.026
2	5	651.72(±4.66)	178.26(±3.71)	0.041(±0.010)	0.093(±0.011)	0.051
3	10	784.60(±7.34)	214.52(±3.83)	0.690(±0.110)	0.158(±0.013)	0.072
4	20	835.38(±7.55)	236.60(±4.11)	0.992(±0.130)	0.217(±0.011)	0.092
5	50	1120.85(±9.25)	286.73(±3.66)	0.138(±0.073)	0.310(±0.087)	0.110
6	100	890.00(±6.82)	241.50(±4.25)	0.167(±0.071)	0.313(±0.090)	0.130

Initial biomass : Fresh weight : 316.92(±1.73) mg
Dry weight : 87.68(±0.81) mg

Data collected on 28th day of inoculation
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Sl.No.1. In all cases $p < 0.05$.

't' values of Table 5A..8

't' values between	't' values of group A	't' values of group B
1 and 2	34.740	7.997
4 and 5	11.521	1.341*
5 and 6	10.200	0.030 *

* not significant at 5% level

Table 5A.9 : Effect of different concentrations of IBA with 1 µM BAP on cell biomass and berberine production in cell suspension cultures of *Coscinium fenestratum* (Medium: B_5 basal + IBA + 1 µM BAP)

Sl. No.	Concentration (µM)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	0	362.23(±2.16)	72.87(±0.98)	0.009(±0.001)	0.019(±0.004)	0.026
2	5	1026.30(±7.28)	270.88(±3.52)	0.049(±0.010)	0.109(±0.033)	0.040
3	10	1169.67(±5.89)	316.82(±5.29)	0.137(±0.012)	0.316(±0.019)	0.097
4	20	1322.80(±7.18)	352.30(±4.22)	0.203(±0.011)	0.422(±0.021)	0.120
5	50	1166.39(±6.77)	324.81(±2.91)	0.218(±0.024)	0.486(±0.040)	0.151
6	100	773.28(±8.33)	212.36(±4.18)	0.163(±0.017)	0.360(±0.015)	0.174

Initial biomass : Fresh weight : 316.92(±1.73) mg
Dry weight : 67.88(±0.81) mg

Data collected on 28th day of inoculation
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Sl.No.1. In all cases $p < 0.05$.

't' values of Table 5A.9

't' values between	't' values of group A	't' values of group B
1 and 2	68.547	3.4246
4 and 5	6.783	1.7919*
5 and 6	27.927	3.730

* not significant at 5% level

Table 5A.10 : Effect of different concentrations of 2,4,5-T with 1 µM BAP on cell biomass and berberine production in cell suspension cultures of *Coscinium fenestratum* (Medium: B_5 basal + 2,4,5-T + 1 µM BAP)

Sl. No.	Concentration (µM)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	0	362.72(±2.60)	72.87(±0.98)	0.009(±0.001)	0.019(±0.004)	0.026
2	5	665.30(±8.12)	192.14(±3.56)	0.021(±0.008)	0.048(±0.013)	0.021
3	10	782.28(±5.77)	208.75(±3.79)	0.033(±0.005)	0.079(±0.015)	0.036
4	20	813.93(±2.81)	214.64(±7.32)	0.058(±0.009)	0.110(±0.016)	0.052
5	50	766.85(±7.11)	166.77(±5.66)	0.072(±0.004)	0.154(±0.011)	0.083
6	100	438.73(±5.33)	112.39(±4.83)	0.071(±0.010)	0.135(±0.018)	0.121

Initial biomass : Fresh weight : 316.92(±1.73) mg
Dry weight : 67.88(±0.81) mg

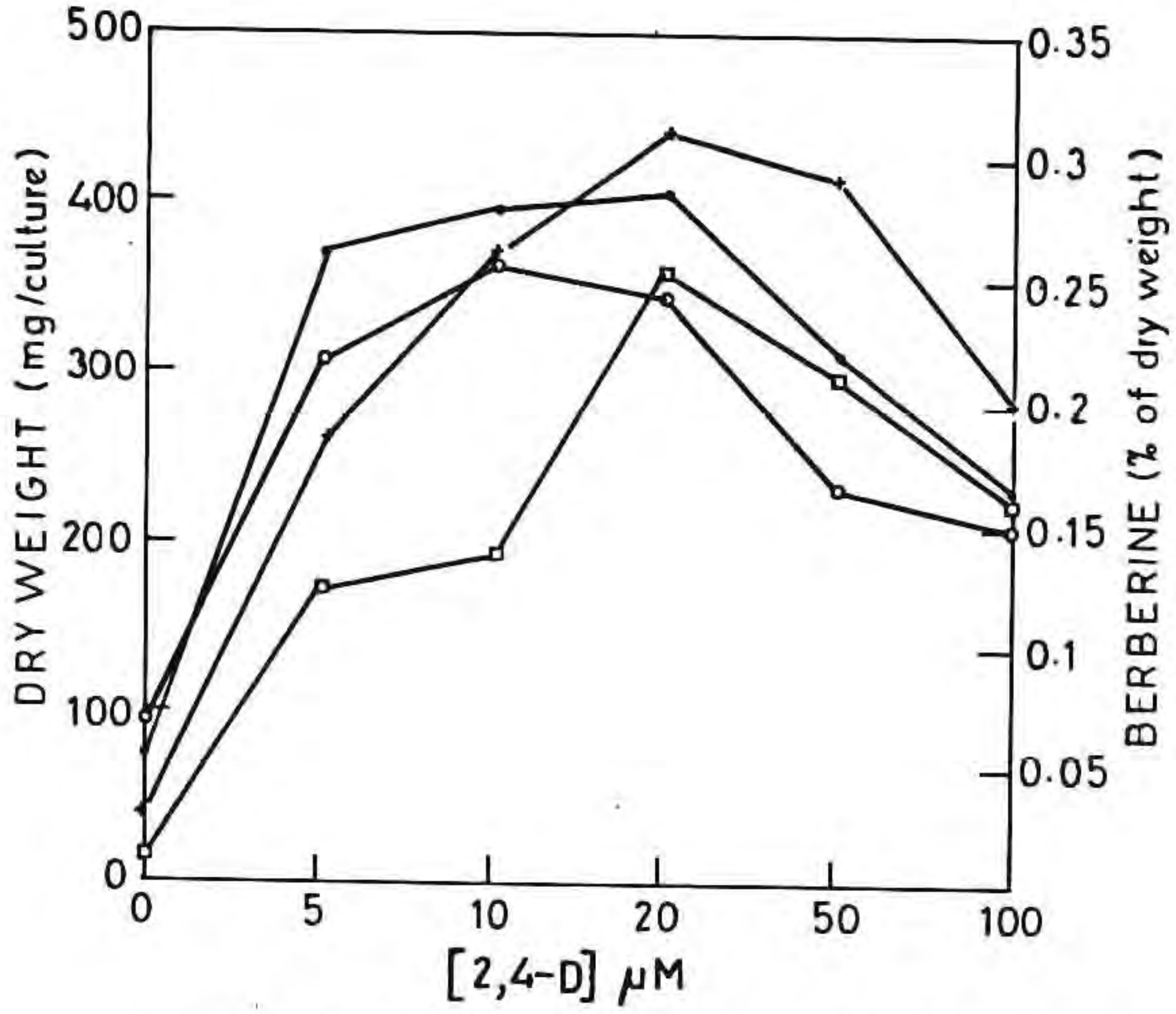
Data collected on 28th day of inoculation
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Sl.No.1. In all cases $p < 0.05$.

't' values of Table 5A.10

't' values betwen	't' values to group A	't' values of group B
1 and 2	40.858	2.696
4 and 5	6.542	2.866
5 and 6	9.244	1.139*

* not significant at 5% level

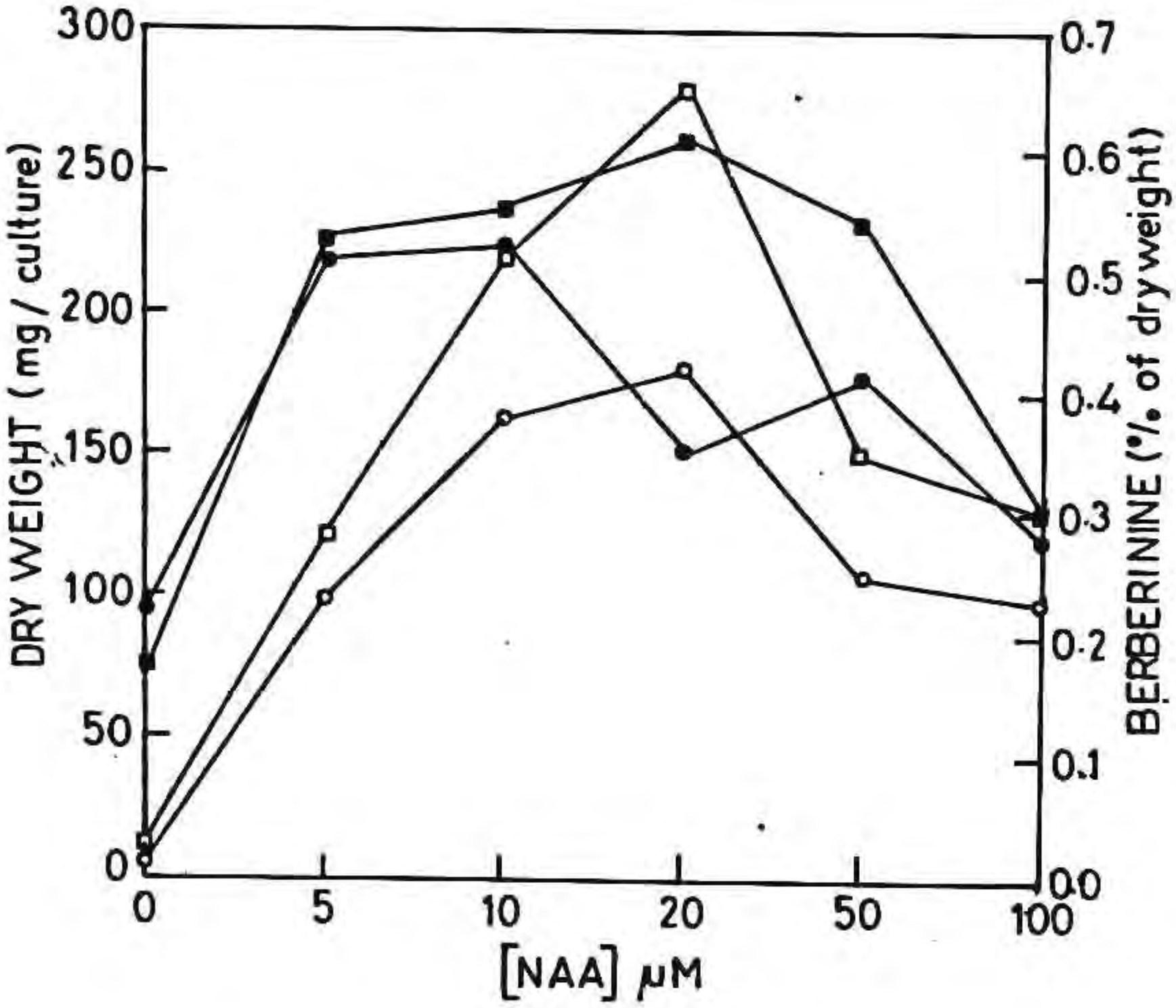
Fig.5A.3 : Effect of 2,4-D in combination with and without BAP on growth and berberine production in cell suspension cultures of *Coscinium fenestratum*



The cells were grown in B_5 basal medium supplemented with growth hormones for 28 days

The values plotted are the mean of five independent determinations

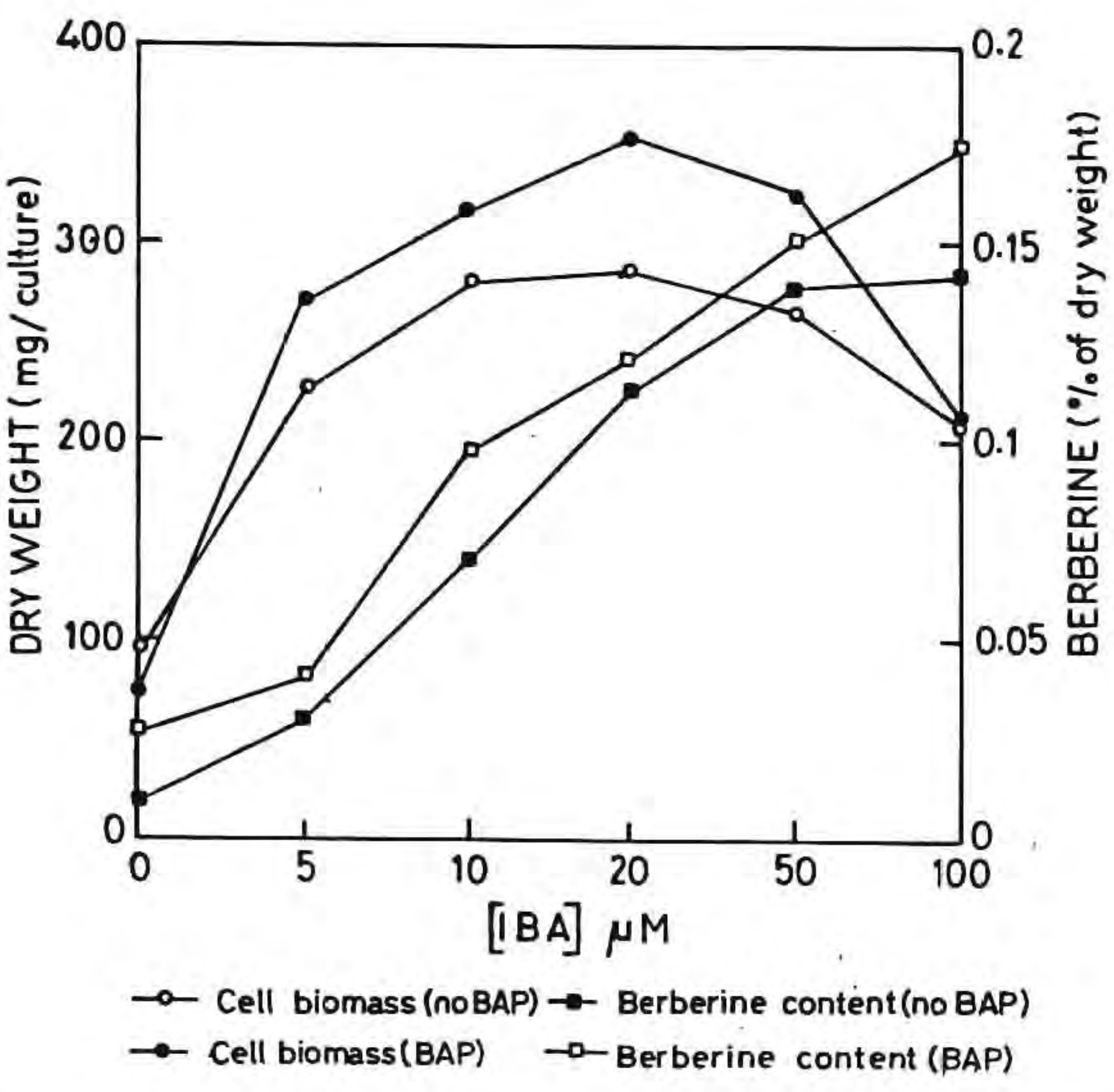
Fig.5A.4 : Effect of NAA in combination with and without BAP on growth and berberine production in cell suspension cultures of *Coscinium fenestratum*



The cells were grown in B_5 basal medium supplemented with growth hormones for 28 days

The values plotted are the mean of five independent determinations

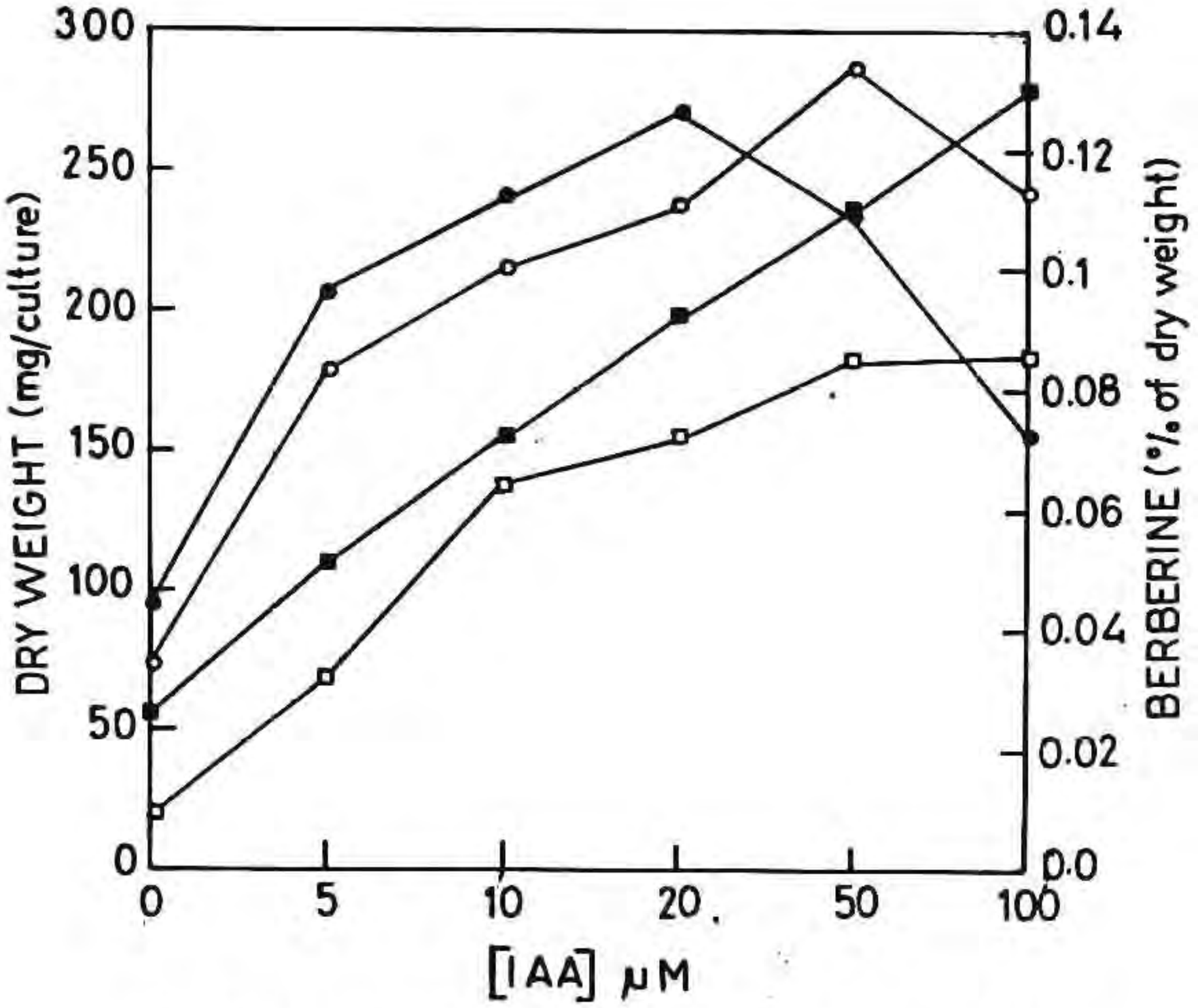
Fig.5A.5 : Effect of IBA in combination with and without BAP on growth and berberine production in cell suspension cultures of *Coscinium fenestratum*



The cells were grown in B_5 basal medium supplemented with growth hormones for 28 days

The values plotted are the mean of five independent determinations

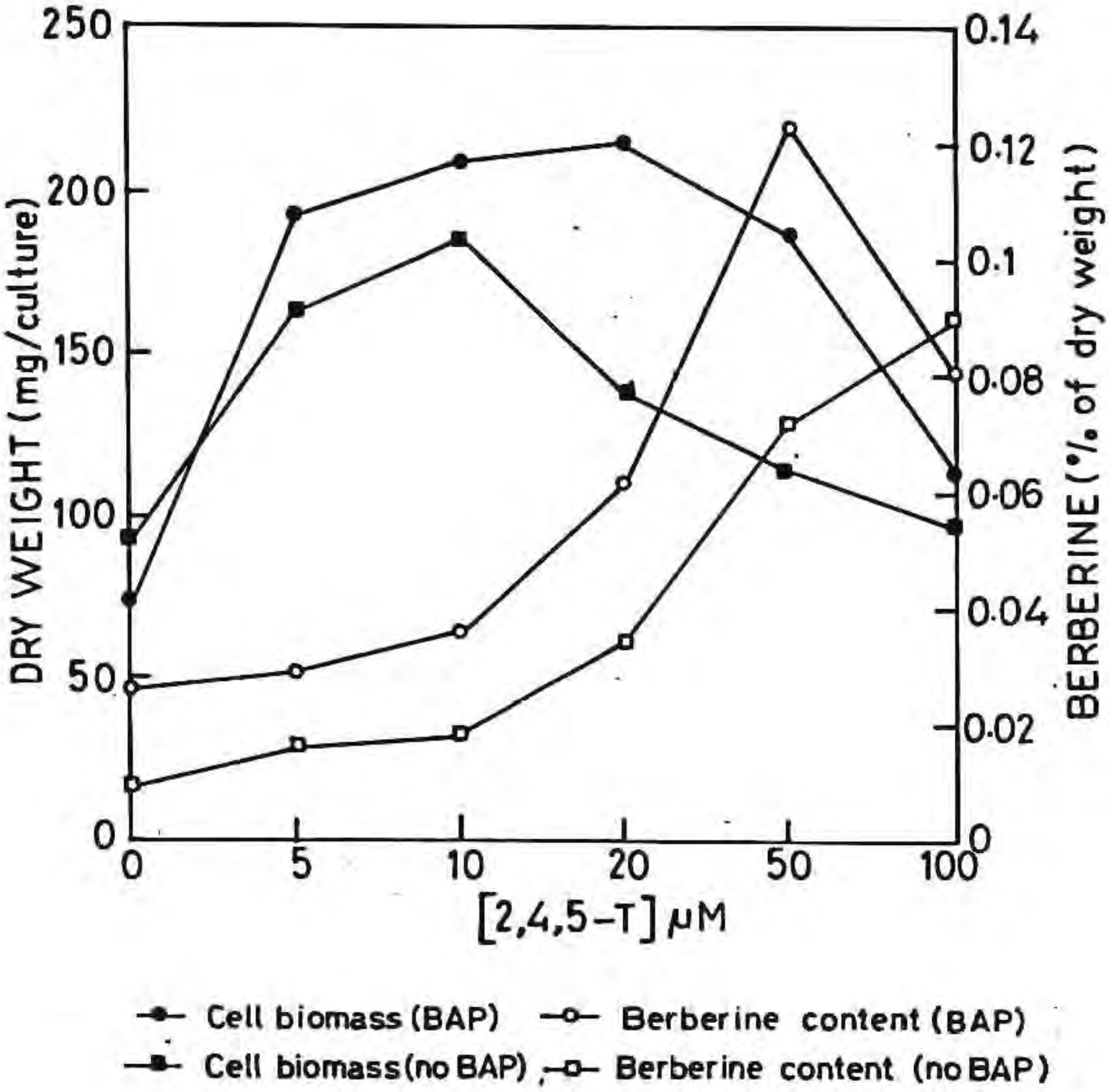
Fig.5A.6 : Effect of IAA in combination with and without BAP on growth and berberine production in cell suspension cultures of *Coscinium fenestratum*



The cells were grown in B_5 basal media supplemented with growth hormones for 28 days

The values plotted are the mean of five independent determinations

Fig.5A.7 : Effect of 2,4,5-T in combination with and without BAP in growth and berberine production in the cell suspension cultures of *Coscinium fenestratum*



The cells were grown in B_5 basal media supplemented with growth hormones for 28 days

The values plotted are the mean of five independent determinations

berberine content. Also in these experiments high concentrations of 2,4-D and NAA (100 µM) were found to be unfavourable for berberine synthesis and growth of cells. The highest berberine content was observed in media containing 20 µM 2,4-D or NAA alone as well as in combination with 1 µM BAP. The results of these experiments indicate that NAA is the most suitable among the auxins used for the enhanced production of berberine and the supplementation of BAP along with NAA was found to enhance the berberine biosynthesis.

Different concentration of NAA from 20 µM to 50 µM along with 1 µM BAP were used and its results are presented in Table 5A.11. Variations in the berberine content and dry cell biomass of the cultures, with respect to the variations in the concentration of NAA supplemented to the media containing 1 µM BAP, is represented in the Fig.5A.8. The highest berberine content was observed when the media contained 35 µM NAA although the cell biomass was considerably reduced at these concentrations compared to that at lower concentrations.

5A.3.4. Effect of cytokinins

The effect of cytokinins-kinetin and BAP along with 35 µM NAA on berberine production was carried out to find out the most effective one. Table 5A.12 shows that there

Table 5A.11 : Effect of different concentrations of NAA with 1 µM BAP on berberine production and growth in cell suspension cultures of *Coscinium fenestratum* (Medium B_5 basal + 1 µM BAP)

Sl. No.	Concentration (µM)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	20	983.55(±3.63)	256.30(±4.32)	0.675(±0.114)	1.630(±0.120)	0.64
2	25	965.36(±5.28)	254.52(±3.74)*	0.798(±0.120)	1.670(±0.080) *	0.66
3	30	922.76(±4.83)	225.80(±3.89)	0.811(±0.051)	1.690(±0.079)	0.75
4	35	795.25(±5.27)	204.73(±5.66)	0.885(±0.160)	1.880(±0.121)	0.92
5	40	736.93(±4.88)	185.28(±3.71)	0.592(±0.081)	1.310(±0.060)	0.71
6	45	643.26(±4.39)	165.54(±3.11)	0.451(±0.080)	0.980(±0.093)	0.59
7	50	482.89(±3.20)	146.59(±2.88)	0.216(±0.110)	0.450(±0.093)	0.36

Initial biomass : Fresh weight : 350.58(±8.17) mg
Dry weight : 69.73(±1.22) mg

Data collected on 28th day of inoculation

Each value is the mean ±SEM of five independent determinations

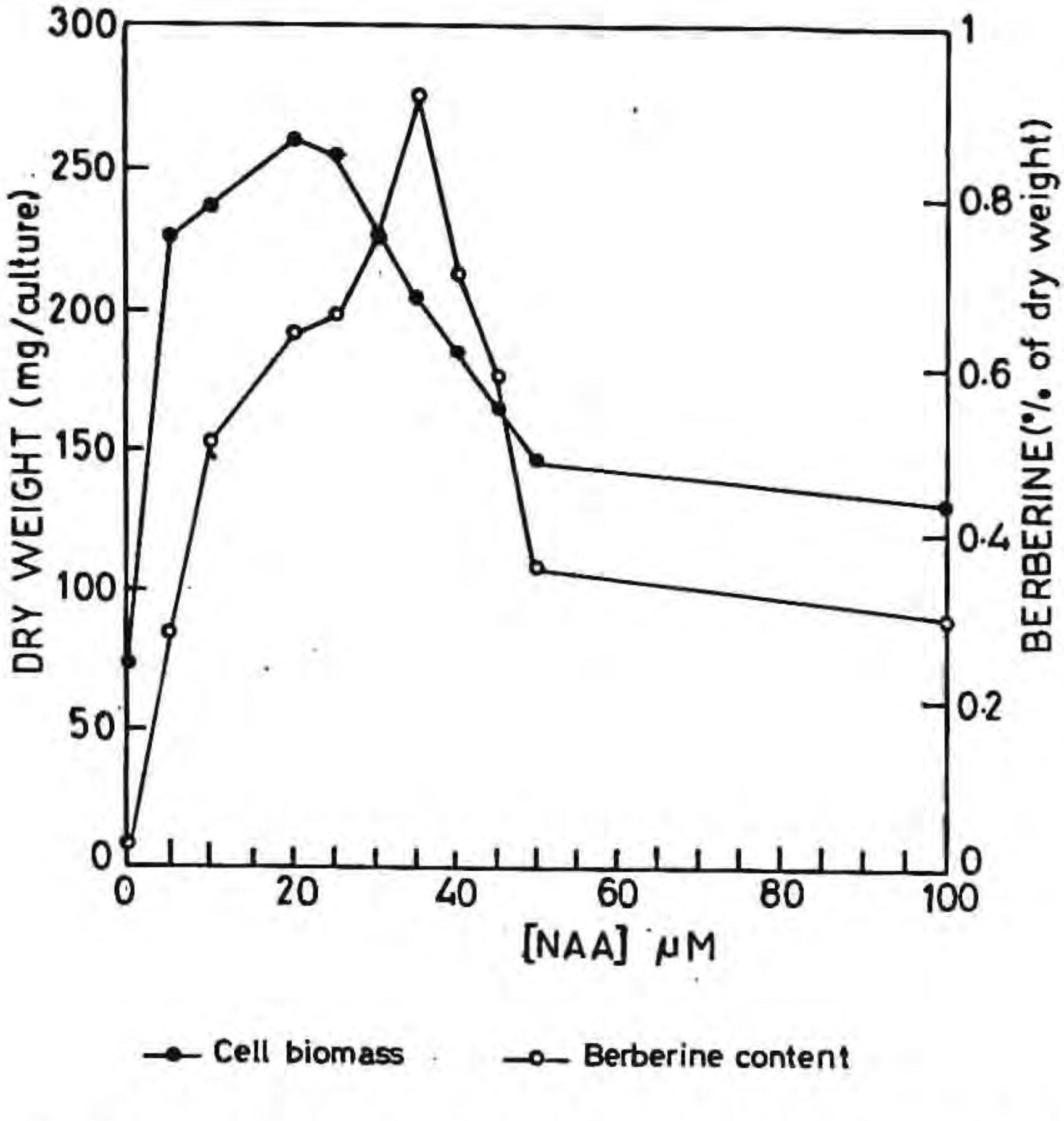
The values were compared with those of Sl.No.1. In all cases $p < 0.05$. *$p > 0.05$.

't' values of Table 5A.11

't' values between	't' values of group A	't' values of group B
1 and 2	0.394*	0.350*
3 and 4	6.732	0.225*
4 and 5	-3.880	1.663 *
5 and 6	3.635	-5.338
6 and 7	5.595	4.909

* not significant at 5% level

Fig.5A.8 : Effect of different concentrations of NAA in combination with BAP on growth and berberine production in the cell suspensin cultures of *Coscinium fenestratum*



The cells were grown in B_5 basal medium supplemented with different amount of NAA and BAP (1 µM)

The values plotted are the mean of five independent determinations

is a significant difference in the total berberine content of the cultures grown in media with equal concentration of BAP and kinetin and the berberine content was higher by 32 percent in the cells grown in media with BAP. Further kinetin supplemented media reduced the cell biomass also. A comparative data is presented in the Fig.5A.9. Thus BAP was selected as the better cytokinin compared to kinetin.

5A.3.5. Effect of different concentrationsof BAP on berberine production

The influence of different concentrations of BAP on berberine biosynthesis is shown in Table 5A.13 and Fig.5A.10. BAP at concentrations of 1 µM to 15 µM was supplemented in B_5 liquid media containing 35 µM NAA. It was observed that addition of BAP at concentrations of 5 µM to 10 µM remarkably increased the berberine content of the cell suspension cultures. Maximum effect was observed at 8 µM concentration, the optimum concentration of BAP in combination with 35 µM NAA. Supplementation of BAP at 10 µM concentration eventhough increased the berberine content (percentage of the dry weight of cell biomass) the total berberine content was reduced due to the low biomass production. It was observed that the alteration in the quality, concentration and combinations of growth regulators did not influence the secretory

Table 5A.12 : Effect of kinetin/BAP on growth and berberine production in cell suspension cultures of *Coscinium fenestratum*
(Medium: B_5 basal + 35 µM NAA + 1 µM BAP/Kinetin)

Sl. No.	Cytokinin	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total Berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	Kinetin	685.76(±7.39)	186.00(±3.25)	0.681(±0.083)	1.458(±0.130)	0.81
2	BAP	749.34(±7.72)	210.75(±4.15)	0.918(±0.056)	1.930(±0.121)	0.91

Initial biomass : Fresh weight : 332.91(±1.73)
Dry weight : 66.28(±1.15)

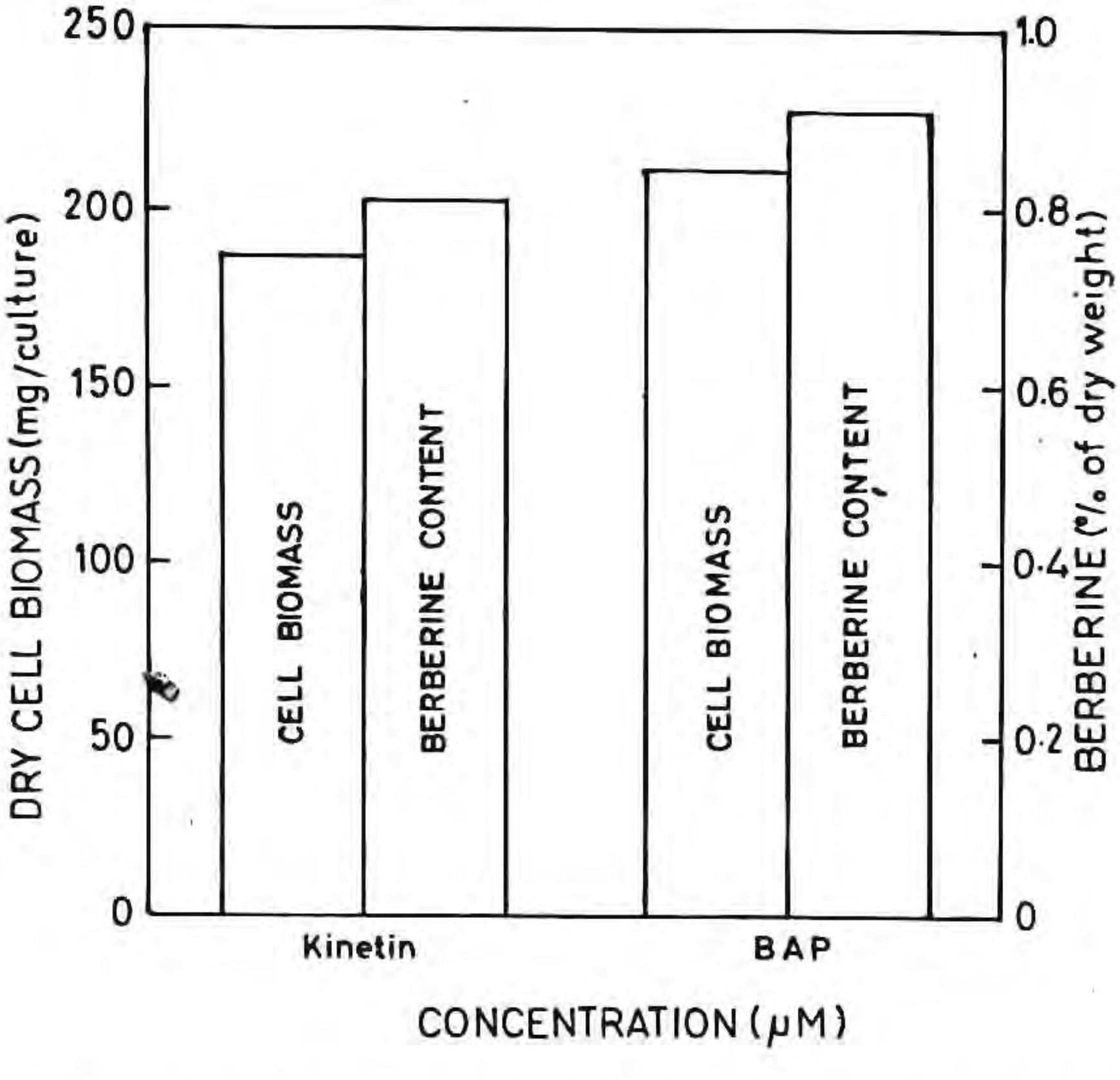
Data collected on 28th day of inoculation

Each value is the mean ±SEM of five independent determinations

The values of Sl.No.1 and 2 were compared. $p < 0.05$

't' values between	't' values to group A	't' values to group B
1 and 2	5.939	3.374.

Fig.5A.9 : Effect of kinetin and BAP on berberine production and growth in cell suspension cultures of *Coscinium fenestratum*



Cells were grown in B_5 basal media supplemented with growth hormones for 28 days

The values plotted are the mean of five independent determinations

Table 5A.13 : Effect of different concentrations of BAP wih 35 µM NAA on growth and berberine production in cell suspension cultures of *Coscinium fenestratum*
(Medium: B_5 basal + 35 µM NAA + 1 µM BAP/Kinetin)

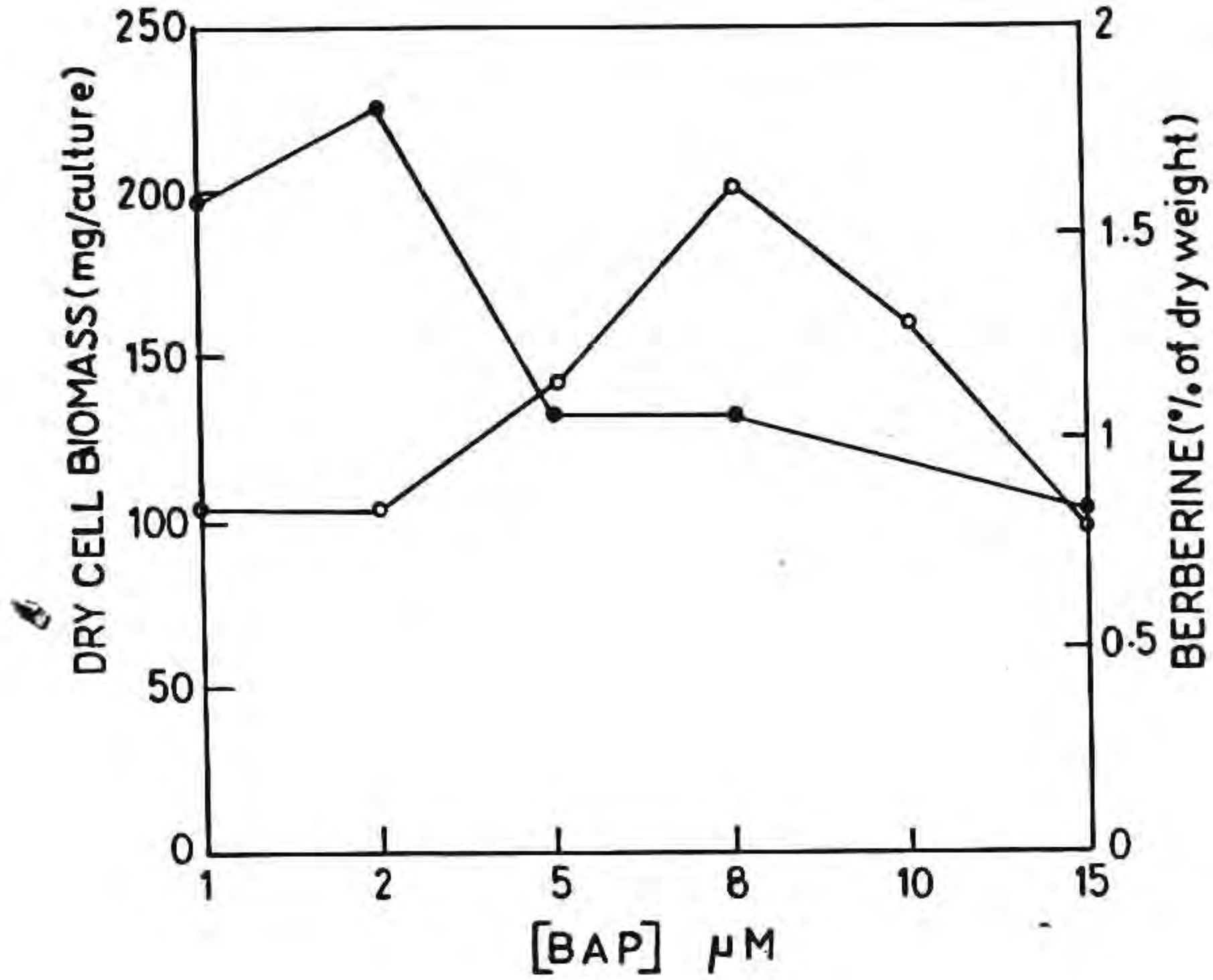
Sl. No.	Concentration (µM)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight
			A		B	
1	1	768.35(±4.77)	197.28(±3.42)	0.794(±0.012)	1.637(±0.013)	0.831
2	2	918.56(±6.39)	225.81(±3.36)	0.912(±0.015)	1.870(±0.011)	0.830
3	5	648.73(±5.28)	132.25(±2.83)	0.971(±0.009)	2.034(±0.036)	1.130
4	8	541.68(±4.52)	132.00(±3.24)	0.978(±0.011)	2.125(±0.018)	1.610
5	10	429.84(±3.37)	116.30(±3.13)	0.781(±0.020)	1.490(±0.033)	1.280
6	15	361.82(±7.16)	103.54(±2.48)	0.391(±0.017)	0.814(±0.020)	0.790

Initial biomass : Fresh weight : 350.72(±3.77) mg
Dry weight : 70.94(±1.44)
The values were compared with those of Sl.No.1. In all cases $p < 0.05$.
Data collected on 28th day of inoculation
The values are the mean ±SEM of five independent determinations

't' values of Table 5A.13

"t' values between	't' values of group A	't' values of group B
1 and 3	18.527	13.117
1 and 5	22.091	5.241
5 and 6	4.041	2.586

Fig.5A.10 : Effect of different concentrationsof BAP on growth and berberine production in the cell suspension cultures of *Coscinium fenestratum*



The cells were grown in B_5 basal media supplemented with 35 µM NNA and different concentrations of BAP, for 28 days

The values plotted are the mean of five independent determinations

behaviour of the cultured cells (Table 5A.1 to 5A.13). In all cases approximately half of the total berberine content of cultures were recovered from the medium of the cell suspension cultures. None of the cultures showed any morphological variations such as formation of large cell clumps or morphogenetic responses such as shoot or root formation during the culture period.

5A.4. DISCUSSION

In these experiments it was demonstrated that the berberine biosynthesis by the cell cultures of *C. fenestratum* was greatly influenced by the quality as well as the concentrations of the auxins present in the medium.

In the cell suspension cultures of *C. fenestratum* it was observed that an increase in the concentration of auxins particularly 2,4-D and NAA stimulated the production of berberine. Nakagawa et al[116] also reported that very high concentration of 2,4-D and NAA stimulated the production of berberine considerably in cell cultures of *Thalictrum minus*. But in the present investigations it was observed that when higher concentration of NAA and 2,4-D (100 µM) was supplemented in the medium of berberine content declined in the cell cultures of *C. fenestratum*. Other auxins such as IAA and IBA at concentrations of 50 µM and 100 µM increased the berberine production

compared to that of low concentration. These observations indicate that the type of auxins has got a specific role in the regulations of secondary metabolism particularly in stimulating berberine biosynthesis which may be due to specific chemical and structural features of the molecules that vary from auxin to auxin[217,268]. Among the different types of auxins used, NAA was found to be most effective for berberine biosynthesis. Similar observation was reported in the case of *T. minus*[116] and *Coptis japonica* cell cultures[113,241] for berberine synthesis. Higher stimulatory effect of NAA over the other types of auxins for the production of secondary metabolites were reported in a number of cases also. Zenk et al[262] reported that NAA was favourable for the production of anthraquinones by the cell cultures of *Morinda citrifolia*. Similar effect was noticed in the production of rosmarinic acid in *Anchusa officinalis*[269]. The cell cultures of *C. fenestratum* differs from that of *T. minus* in that very high concentration of auxins such as 2,4-D and NAA are not favourable for berberine biosynthesis. Inhibitory effect of 2,4-D on biosynthesis of secondary metabolites has also been reported in a number of cases[217,264].

Addition of BAP along with auxins enhanced the production of berberine. The combined effects of auxins

and cytokinins on secondary metabolite production are difficult to assess since there is a lack of data on endogenous levels of these regulators in the cultured cells. The combination of the same concentration of BAP with different concentration of the same type of auxins showed different response with respect to berberine biosynthesis. Almost similar effect of auxins-cytokinin combination on berberine production was reported by Nakagawa et al[116] in the cell cultures of *Thalictrum minus*. In combination with 1 µM BAP also, NAA was found to be more effective than other auxins. Since there is a combined action of auxins and cytokinin on berberine biosynthesis, different concentration of NAA along with 1 µM BAP were used to find out the optimum concentration and the maximum product synthesis was observed at 35 µM NAA.

Different concentrations of BAP in combination with 35 µM NAA also showed great variation in product formation. Here also increase in the concentration of BAP increased the berberine production but only upto 10 µM. Concentrations above this were not found favourable for product synthesis. A similar kind of NAA, BAP interaction on the regulation of berberine synthesis was also reported in *Thalictrum minus* cell culture, the difference being the optimum concentrations of NAA and BAP for higher berberine biosynthesis. The optimum hormonal conditions for higher berberine

production was not found suitable for higher cell biomass production. Similarly the optimum hormonal condition for higher cell growth rate did not produce higher quantity of berberine in the cultures. Therefore it is advisible to adopt a two stage culture system for berberine biosynthesis which will be advantageous in the industrial application.

The optimum concentration of hormones for high rate of berberine production in *C. fenestratum* differs from other reports as shown above.It can be due to the specific endogenous level of growth substance in the inoculum cells or the ability of the cell to synthesize its own growth regulators. The rate of uptake of hormone supplemented in the medium by the cells can vary from culture to culture or even cell to cell. Another probable reason is that the presence of some antisubstances within the cells can alter the effect of growth substances absorbed into the cells from the medium.

The exact mechanism behind the varied action of these different auxins and cytokinins on secondary metabolism and production of compounds like berberine is not clear. Even then the probable mode of action of hormones on increasing the product rate can be by enhancing the transcription and translation frequency of the appropriate

genes of secondary metabolisms and thereby increasing the enzyme quantities of the biosynthetic pathway. There are reports[270] in which plant hormones are being found to associate closely with DNA or RNA. Low molecular weight plant hormones have the ability to intercalate with nucleic acids thus conceivably may also be capable of acting as modulators of gene transcription or translation process.

SECTION B

TWO STAGE SYSTEM FOR BERBERINE PRODUCTION

5B.1. INTRODUCTION

The relationship between growth and secondary product synthesis is one of the very important factors to be considered for developing a process strategy to produce the compound very efficiently and economically. In most circumstances natural product accumulation occurs in maximum amount at the stationary phase or towards the end of the lag phase when the growth rate decreases[238]. But there are cell culture systems where the synthesis of secondary metabolite accompanies growth as observed in the case of *Coscinium fenestratum*. When growth of cells and product formation occurs simultaneously in the same medium a single stage continuous culture can be very easily adopted. In *C. fenestratum* the product synthesis and cell multiplication

occurs simultaneously. Even then it was observed from the earlier studies that the basal media and hormonal conditions ideal for growth and berberine production are different. The growth and berberine production showed an inverse relationship.

Based on the observations of the previous experiments, the feasibility of adopting a two stage culture system for the enhanced production of berberine was examined.

5B.2. MATERIALS AND METHOD

5B.2.1. Stock culture

The cell suspension cultures of *C. fenestratum* were maintained in B_5 basal medium supplemented with 10 µM 2,4-D and 1 µM BAP. The stock cultures were grown in 250 ml Erlenmeyer flasks with 50 ml medium and subcultured as described earlier.

5B.2.2. Two stage culture systems

Based on the results described earlier, Gamborg's B_5 basal media supplemented with 10 µM 2,4-D and 1 µM BAP was selected as the "growth medium" as the cells in suspension cultures proliferated rapidly and produced more biomass. Cells were grown in this medium as a source of biomass. The cells were separated by filtration on 25th day of inoculation and transferred to the production medium after a wash in the same medium. Murashige and Skoog

medium containing 35 µM NAA and 8 µM BAP was used as the "production medium". Cell growth and berberine production were monitored as described earlier.

5B.3. RESULTS

The growth of cells in the growth media was rapid with a lag phase of 8 to 10 days and produced comparatively good cell biomass with four to five fold increase after 28 to 32 days as has been reported in the previous chapter. On 25th day the cells were filtered out and about 350 mg (fresh weight) of cells were transferred to the production medium and incubated as described above. For comparison the berberine production by cells maintained in the growth medium was also studied.

The biomass accumulation and berberine production by the suspension cultures grown in "production medium" are shown in Table 5B.1. The berberine content of the cultures were considerably increased compared to that of the growth medium (Fig.5B.1) but the rate of cell biomass increase was considerably reduced (Fig.5B.2).

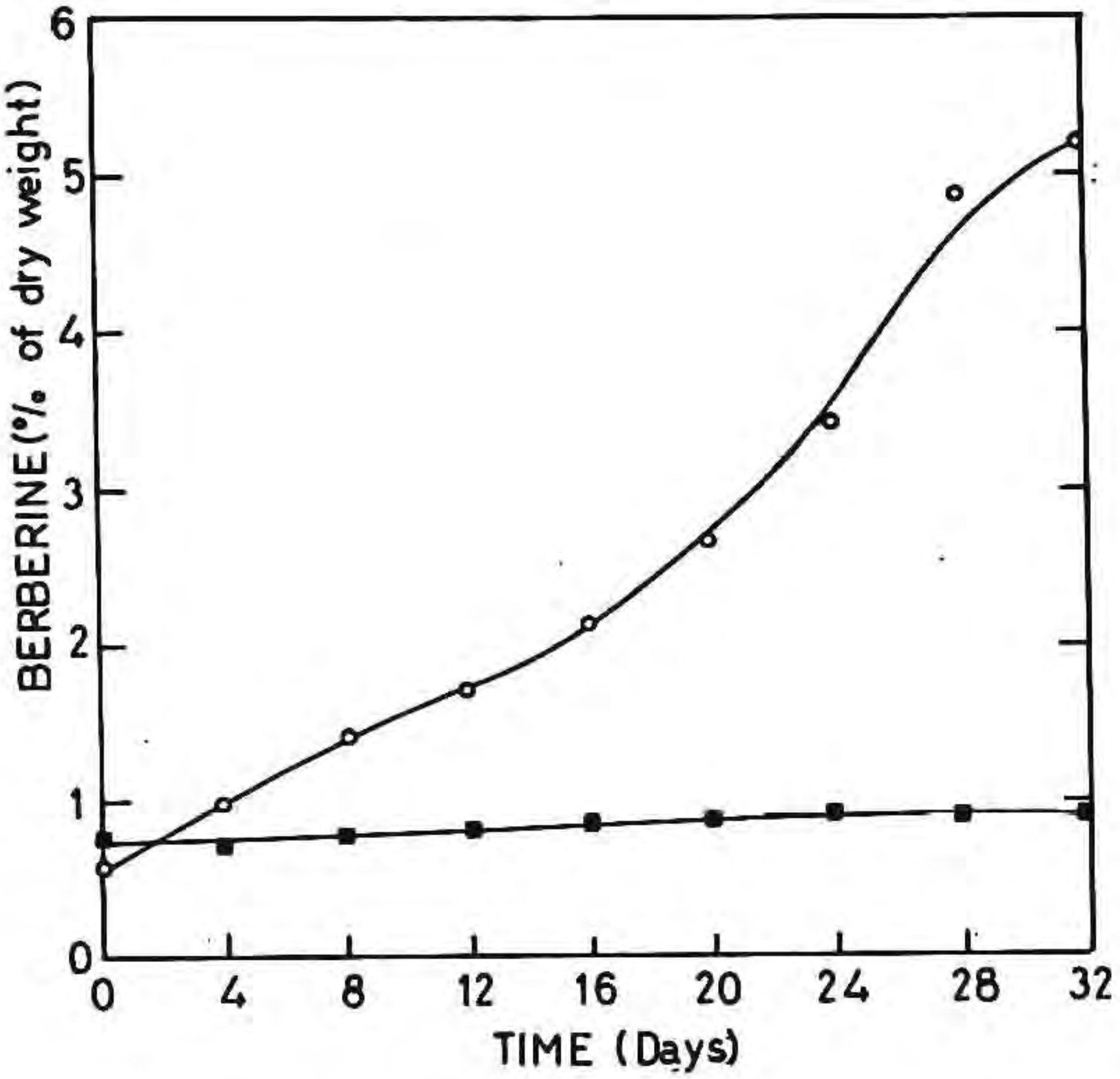
Berberine synthesis and its secretion into the medium started from the fourth day of inoculation itself and there was a four to five fold increase in the total berberine content during the logarithmic phase of growth before coming to stationary phase. Berberine synthesis

Table 58.1 : Growth and berberine production in MS medium supplemented with 35 µM NAA and 8 µM BAP (Production media)

Sl. No.	Time (days)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
1	0	325.26(±6.13)	65.12(±3.34)	0.172(±0.008)	0.371(±0.016)	0.57
2	4	331.84(±4.86)	68.29(±2.82)	0.341(±2.011)	0,670(±0.011)	0.98
3	8	358.55(±7.90)	81.75(±1.92)	0.811(±0.009)	1.441(±0.027)	1.76
4	12	362.43(±5.86)	80.38(±1.56)	0.960(±0.014)	1.700(±0.022)	2.12
5	16	388.92(±2.62)	85.29(±3.18)	1.340(±0.014)	2.260(±0.038)	2.66
6	20	411.87(±3.53)	85.20(±1.15)	1.520(±0.031)	2.898(±0.023)	3.41
7	24	431.66(±3.71)	98.00(±2.71)	2.451(±0.026)	4.760(±0.014)	4.86
8	28	453.79(±3.68)	106.73(±2.45)	2.875(±0.015)	5.548(±0.040)	5.20
9	32	489.25(±2.77)	109.31(±3.11)	2.979(±0.033)	5.480(±0.037)	5.01

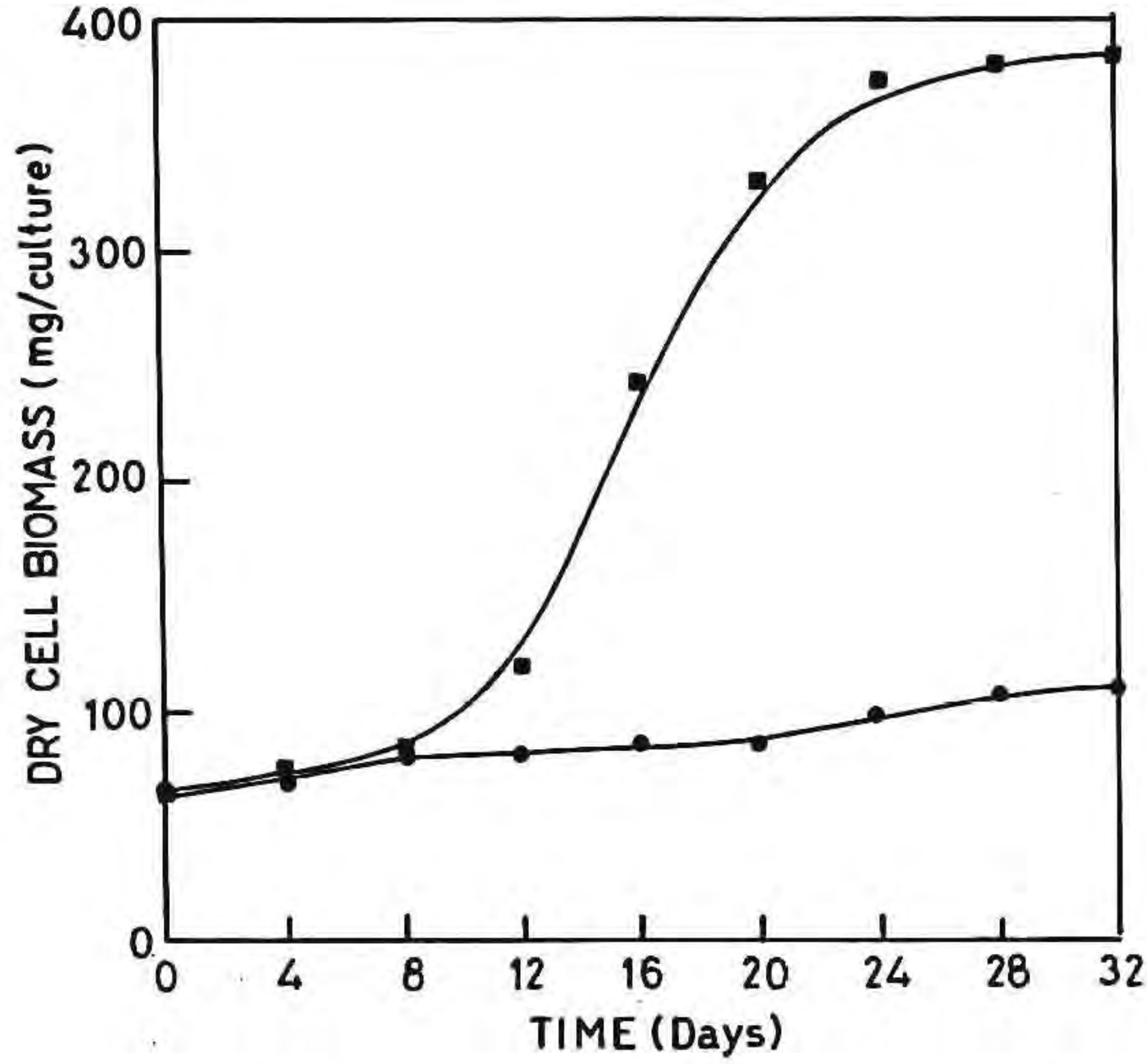
Data collected on 20th day of inoculation
Each value is the mean ±SEM of five independent determinations

Fig.5B.1 : Production of berberine by cell suspension cultures of *Coscinium fenestratum* grown in production medium and growth medium



The values plotted are the mean of ten independent determinations

Fig.5B.2 : Growth of cells in suspension cultures of *Coscinium fenestratum* in production medium and growth medium



The values plotted are the mean of five independent determinations

was reduced when the cell entered into the stationary phase. The berberine content of the culture media was found to be around 3 mg/50 ml which is approximately 5o to 55 percent of the total berberine content of the suspension culture (Table 5B.1).

5B.4. DISCUSSION

The feasibility of using a two stage culture system for the enhanced production of berberine by the cell cultures of *Coscinium fenestratum* was tested. Maximum berberine production has been achieved by adopting a strategy for maximum biomass production in the first stage using a growth medium and having optimal condition for maximum product formation in the second stage in the production medium. The results presented above indicate that the production of berberine in both the "growth medium" and "production medium" occurs during the cell growth although the berberine content is different. When the product formation is growth associated the promotion of cell growth usually results in the formation of more product. But in *Coscinium fenestratum* cell cultures, when cell growth was promoted by culturing in the growth medium, only small quantities of berberine was produced. The formation of berberine when cultured in both media indicates that there may not be any metabolic block in the pathway of berberine synthesis. Probably, when the cells are cultured in growth media

a major portion of the energy and nitrogenous compounds may be channeled to the berberine biosynthesis. Since the cell biomass influences the product formation and at the same time there is a type of uncoupling of rates of growth and berberine production in a particular medium, the two stage culture system was found useful.

When a two stage culture is used for a product synthesis, the product formation in the second stage depends on the previous biomass synthesis stage since maximum formation of cell biomass increases the product synthesis.

In the nongrowth associated situations where the product formation occurs at the stationary phase, biomass production is uncoupled from secondary product synthesis. A careful balance has to be achieved for biomass production without reducing the secondary metabolite production[271]. Uncoupling of cell growth and product formation stages by use of two stage batch or continuous culture may be essential for optimal biosynthesis of a nongrowth associated product[272]. In *Coscinium fenestratum* cell cultures increased product synthesis was observed when growth rate was reduced.

Two stage culture system has proved highly successful with a number of secondary products[273]. This has been applied very successfully for the industrial production of shikonin derivatives, one of the first secondary metabolites to be produced commercially by *Lithospernum*

erythrorhizone cultures which is an example for nongrowth associated product formation[34,274,275].

An observation similar to that of *C. fenestratum* has been reported in the case of berberine production by *Thalictrum minus*[116]. It was found that the optimum nutritional conditions for cell growth and those for berberine production are different. Therefore the possibility of adopting a two stage culture system has been discussed as similar to that of *Coscinium fenestratum*, but they have not developed a two stage culture system for berberine production in *T. minus*. Kim et al[276] examined a two stage culture system for berberine production by using the cell cultures of *Thalictrum rugosum* but this was not found successful. They observed a reduction in both biomass and berberine production when the cells were transferred to the production media.

The two stage culture system described above may be useful for the continuous or semi-continuous production of berberine in an industrial process.

CHAPTER VI

CHAPTER VI

EFFECT OF AMINO ACIDS AND PRECURSORS ON BERBERINE PRODUCTION

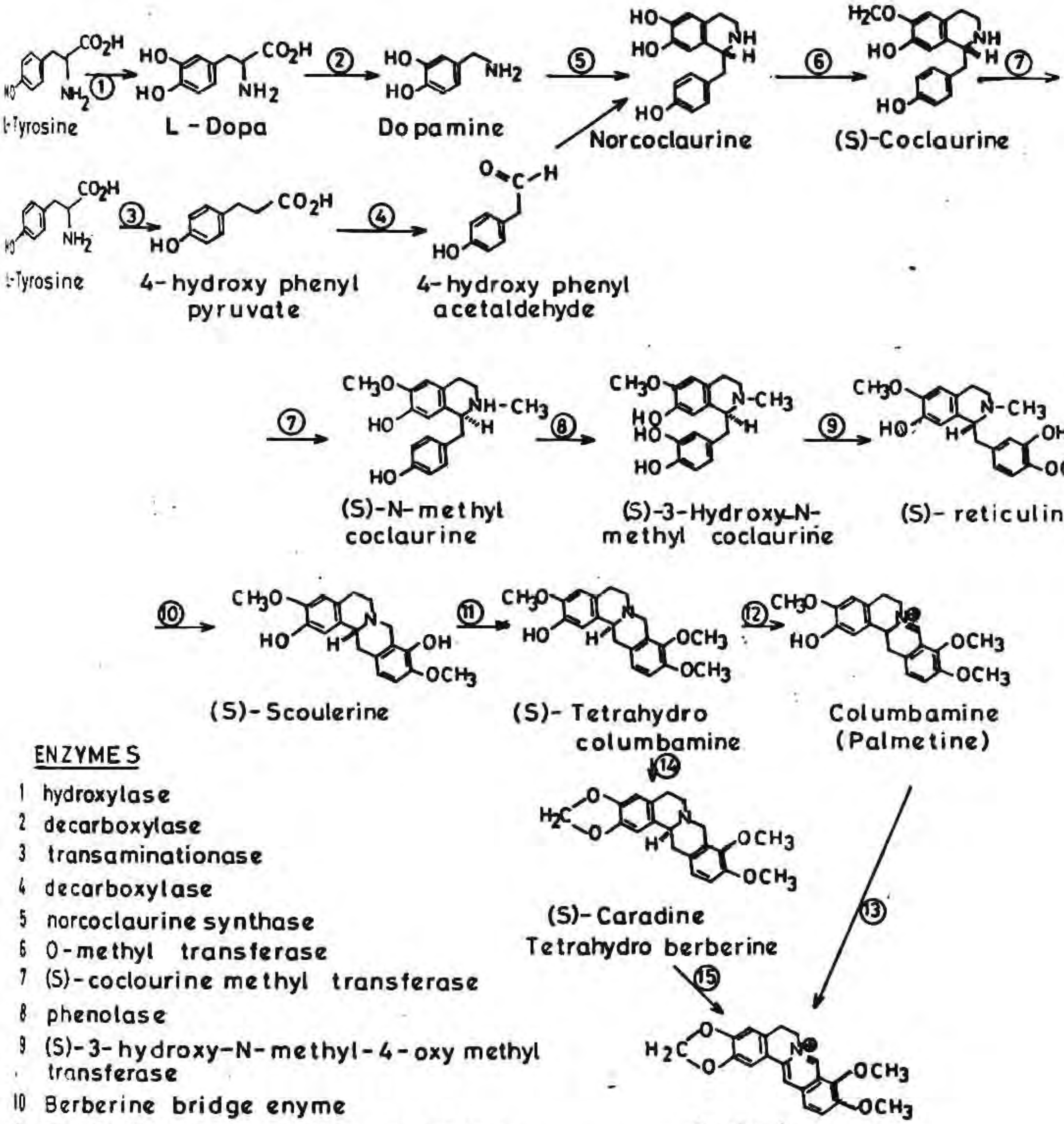
6.1. INTRODUCTION

Alkaloids, in most cases are formed from a group of amino acids such as lysine, ornithine, phenylalanine, tyrosine and tryptophane. The basic skeletons of these amino acids are retained largely intact in the derived alkaloids. Isoquinoline alkaloids are one of the major groups, which are derived from the amino acid L-tyrosine and Berberine is one of the important isoquinoline alkaloids.

The biosynthetic pathway of berberine has been worked out mainly using cell cultures of different berberis species as the experimental material[81]. The different steps involved in the biosynthetic pathway from tyrosine to berberine through reticuline (Fig.6.1) have been established. The biosynthesis of this intermediate has been suggested to proceed through the formation of the intermediate norcoclaurine.[277] The benzyl isoquinoline skeleton is believed to be elaborated from two molecules of L-tyrosine[278].

A total of 13 enzymes are believed to be involved in transforming two molecules of L-tyrosine to one molecule of berberine. Tyrosine on hydroxylation yields

Fig.6.1 : Biosynthetic pathway of berberine



L-dihydroxy phenylalanine (L-dopa) which on decarboxylation forms Dopamine. Another molecule of L-tyrosine is first transaminated to form 4-hydroxyphenylpyruvate which on subsequent decarboxylation yields 4-hydroxyphenyl acetaldehyde.

Dopamine and 4-hydroxyphenyl acetaldehyde undergo condensation to yield (S)-norcoclaurine which is catalysed by the enzyme (S)-norcoclaurine synthase. This product undergoes o-methylation at C-6 to give (S)-coclaurine. The enzyme here is a o-methyl transferase. Like reticuline (S)-coclaurine is also a key benzyl isoquinoline precursor. Methylation at N-position of coclaurine leads to the formation of (S)-N-methyl coclaurine, which is catalysed by the enzyme (S)-coclaurine methyl transferase; this follows hydroxylation at C-3 to yeld (S)-3-hydroxy N-methyl-coclaurine by the enzyme phenolase. This product again undergoes methylation to form (S)-reticuline; the enzyme involved is (S)-3-hydroxy N-methyl-4-oxymethyl transferase. The synthesis of reticuline is one of the important steps as it forms the common starting material for a number of isoquinoline alkaloids.

The elaboration of the benzyl isoquinoline skeleton, (S)-reticuline for the synthesis of berberine involves the inclusion of an extra carbon atom (C-8) for the formation of berberine bridge. This occurs by oxidative

cyclization of N-methyl group of the benzyl-isoquinoline precursor, (S)-reticuline to form (S)-scoulerine[278,280]. The enzyme involved here is designated as the berberine bridge enzyme. The pathway from the nonalkaloidal precursors to (S)-reticuline is catalysed by soluble cytosolic enzymes,but (S)-reticuline has to enter a specific vesicle which houses the berberine bridge enzyme responsible for the transformation of the universal isoquinoline precursor (S)-reticuline to (S)-scoulerine. This forms the parent compound for a large number of tetrahydro protoberberine alkaloids. In the next step, (S)-scoulerine is transformed to (S)-tetrahydro columbamine by the enzyme scoulerine-9-oxy methyl transferase. Experiments with tissue culture has led to the isolation of a stereo and regiospecific-o-methyl transferase which functions in the presence of S-adenosyl-L-methionine[281]. But it was observed that this enzyme is not present in the alkaloid vesicle but is cytosolic[83]. Therefore the (S)-scoulerine probably leaves the vesicle to be transformed to (S)-tetra hydrocolumbamine and then this may be reinserted into the vesicle. It is finally converted to columbamine (Palmetine) by tetrahydro protoberberine oxidase (STOX)[282]. Columbamine gives rise to berberine by the formation of methylene dioxy group. But Galnedar et al[82] demonstrated an alternative final step in berberine biosynthesis using

the cultured cells of *Coptis japonica*. According to this tetrahydro columbamine is converted to (S)-canadine or tetrahydro berberine by the enzyme canadine synthase. (S)-canadine in turn is stereospecifically dehydrogenated to berberine by an enzyme tetrahydro berberine oxidase or canadine oxidase.

In the biosynthesis of a secondary metabolite by cells in cultures, the primary metabolite precursor and intermediates will have an important role in the regulation of the product formation directly or indirectly. This may be by activating or by repressing an enzyme of the biosynthetic pathway of the secondary metabolite. Sometimes the insufficiency of the primary metabolic precursor may limit the rate of product formation. Therefore studies were undertaken to investigate the influence of some primary metabolite precursors and intermediates such as L-tyrosine, L-dopa and other amino acids like L-tryptophan and L-phenylalanine on berberine production by the cell cultures of *Coscinium fenestratum*. These primary metabolites were supplemented to the optimized "production media" with a view to increase the production of berberine in the cell cultures and also to see whether these cell cultures can be used as a biotransformation system which can transform the supplements continuously and completely into products.

6.2 MATERIALS AND METHOD

The cell suspension cultures of *C. fenestratum* were grown and maintained in "growth medium" as described earlier and were used as stock cultures for the present experiments. The cells were filtered out from the media at the late logarithmic phase (25th day) and a known weight of cells were inoculated into the "production medium". This was supplemented with amino acids, L-tyrosine or L-tryptophan or L-phenylalamine or the intermediate L-dopa at various concentrations. The cultures were incubated on a rotary shaker (140±5 rpm) in the dark at 25±2°C. The cultures were harvested in five replicates on 28th day of inoculation and fresh weight, dry weight and total berberine content of the cultures were determined.

Quantitation of the total berberine and biomass of cultures was done as described earlier.

6.3 RESULTS

The influence of each amino acid on the production of berberine in the cell cultures was studied separately and the results are presented here. Each amino acid was supplemented at various concentrations from 0.5 to 5.0 mg/l.

6.3.1. Effect of addition of L-tyrosine

Table 6.1 shows the effect of L-tyrosine supplementation at different concentrations on berberine production by the cell cultures. A decrease in the fresh weight

Table 6.1 : Effect of different concentrations of L-Tyrosine on cell biomass and berberine production in cell suspension cultures of *Coscinium fenestratum* (Medium: Production medium + L-Tryptophan)

Sl. No.	Concentration (mg/l)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	0.00	615.73(±3.52)	126.05(±1.04)	2.91(±0.27)	6.09(±0.12)	4.83
2	0.50	629.49(±3.25)	136.00(±1.51)	3.81(±0.22)	7.22(±0.73)	5.31
3	1.00	557.25(±6.34)	128.42(±1.30)*	3.74(±0.53)	7.64(±0.54)	5.95
4	2.00	542.83(±4.28)	118.77(±2.44)	3.65(±0.46)	7.03(±0.92)	5.92
5	3.00	540.50(±4.11)	120.56(±1.56)	3.42(±0.39)	6.75(±0.88)*	5.60
6	5.00	576.00(±3.29)	121.09(±1.25)	2.58(±0.66)	5.73(±0.71)	4.73

Initial biomass : Fresh weight : 399.42(±1.88) mg
Dry weight : 68.28(±1.13) mg

Data collected on 28th day of inoculation

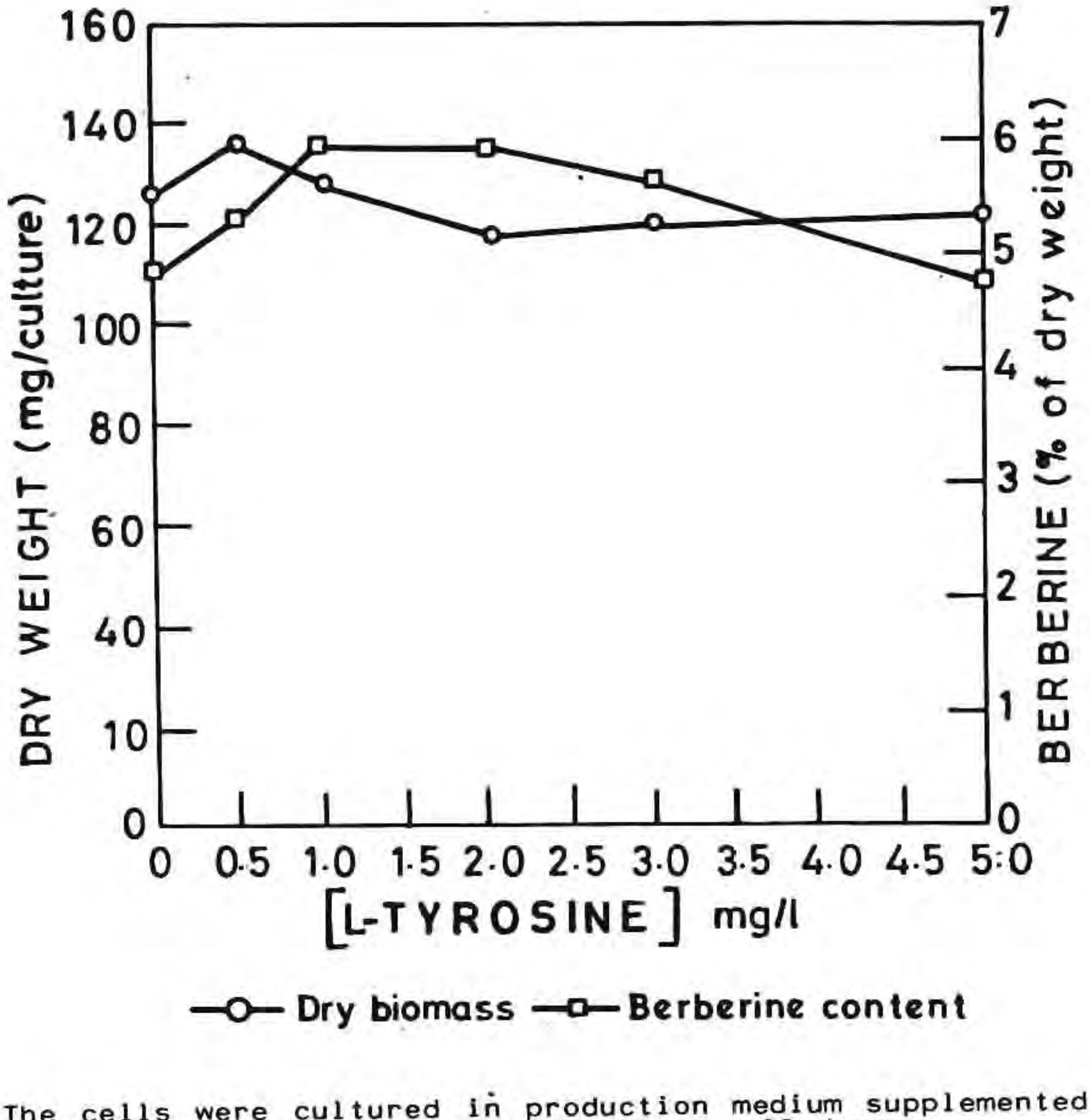
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Sl.No.1. In all cases $p < 0.05$. *$p > 0.05$.

't' values of Table 6.1

't' values between	't' values of group A	't' values of group B
1 and 2	6.864	1.932
1 and 3	1.800*	3.544
1 and 5	3.703	0.939*

* not significant at 5% level

Fig.6.2 : Effect of L-tyrosine supplementation on growth berberine production in the cell cultures of *Coscinium fenestratum*



The cells were cultured in production medium supplemented with various concentrations of L-tyrosine for 28 days

The values plotted are the mean of five independent determinations

of cells was observed when the concentration of tyrosine increased,eventhough the difference was not significant at low concentrations. The dry weight of the cell biomass appeared to be identical in all concentrations of tyrosine supplementation except for 0.5 mg/l tyrosine supplementation where a significant increase in dry weight was observed. With increase in tyrosine content there was increase in berberine production. Berberine content of the cell cultures was increased by approximately 25 percent when tyrosine was supplemented in the production medium at a concentration of 1 mg/l. On further increase in concentration, there was a gradual increase in the total berberine content of the cell cultures (Fig.6.2). But higher concentration (above 3 mg/l) reduced the berberine content significantly when compared to that of control (Table 6.1).

6.3.2. Effect of addition of L-dopa

The effect of L-dopa supplementation on berberine production was studied and the results are given in Table 6.2 and in Fig.6.3. Administration of L-dopa in the production medium increased the total berberine content of the cell cultures with a slight decrease in the biomass content both in fresh weight and dry weight. The dry weight of biomass increased to a maximum at 0.5 mg/l L-dopa supplementation and then ~~decreased~~ ~~gradually~~y ~~with~~

Table 6.2 : Effect of different concentrations of L-Dopa on cell biomass and berberine formation in cell suspension cultures of *Coscinium fenestratum* (Medium: Production medium + L-Dopa)

Sl. No.	Concentration (mg/l)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	0.00	615.73(±3.13)	126.05(±1.04)	2.91(±0.27)	6.09(±0.12)	4.83
2	0.50	635.50(±5.11)	140.42(±1.25)	3.41(±0.18)	7.34(±0.75)	4.91
3	1.00	619.35(±3.27)	135.28(±2.26)	3.83(±0.18)	7.82(±0.03)	5.78
4	2.00	588.20(±5.28)	127.53(±1.73)	3.85(±0.44)	7.50(±0.84)	5.88
5	3.00	587.29(±4.92)	127.50(±2.43)	3.58(±0.72)	7.52(±1.13)	5.96
6	5.00	480.55(±3.73)	124.39(±2.11)*	3.62(±0.57)	7.43(±0.89)	5.97

Initial biomass : Fresh weight : 352.18(±1.44) mg
Dry weight : 69.72(±1.30) mg

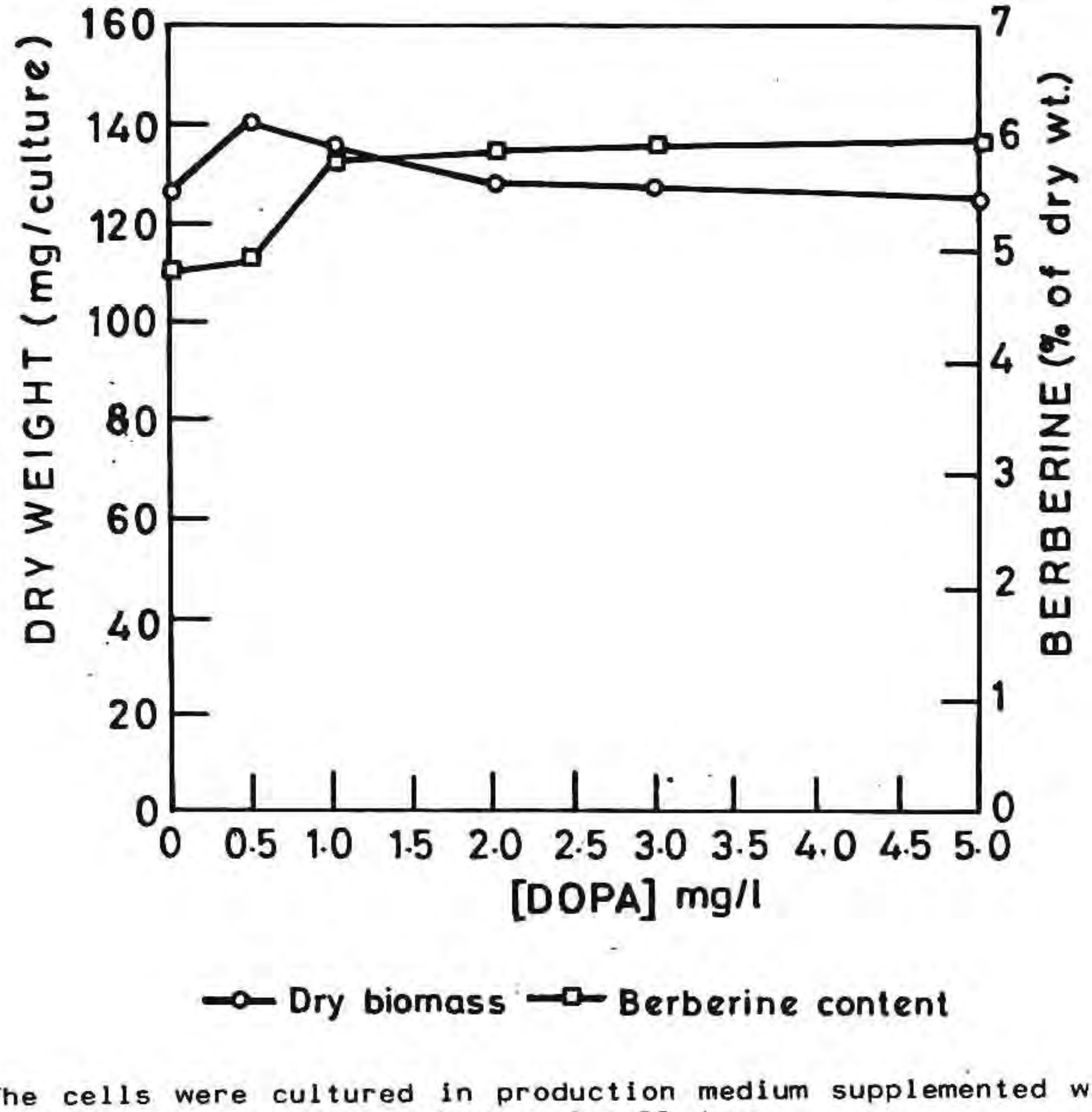
Data collected on 28th day of inoculation
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Sl.No.1. In all cases $p < 0.05$. *$p > 0.05$

't' values of Table 6.2

't' values between	't' values of group A	't' values of group B
1 and 2	10.478	2.081
1 and 3	4.235	17.691
1 and 6	1.376*	1.887

* not significant at 5% level

Fig.6.3 : Effect of L-dopa supplementation on growth and berberine production in the cell suspension cultures of *Coscinium fenestratum*



The cells were cultured in production medium supplemented with various concentration of L-dopa for 28 days.

The values plotted are the mean of five independent determinations

decreased gradually with increase in L-dopa concentration. Berberine content of the cultures increased significantly with the increase in the concentration of L-dopa giving a maximum effect at 1 mg/l and on further increase there was no increase in berberine production. At 1 mg/l L-dopa, there was about 28 percent increase in the total berberine content.

6.3.3. Effect of addition of tryptophan

The effect of tryptophan supplementation on berberine accumulation was studied and the results are given in Table 6.3. There was a reduction in the total berberine content of the cell cultures when tryptophan was supplemented to the production medium. A progressive reduction in the total berberine content of the cell cultures was observed with increase in the concentrations of tryptophan in the medium. A more or less same effect was observed for the biomass too. These results are also represented in the Fig.6.4.

6.3.4. Effect of addition of L-phenylalanine

The effect of phenylalanine on the production of berberine was studied and the results are given in Table 6.4. Berberine content of the cell cultures was considerably reduced when phenylalanine was supplemented in the production medium. This berberine lowering effect

Table 6.3 : Effect of different concentrations of L-Tryptophan on cell biomass and berberine production in cell suspension cultures of *Coscinium fenestratum* (Medium: Production medium + L-Tryptophan)

Sl. No.	Concentration (mg/l)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight
			A		B	
1	0.00	615.73 (±3.52)	125.85(±1.04)	2.91(±0.27)	6.09(±0.12)	4.83
2	0.50	586.17(±3.89)	141.25(±1.77)	2.39(±0.78)	5.16(±0.83)	3.66
3	1.00	538.30(±6.29)	137.00(±2.55)	2.41(±0.35)	5.08(±0.72)*	3.71
4	2.00	540.75(±6.72)	126.75(±1.70)	2.32(±0.25)	4.53(±0.68)	3.58
5	3.00	496.65(±4.84)	114.63(±1.38)	1.53(±0.32)	3.36(±0.80)	2.93
6	5.00	481.63(±3.97)	98.87(±2.13)	1.26(±0.33)	2.68(±0.77)	2.71

Initial biomass : Fresh weight : 349.42(±1.88) mg
Dry weight : 68.28(±1.137)mg

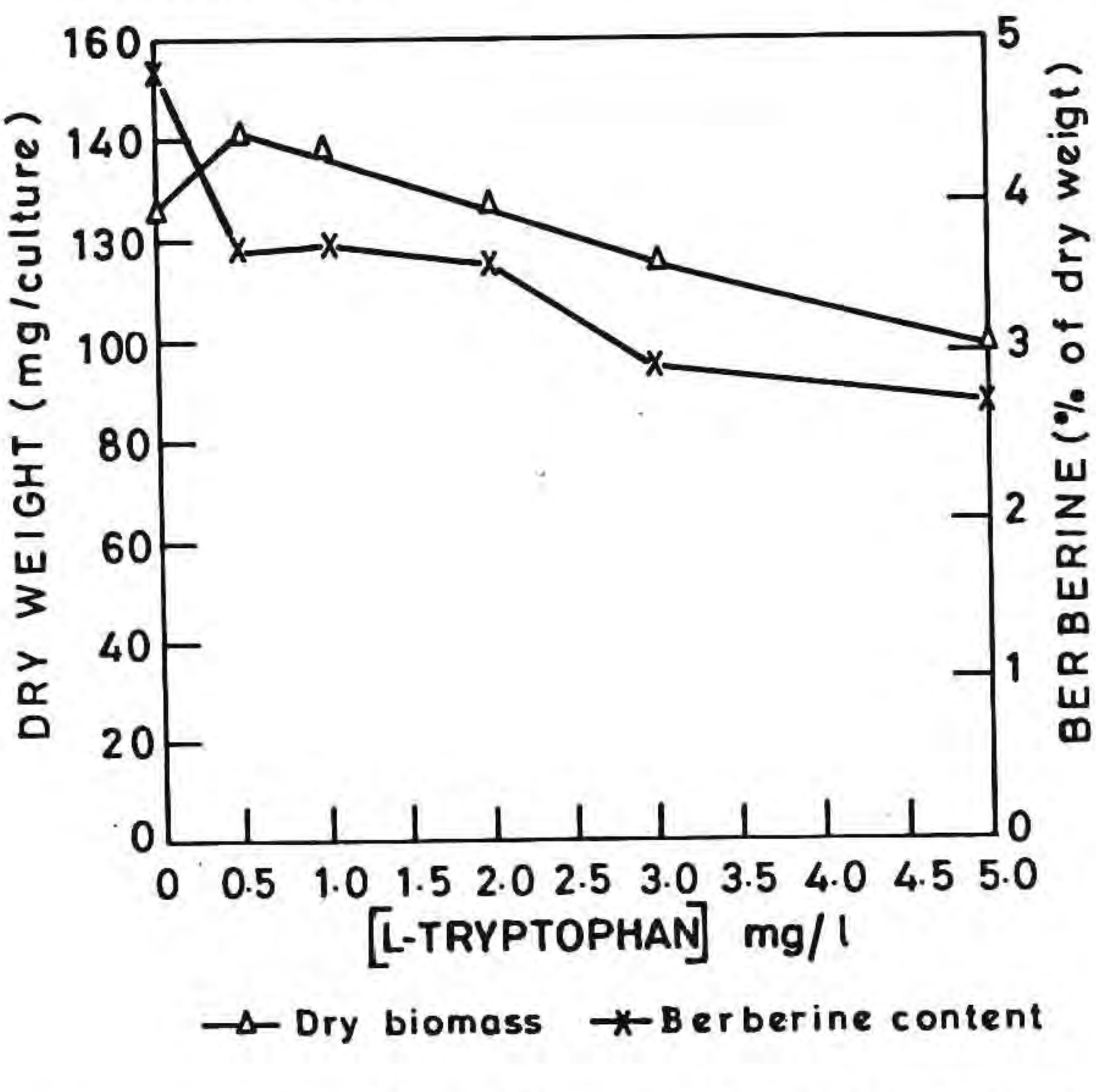
Data collected on 28th day of inoculation
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Sl.No.1. In all cases $p < 0.05$.

't' values of Table 6.3

't' values between	't' values of group A	't' values of group B
1 and 2	8.810955	-1.40272*
1 and 3	4.616087	-1.75024*
1 and 6	-14.984600	-5.53493

* not significant at 5% level

Fig.6.4 : Effect of L-tryptophan on growth and berberine production in cell suspension cultures of *Coscinium fenestratum*



The cell suspension cultures are grown in production medium

The values plotted are the mean of five independent determinations

Table 6.4 : Effect of different concentrations of L-Phenylalanine on cell biomass and berberine production in cell suspension cultures of *Coscinium fenestratum* (Medium: Production medium + L-Phenylalanine)

Sl. No.	Concentration (mg/l)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
			A		B	
1	0.00	615.73(±3.52)	126.05(±1.04)	2.81(±0.27)	6.09(±0.12)	4.83
2	0.50	457.88(±2.11)	97.31(±1.14)	1.23(±0.78)	2.87(±0.61)	2.95
3	1.00	362.53(±3.39)	85.26(±1.22)	1.91(±0.91)	1.96(±0.39)	2.31
4	2.00	377.00(±3.39)	85.61(±1.53)	0.79(±0.21)	1.69(±0.52)	1.98
5	3.00	361.28(±4.21)	78.25(±2.11)	0.78(±0.38)	1.40(±0.45)	1.79
6	5.00	353.20(±2.13)	76.80(±1.92)	0.54(±0.17)	1.29(±0.50)	1.68

Initial biomass : Fresh weight : 352.18(±1.44) mg
Dry weight : 69.72(1.30) mg

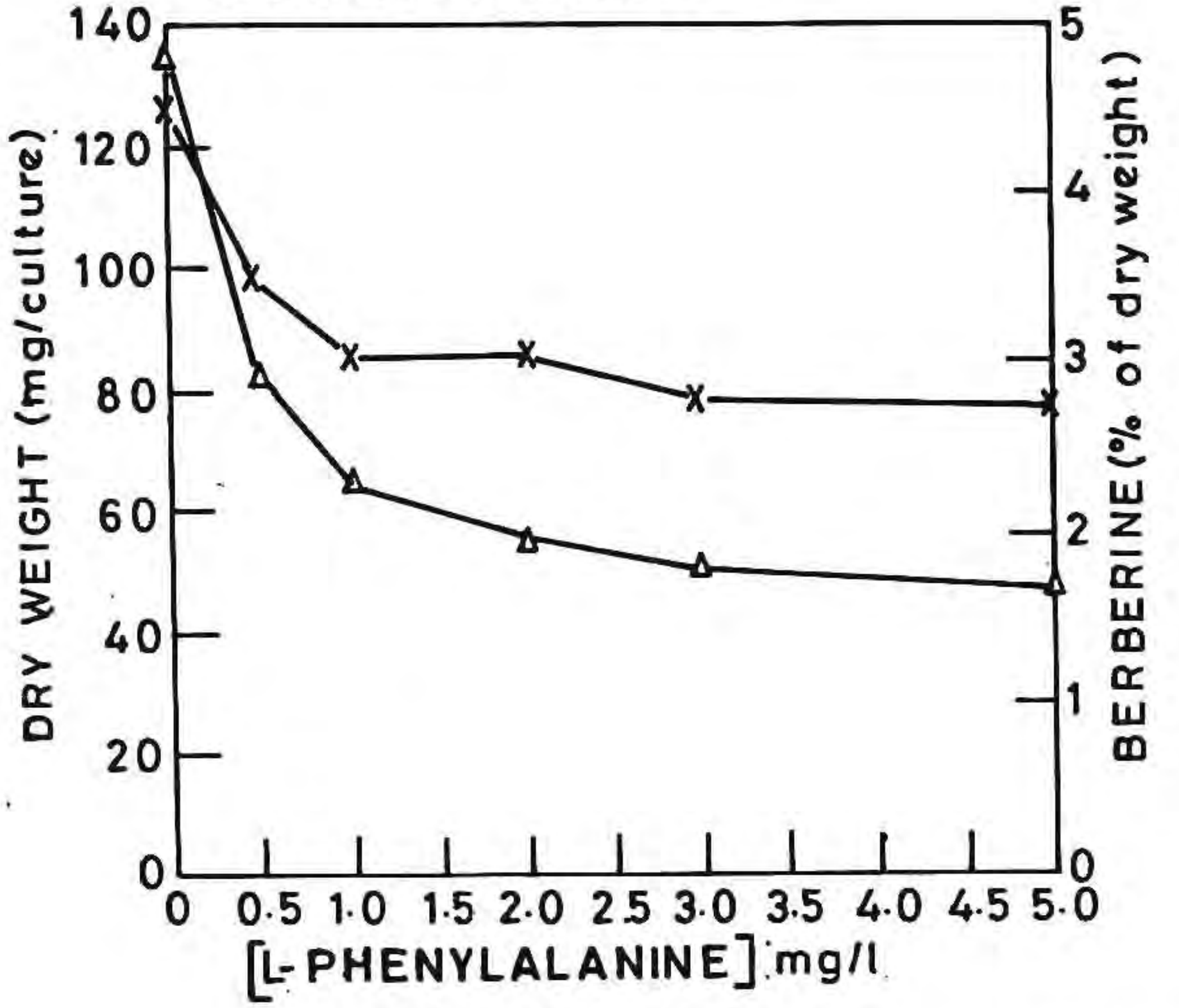
Data collected on 28th day of inoculation
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Sl.No.1. In all cases $p < 0.05$

't' values of Table 6.4

t values between	't' values of group A	't' values of group B
1 and 2	-32.184	-12.802
3 and 4	- 3.571	- 0.533*

* not significant at 5% level

Fig.6.5 : Effect of L-phenylalanine supplementation on growth and berberine production in cell suspension cultures of *Coscinium fenestratum*



The cell suspension cultures were grown in production medium (MS basal medium supplemented with 35 µM NAA and 8 µM BAP)

The values plotted are the mean of five independent determinations

was observed at 1 mg/l of phenylalanine and on further increase in its concentration there was no further significant reduction in berberine production. A reduction in the biomass formation was also observed in all concentrations of phenylalanine supplementation. A progressive reduction in the biomass and berberine content of the cell cultures was noticed with increase in the concentration of phenylalanine present in the production media. The results obtained are also presented in Fig.6.5.

6.4. DISCUSSION

The rate of production of a secondary metabolite depends on (a) availability of precursor substances (b) activity of different enzymes of the pathway which in turn will depend on the presence of activators or inhibitors and the concentration of the enzyme and (c)rate of transport or secretion.

Therefore the rate of production of berberine will depend, among other factors, on the availability of precursor substances. Tyrosine is the precursor amino acid. The supplementation of the amino acid precursor tyrosine increased the total berberine content of the cell cultures. L-dopa also had a similar effect. By providing amino acid precursors the interplay of the regulation of synthesis of tyrosine and its confounding effect on berberine production is eliminated and therefore whatever effects are

seen are due to changes in the berberine pathway. Even-though the biosynthetic pathway of berberine at enzyme level is clear with minor changes in some of the steps and sequence of reactions and intermediates, its regulatory aspects at the enzyme level is not elucidated.

The biosynthesis of alkaloids is usually controlled by the availability of amino acid precursors. But the over production of amino acid precursors or supplementation of the amino acid to the cell cultures or tissues may not cause over production of the end-products in all the cases. This was shown in the case of indole alkaloid production by tryptophan over producing *Catharanthus roseus* cell cultures[283,284]. Although the free tryptophan (the precursor of indole alkaloids) level within the cell was very high, it did neither result in an increased accumulatin of tryptamine nor of indole alkaloids in the case of a cell line resistant to 5-methyl tryptophan. Increased alkaloid production was obtained by feeding L-tryptophan, tryptamine, secologanin, loganin, loganic acid and shikimic acid[152]. In the present investigation it was found that there was an initial increase in the production of berberine with the supply of tyrosine into the production medium. Highest berberine content was observed when 1 mg/l tyrosire was supplemented to the production medium. But

when its concentration was increased there was no corresponding increase in the production of berberine. This can be attributed to the tyrosine mediated regulation of some enzymes of berberine biosynthetic pathway. Another possibility is that in the cell culture, the rate of berberine synthesis may be limited by the insufficient supply of L-tyrosine and this may be the reason for the initial increase due to the additional supply of tyrosine. The reduction in the berberine production in the presence of excess tyrosine may also be due to an inhibitory action of some enzymes of the biosynthetic pathway of berberine. Another possible reason for reduced berberine content at high concentration of tyrosine may be a possible interference with the secretary behaviour by the amino acids. An interference with the secretion can lead to accumulation in the cells and thereby can result in a reduced synthesis of berberine.

When L-dopa was supplemented there was an increase in the berberine content as observed in the case of L-tyrosine supplementation. The effect of L-dopa is not similar to that of L-tyrosine, eventhough the former is formed from L-tyrosine. L-dopa appeared to have a saturating effect causing a maximum production of berberine at 1 mg/l and on further increase, there was no further increase in berberine production which is different from that observed for tyrosine at high concentration.

The other two amino acids tested viz. phenylalanine and tryptophan are not involved in the biosynthesis of berberine. These were supplemented in the production media to see whether these aromatic amino acids have any indirect role in regulating the formation of berberine so that it can be used to increase the production in the cell cultures. But the results obtained indicate that the supplementation of tryptophan decreased the product formation and biomass formation compared to the cultures grown in normal production media.

In the case of phenylalanine, its supplementation caused reduction in the total berberine content of the cultures. High concentration of phenylalanine caused more reduction in the product formation.

Biosynthesis of tyrosine takes place by hydroxylation of phenylalanine. But addition of phenylalanine does not produce any effect similar to that of tyrosine. It is not known whether phenylalanine is converted to tyrosine in *C. fenestratum*. It may be possible that this reaction may not occur or may be taking place at very low rate. There is another possibility that phenylalanine competes with tyrosine for uptake whereby inhibiting the conversion of tyrosine which can lead to reduced synthesis of berberine. Further phenylalanine may also compete with tyrosine or with any of its products in the biosynthesis

of berberine with the result that the available tyrosine is not effectively converted to berberine.

In the cell culture system there may be a balance between different primary metabolite amino acids which regulates the biosynthesis of secondary metabolites by triggering or regulating specific enzymes. Lockwood and Essa[143] have shown that administration of certain group of amino acids and primary metabolites like ornithine, arginine, glutamic acid and nicotinic acid respectively reduced the nicotine level in the cell cultures in *Nicotiana tabacum*. It also reduced the cell growth and increased the level of another alkaloid anatabine. The feeding of the precursors of these also reduced growth and nicotine content, whereas it increased the presence of other alkaloids notably anatabine. There are some other examples also for the inhibitory or regulatory effect of some amino acids on the biosynthesis of secondary products. Yazaki et al[285] observed that the shikonin free cells cultured in LS medium accumulated large amounts of different amino acids such as glutamine, glutamic acid, asparagine,aspartic acid etc than shikonin producing cell cultures. They demonstrated that supplementation of excess of glutamine in the medium repressed the shikonin production by the shikonin producing cell cultures also. The production

of shikonin was under the strong influence of glutamine, a remote primary metabolite. Other amino acids like glutamic acid show inhibitory effect to a lesser extent.

All these indicate that certain amino acids in excess concentration can inhibit specific secondary metabolic pathway. These primary metabolites may not directly involve in the biosynthetic pathway. Thus from the present study it is concluded that supplementation of precursor amino acids, tyrosine or similar primary metabolites like L-dopa of the biosynthetic pathway, though results in an increase in berberine production, the increase does not appear to be commensurate with the amount of precursors supplemented to the medium. Amino acids like L-tryptophan and L-phenylalanine eventhough are not involved directly in the biosynthesis of berberine have some inhibitory or regulatory effect on its biosynthesis.

CHAPTER VII

CHAPTER VII

ACTIVITY OF ENZYMES OF NITROGEN METABOLISM AND ATPase OF CELL CULTURES OF *COSCINIUM FENESTRATUM*

7.1. INTRODUCTION

The optimization of media and hormonal conditions for increased production of berberine has led to the formulation of two media viz. "Production medium" and "growth medium". The growth medium has been optimized for getting rapid growth and maximum biomass whereas in production medium the biomass shows less growth but synthesizes large quantity of berberine. This altered berberine biosynthesis and growth strategies of the cultures grown in the two media will depend among other factors, on the primary metabolism induced by the nutritional status or the chemical environment of the cultured cells.

One of the methods to monitor the level and quality of biochemical reactions of the primary metabolism is to study the activity of certain specific key enzymes which are involved in the normal primary metabolic pathways. Nitrogen metabolism is one of the important events among the primary metabolic pathways. It starts with inorganic nitrogen and leads through amino acids to proteins

and other nitrogenous organic compounds. It is linked to other metabolic pathways such as synthesis of vitamins, nucleic acids etc. The most important point as far as alkaloid biosynthesis is concerned is that the nitrogen metabolism is also extended through amino acid precursors to the secondary metabolism and terminates by the formation of specific alkaloids. Therefore certain important enzymes of nitrogen metabolism such as nitrate reductase, glutamine synthetase, glutamate dehydrogenase and glutamate synthase were selected for monitoring the level of primary metabolism by studying their activity. Another important enzyme which participates in the primary metabolism is ATPase which is a key enzyme in the process of energy transactions within the cells during both primary and secondary metabolism.

7.1.1. Nitrate Reductase (NR) (NADPH: Nitrate oxido reductase EC. 1.6.6.2)

Ammonium ions and ammonia are toxic and cannot be accumulated to any appreciable extent without damage to the cells. But in the cells, NH_4^+ is the usable form of inorganic nitrogen. Nitrate is reduced *in vivo* to ammonia and incorporated into organic acids, which are the intermediates of carbohydrate metabolism to form amino acids[286]. Nitrate reductase catalyses the reduction of nitrate and forms the first step in the utilization

of nitrate and further assimilation of ammonia. An excellent discussion of the biochemical and physiological aspects of nitrate metabolism was made by Hewitt *et al*[287].

7.1.2. Enzymes of glutamate formation

Primary incorporation and recycling of ammonia into organic compounds is generally accepted to occur through glutamatedehydrogenase-glutamine synthetase-glutamte synthase pathway[288].

Glutamate dehydrogenase (GDH)

(L-glutamate: NAD(P) Oxidoreductase (deaminating) (C. 1.4.1.3) glutamate dehydrogenase facilitates entry of NH_3 for forming amino nitrogen (eqn.1) by the process known as reductive amination.

$$\alpha\text{-Ketoglutarate} + NH_3 + NADPH\ (\text{or}\ NADH) \xrightarrow{\text{GDH}} L\text{-glutamate} + H_2O + NADP^+\ (\text{or}\ NAD) \quad (1)$$

Glutamine synthetase (GS) (EC. 6.3.1.24)

It is another important NH_3 assimilating enzyme which synthesizes glutamine from glutamate and NH_3 (eqn.2).

$$\text{Glutamate} + NH_3 + ATP \xrightarrow{\text{glutamine synthetase}} \text{Glutamine} + \text{orthophosphate} + ADP \quad (2)$$

Glutamate synthase (GOGAT) (L-glutamate: $NADP^+$ Oxidoreductase (transaminating) (EC. 1.4.1.13)

Glutamate synthase catalyses transamination of glutamine to form glutamate and other amino acids as shown below

$$\text{Glutamate + L-glutamine + NADPH + Ferredoxin(reduced)} \longrightarrow \text{2L-glutamate + } NADP^{+} \text{ + Ferredoxin (oxidised)} \quad (3)$$

The glutamine synthetase and glutamate synthase together act as a cyclic pathway. In glutamate synthase cycle the glutamate acts both as an acceptor and product of ammonia assimilation[289] (Fig. 7.1). Since the nitrate reductase and enzymes of glutamate metabolism are very important in nitrate metabolism and synthesis of amino acids their activity in the cells can indicate the overall primary metabolic level.

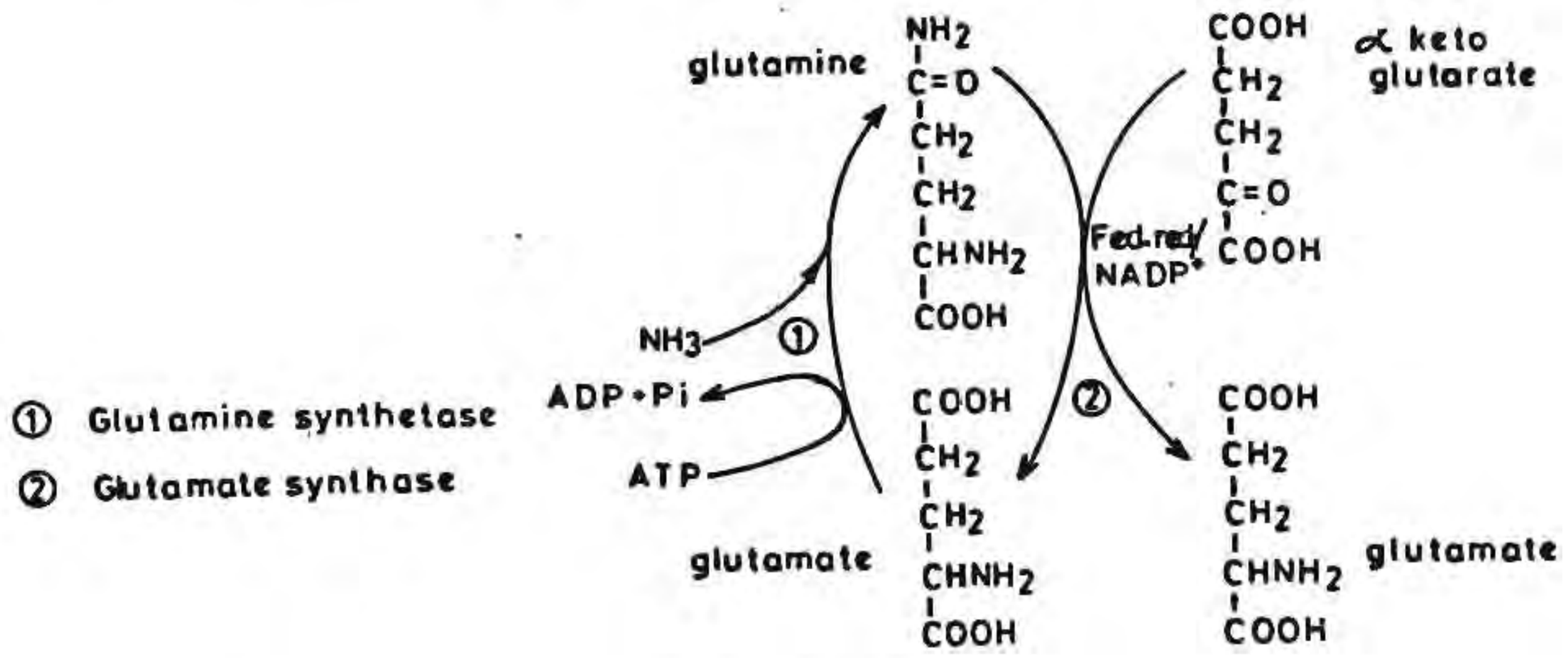


Fig. 7.1

7.1.3. ATPase

ATPase is an important enzyme in the cellular metabolism associated with the consumption or release of energy. ATP is required for the active absorption of nutrients and other materials by the cultured plant cells.

In the present study, the activity of the above said enzymes of the cells, cultured in the growth medium and production medium were determined as a measure of the level of primary metabolism of the cells under these varied conditions of growth and berberine production. Studies were also carried out to find out the change in the activity of the enzymes brought about by the addition of amino acids to the production medium.

7.2. MATERIALS AND METHOD

The stock cell suspension cultures were maintained in B_5 medium containing 10 µM NAA and 1 µM BAP as described earlier. Abut 350 mg of cells were filtered out and transferred to flasks containing growth medium (medium of the stock culture) or production medium after a wash with the same medium and incubated on a rotary shaker (140±5 rpm) at 25±2°C for 32 days. The cultures were harvested every four days and the assay of the different enzymes of nitrogen metabolism and ATPase were carried out as described in Chapter II.

In order to study the effect of amino acids such as tyrosine, tryptophan, phenylalanine and L-dopa whose effects on berberine synthesis was studied, the production medium was supplemented with amino acids and maintained for different periods. Cells were harvested at different periods and the activity of nitrate reductase, glutamate

dehydrogenase, glutamine synthetase, glutamate synthetase and ATPase were determined as described in Chapter II. Protein was also estimated as described in Chapter II.

7.3. RESULTS

7.3.1. Activity of Nitrate Reductase (NR)

The activity profile of *in vivo* NR of cells grown in growth medium and production medium is given in Fig.7.2 and Table 7.1. The activity profile was almost identical in both cultures. There was an initial decline in the enzyme activity in both cultures upto four days in the production medium and upto eight days in the growth medium. Thereafter it increased gradually upto 18 to 20 days of the culture period. After that it declined gradually. In cultures of production medium, NR activity decreased and attained a steady state by 24th day after which no further decline in the NR activity was observed . Whereas in the case of cultures in growth medium, the NR activity continued to decline till the end of the culture period. Inspite of the similarity in the activity profile of nitrate reductase, the cells cultured in production medium are characterised by a higher level of activity throughout the growth period. The period of gradual increase in the NR activity and its high peak around 18 to 20th day of culture period was concomitant . with the logarithmic

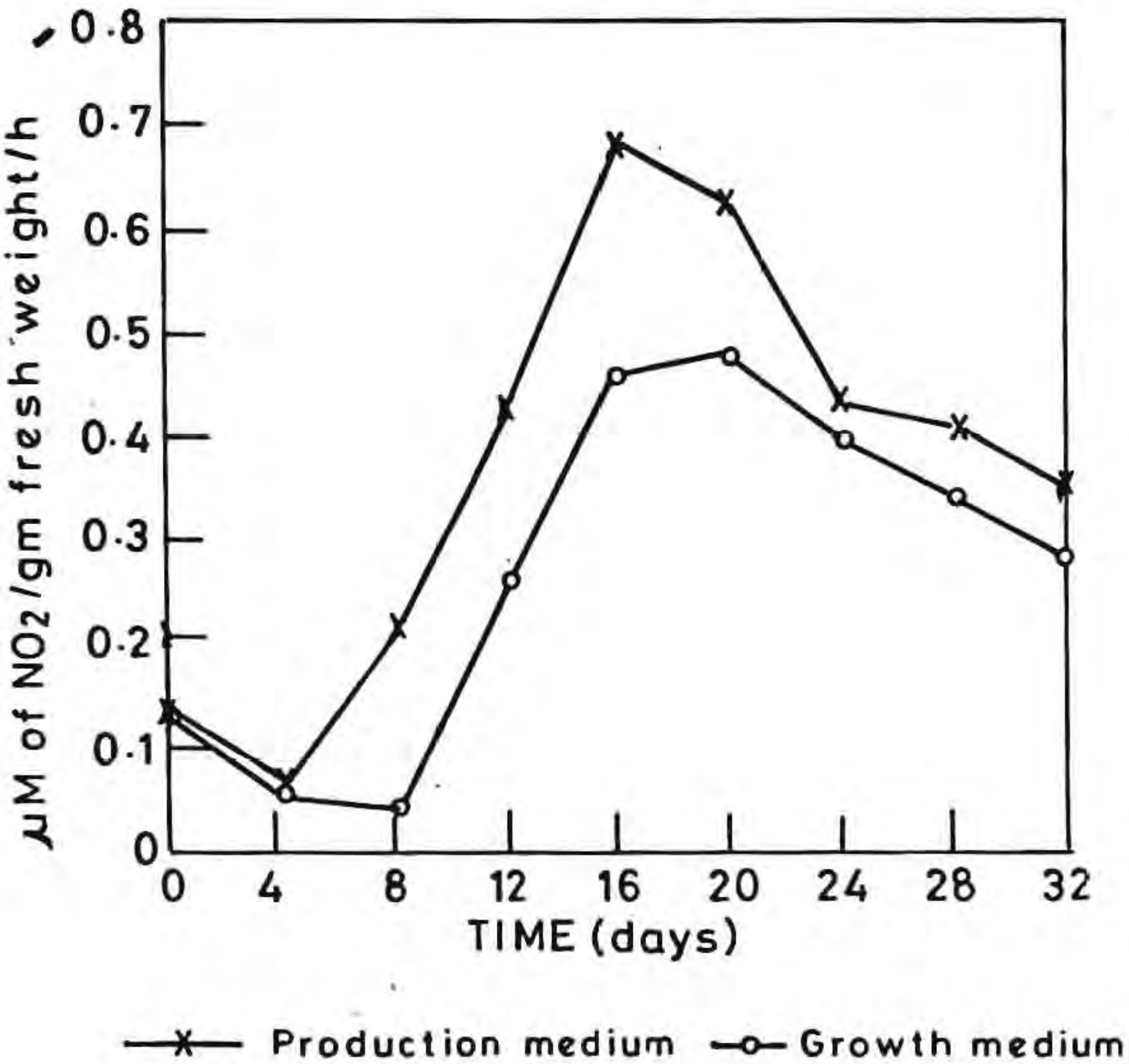
Table 7.1 : Nitrate reductase (NR) activity of *Coscinium fenestratum* cells cultured in growth and production media

Sl. No.	Time (days)	Growth medium (µ moles of NO_2/gm fresh wt/hr)	Production medium (µ moles of NO_2/gm fresh wt/hr)
		A	B
1	0	0.128(±0.004)	0.125(±0.001)*
2	4	0.054(±0.005)	0.063(±0.004)
3	8	0.038(±0.003)	0.211(±0.002)
4	12	0.259(±0.004)	0.422(±0.001)
5	16	0.463(±0.005)	0.683(±0.008)
6	20	0.481(±0.003)	0.625(±0.010)
7	24	0.395(±0.003)	0.431(±0.014)
8	28	0.342(±0.004)	0.408(±0.020)
9	32	0.281(±0.004)	0.352(±0.013)

Each value is the mean ±SEM of five independent determinations

The values of group A and B compared
In all cases $p < 0.05$. *$p > 0.05$

Fig.7.2 : Assay of Nitrate Reductase



Cell cultures of *Coscinium fenestratum* grown in production medium and growth medium were harvested at different time intervals and the enzyme activity was measured.

The values plotted are the mean of five independent determinations.

period of growth cycle and active production of berberine both in growth medium and production medium.

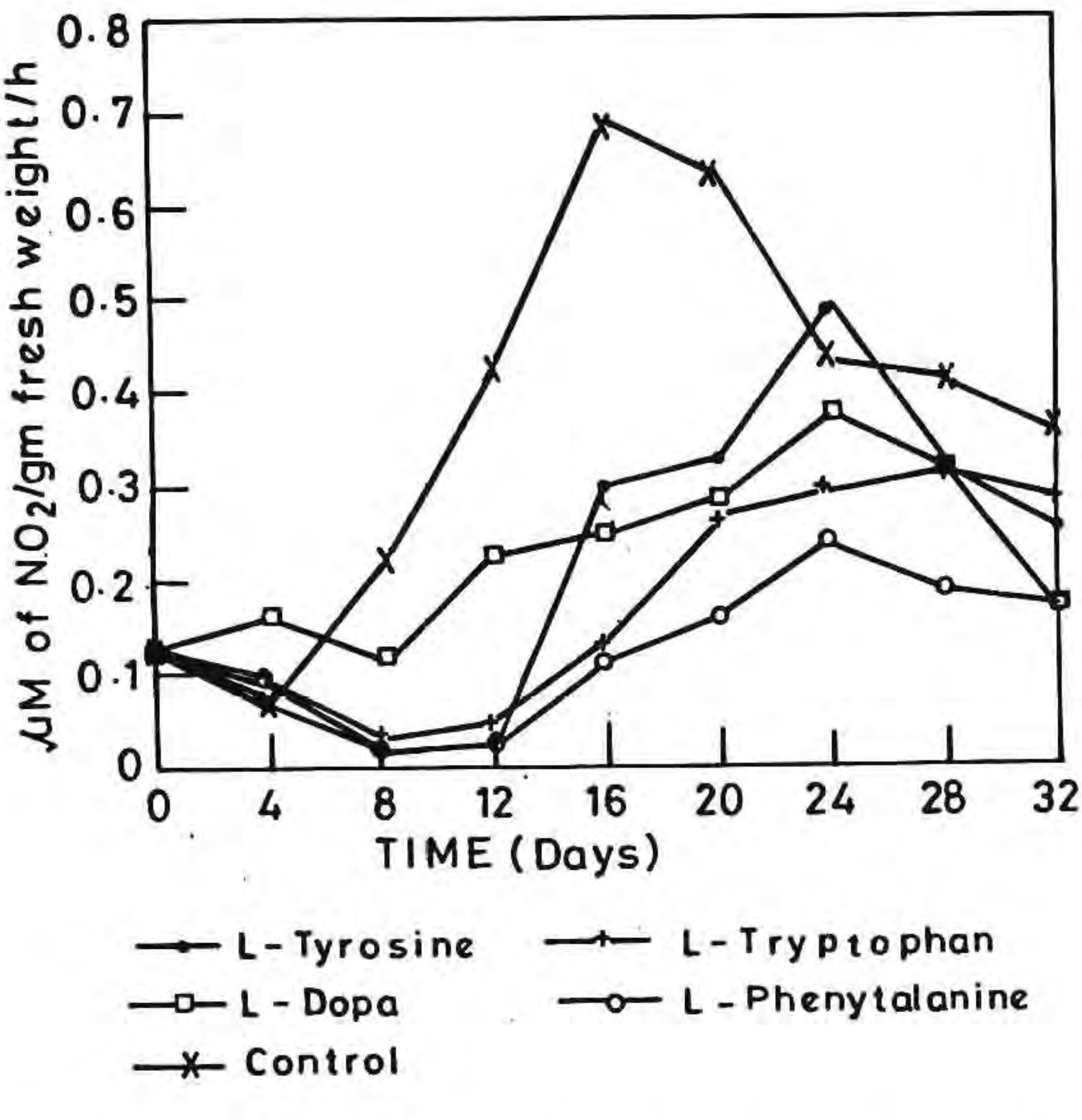
The effect of addition of individual amino acids at a concentration of 2 mg/L to production medium on Nitrate Reductase activity in cell culture of *C. fenestratum* is shown in Table 7.2. NR activity profile of these cultures on amino acid supplementation was compared in Fig.7.3. Supplementation of individual amino acids reduced the nitrate reductase to varying degrees particularly at the early stages of culture periods. Among the amino acids used L-tryptophan and L-phenylalanine strongly suppressed and then slowly came upto the normal level as observed in the control. L-tyrosine also strongly suppressed the NR activity in the early stages of the culture period but after 12th day it increased suddenly and reached a peak around 24th day. L-dopa an intermediate in the biosynthesis of berberine when added into the production medium also reduced the NR activity but the degree of reduction was less compared to the other amino acids supplemented. Thus the result indicated that NR activity was suppressed by all amino acids and the activity was increased to near normal level after 12 to 16 days eventhough the degree of inhibition of NR activity varied with the type of amino acids.

Table 7.2 : Effect of amino acid supplementation on Nitrate Reductase (NR) activity of *Coscinium fenestratum* cells cultured in production medium

Sl. No.	Time (days)	L-Tyrosine (µmoles of NO_2 formed/gm fresh wt. of cells/hr)	L-Tryptophan	L-Phenylalanine	L-Dopa
		C	D	E	F
1	0	0.126(±0.008)*	0.128(±0.003)*	0.131(±0.010)*	0.128(±0.005)*
2	4	0.084(±0.003)	0.069(±0.001)	0.091(±0.008)	0.158(±0.003)
3	8	0.015(±0.003)	0.024(±0.006)	0.018(±0.003)	0.113(±0.008)
4	12	0.026(±0.005)	0.041(±0.005)	0.020(±0.004)	0.224(±0.010)
5	16	0.299(±0.011)	0.124(±0.003)	0.114(±0.009)	0.249(±0.004)
6	20	0.324(±0.009)	0.263(±0.008)	0.157(±0.005)	0.283(±0.011)
7	24	0.485(±0.013)	0.287(±0.011)	0.233(±0.011)	0.376(±0.007)
8	28	0.319(±0.010)	0.308(±0.010)	0.183(±0.010)	0.311(±0.009)
9	32	0.250(±0.009)	0.281(±0.007)	0.165(±0.004)	0.172(±0.010)

Each value is the mean ±SEM of five independent determinations
The values were compared with those of group B in Table 7.1.
In all cases $p < 0.05$. *$p > 0.05$.

Fig.7.3 : Effect of amino acid supplementation on Nitrate Reductase activity in the cell cultures of *Coscinium fenestratum*



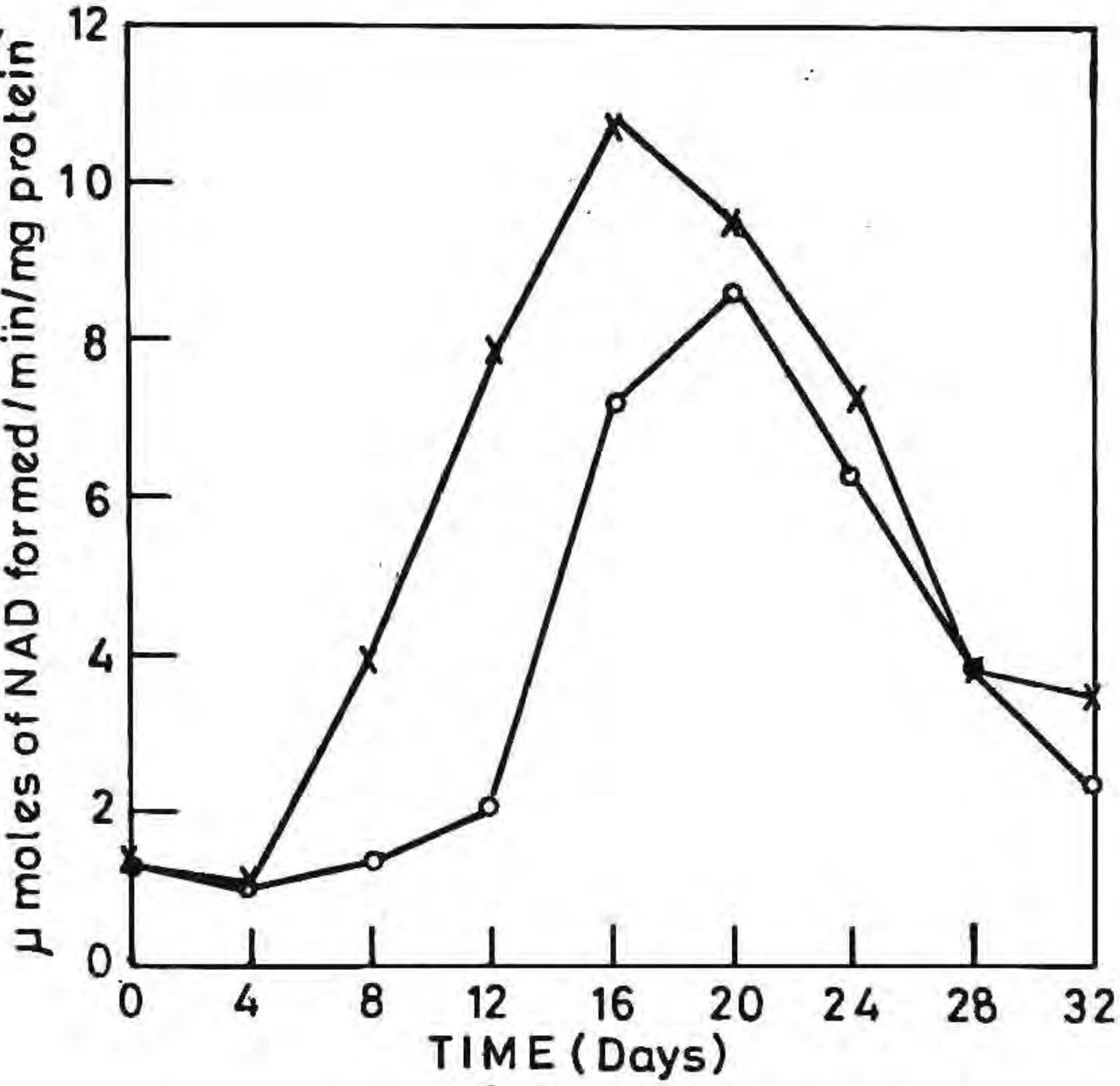
Enzyme activity in the cells cultured in production medium in the presence of different amino acids (50 µ g/l) were determined at different periods

7.3.2. Activity of Glutamate Dedhydrogenase (GDH)

The activity profile of GDH in the cells cultured in growth medium and production medium showed similarity with minor variations. In growth medium, GDH activity increased from fourth to twelfth day gradually and after that it attained a peak around 18 to 20th day, thereafter a gradual decline in the activity was observed. In the production medium, GDH activity increased from the fourth day itself and attained maximum around 16 to 20th day and decreased thereafter. Inspite of the similarity in the GDH activity in the two cultures, the cells grown in production medium were characterized by high level of activity compared to the cells grown in the growth medium. The Fig.7.4 shows that in both cultures GDH activity was maximum during the logarithmic phase of growth and berberine production which fell between 8th to 24th or 25th day of culture period (Fig.5B.1).

The variation in the GDH activity on amino acids supplementation individually to the production medium is shown in Fig.7.5. It is evident from the results that the GDH activity was reduced by the supplementation of amino acids. Though in all the cases, there was a decline in the enzyme activity due to the extracellular supplementation of amino acids, the pattern of GDH activity appeared

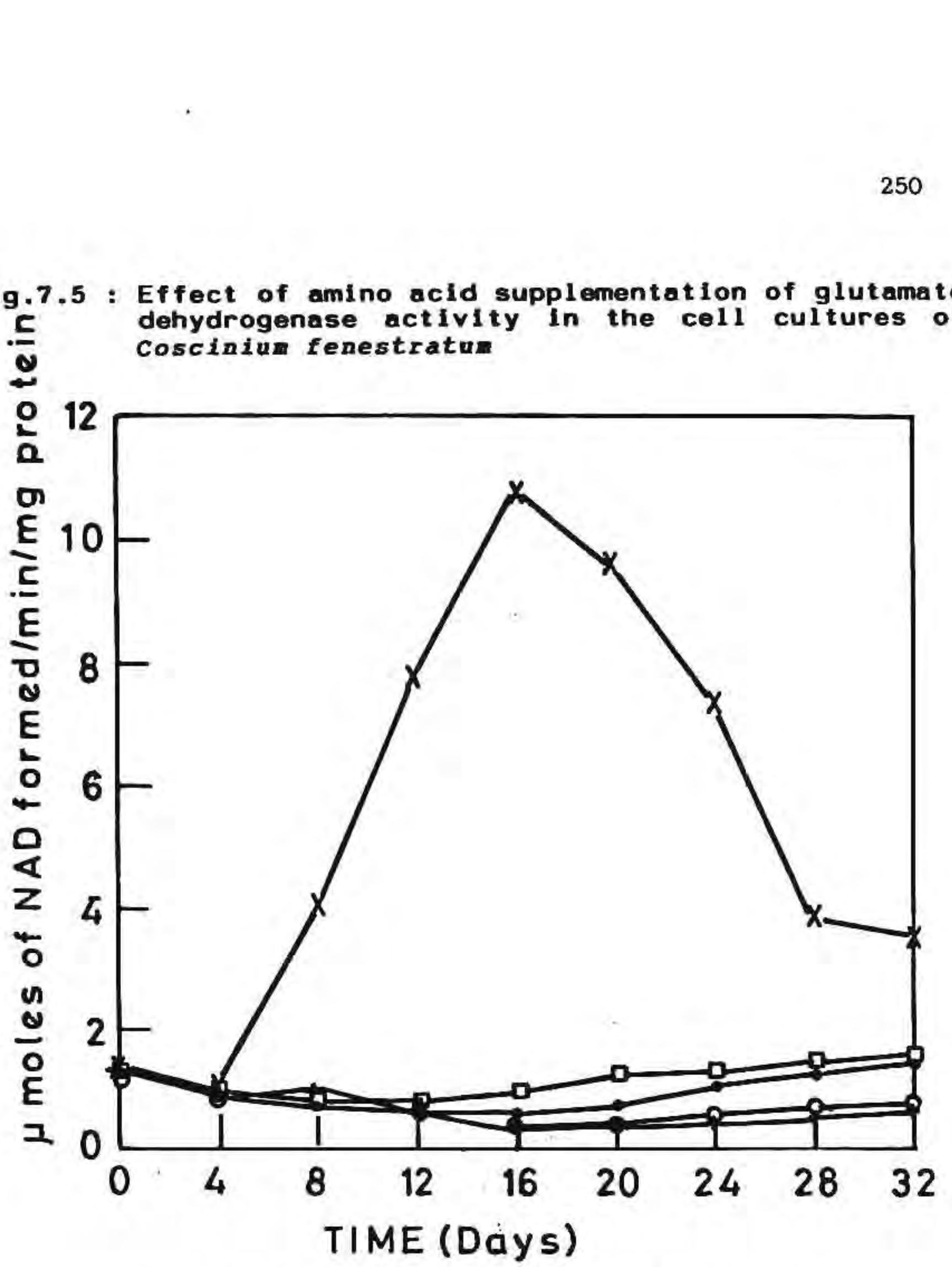
Fig.7.4 : Assay of glutamate dehydrogenase



Cell cultures of *Coscinium fenestratum* grown in production medium and growth medium for different time intervals were used for enzyme assay.

The values plotted are the mean of five independent determinations.

Fig.7.5 : Effect of amino acid supplementation of glutamate dehydrogenase activity in the cell cultures of *Coscinium fenestratum*



Enzyme activity of cells cultured in production medium

The values plotted are the mean of five independent determinations.

Table 7.3 : Glutamate dehydrogenase (GDH) activity of *Coscinium fenestratum* cells cultured in growth medium and production medium

Sl. No.	Time (days)	Growth medium (µmoles of NAD^+ formed/mg protein/min) A	Production medium B
1	0	1.126(±0.003)	1..130(±0.080)*
2	4	0.092(±0.010)	1.034(±0.033)
3	8	1.332(±0.002)	3.920(±0.018)
4	12	1.990(±0.010)	7.752(±0.081)
5	16	7.260(±0.030)	10.805(±0.052)
6	20	8.580(±0.030)	9.510(±0.011)
7	24	6.210(±0.031)	7.275(±0.037)
8	28	3.820(±0.220)	3.781(±0.037)
9	32	2.250(±0.130)	3.450(±0.071)

Each value is the mean ±SEM of five independent determinations

The values of group A and B compared.

In all cases $p < 0.05$. *$p > 0.05$.

Table 7.4 : Effect of amino acid supplementation on glutamate dehydrogenase (GDH) activity of *Coscinium fenestratum* cells cultured in Production medium

Sl. No.	Time (days)	L-tyrosine	L-tryptophan	L-phenylalanine	L-dopa
		(µ moles of NAD+ formed/mg protein/min)			
		C	D	E	F
1	0	1.330(±0.314)*	1.425(±0.171)*	1.187(±0.101)*	1.278(±0.091)*
2	4	0.872(±0.051)	0.729(±0.221)	0.940(±0.083)	0.922(±0.040)
3	8	0.624(±0.080)	0.990(±0.210)	0.615(±0.032)	0.749(±0.081)
4	12	0.597(±0.044)	0.631(±0.101)	0.590(±0.056)	0.693(±0.054)
5	16	0.538(±0.152)	0.286(±0.091)	0.449(±0.081)	0.880(±0.031)
6	20	0.651(±0.060)	0.374(±0.055)	0.452(±0.041)	1.169(±0.081)
7	24	0.983(±0.147)	0.497(±0.110)	0.418(±0.013)	1.204(±0.131)
8	28	1.249(±0.170)	0.530(±0.073)	0.577(±0.034)	1.389(±0.091)
9	32	1.372(±0.293)	0.579(±0.048)	0.695(±0.032)	1.448(±0.140)

Each value is the mean ±SEM of five independent determinations

The values were compared with those of group B in Table 7.3. In all cases $p < 0.05$

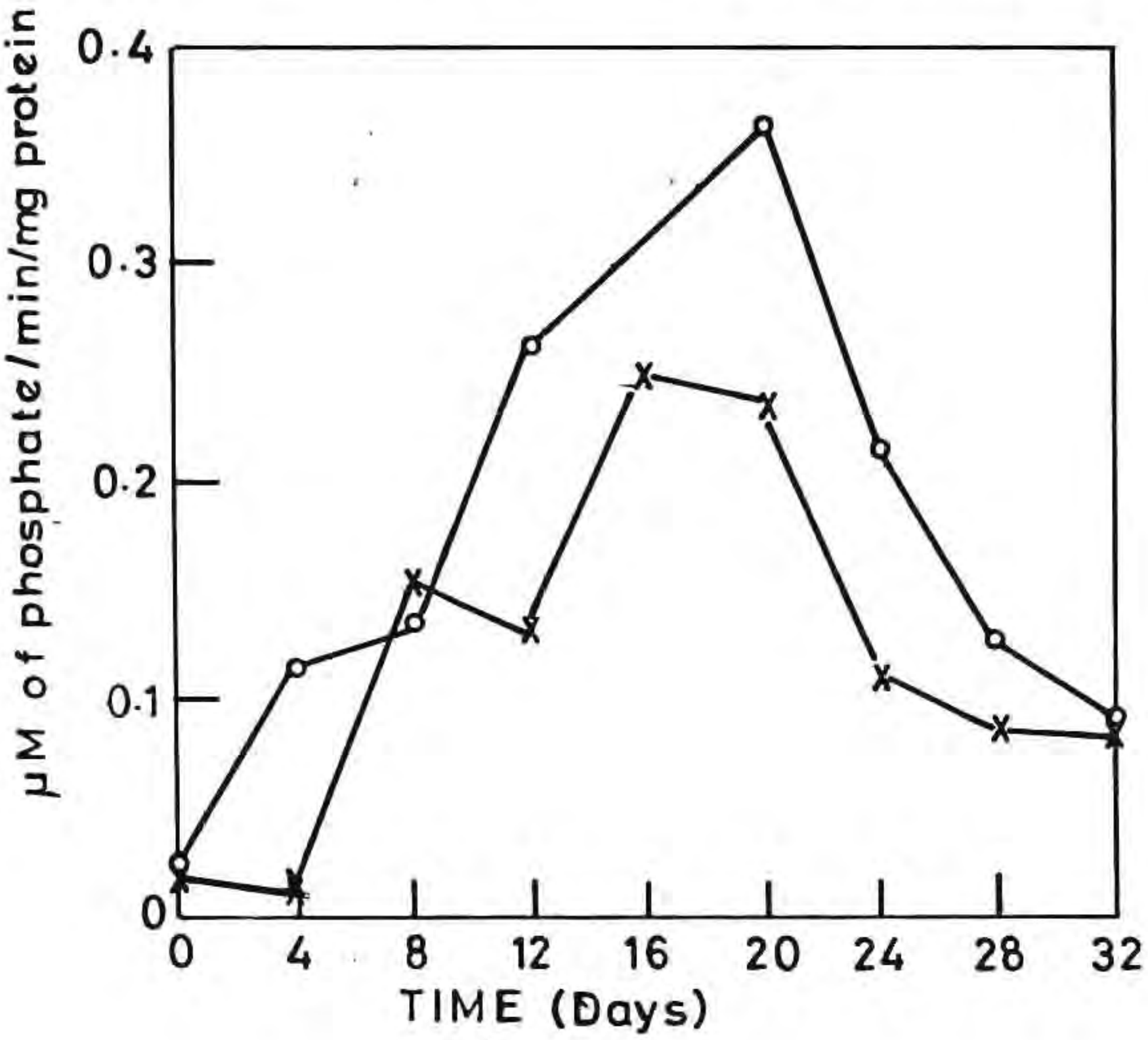
*$p > 0.05$.

to be different under these conditions. The presence of tyrosine reduced the GDH activity in the initial period but towards the mid period there observed a slight increase in the activity and it came to the normal level as seen in Fig.7.5. The same was observed when L-dopa was supplemented in the medium. But when tryptophan or phenylalanine was supplemented a gradual decline in the GDH activity was observed which reached a minimum and remained at the lower level without any increase in the enzyme activity thereafter. The results obtained are also presented in the Tables 7.3 and 7.4.

7.3.3. Activity of Glutamine Sythetase (GS)

The activity profile of GS in cells cultured in production medium and growth medium are represented in Fig.7.6 and the values are given in the Table 7.5. The activity pattern shows little difference in both cultures. GS activity gradually increased and reached maximum on 20th day and then declined gradually in production medium whereas in growth medium GS activity increased from fourth day and followed the pattern similar to that of production medium. The cells grown in production medium showed higher GS activity compared to the growth medium. Here also, the activity peak of GS was observed during the logarithmic period of cell growth i.e. between 8th to 24th day of culture period, where the berberine production was maximum.

Fig.7.6 : Assay of glutamate synthetase in the cell suspension cultures of *Coscinium fenestratum* grown in production medium and growth medium



The values plotted are the mean of five independent determinations

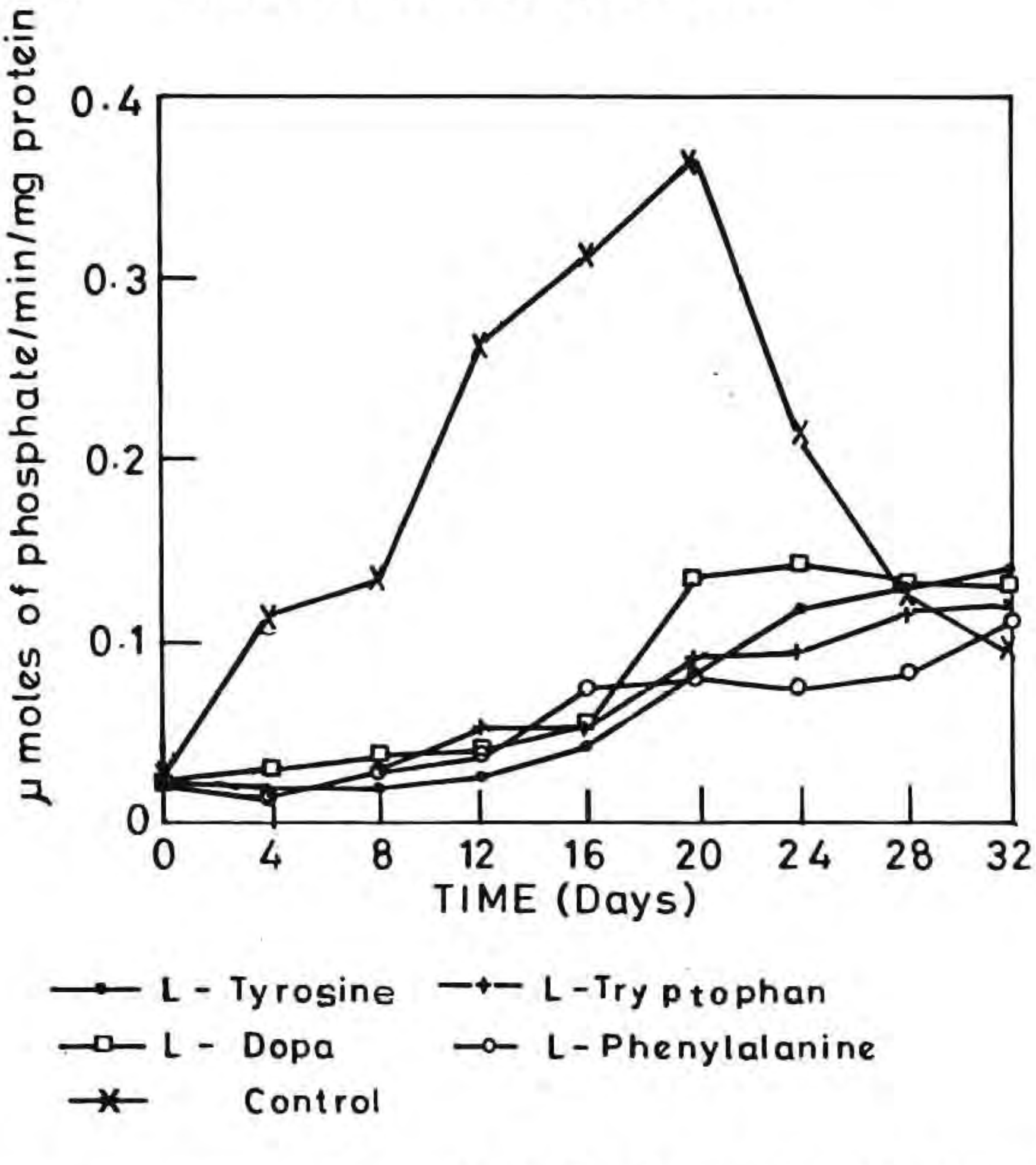
Table 7.5 : Glutamine Synthetase (GS) activity of *Coscinium fenestratum* cells cultured in growth medium and production medium

Sl. No.	Time (days)	Growth medium (µmoles of phosphate released/min/mg protein)	Production medium (µmoles of phosphate released/min/mg protein)
		A	B
1	0	0.019(±0.004)	0.021(±0.008)*
2	4	0.012(±0.003)	0.115(±0.010)
3	8	0.155(±0.009)	0.134(±0.012)
4	12	0.132(±0.011)	0.262(±0.005)
5	16	0.251(±0.007)	0.311(±0.008)
6	20	0.235(±0.010)	0.363(±0.014)
7	24	0.113(±0.005)	0.213(±0.013)
8	28	0.085(±0.012)	0.125(±0.011)
9	32	0.083(±0.004)	0.092(±0.010)

Each value is the mean ±SEM of five independent determinations.

The values of group A and B were compared.
In all cases $p < 0.05$. *$p > 0.05$

Fig.7.7 : Effect of amino acid supplementation on glutamate synthetase activity in the cell suspension cultures of *Cosˋcinium fenestratum*



Enzyme activity of cells cultured in production medium in presence of different amino acids (50 µg/l) were determined at different time intervals.

The values plotted are the mean of five independent determinations.

Table 7.6 : Effect of amino acids supplementation on Glutamine Synthetase (GS) activity of *Coscinium fenestratum* cells in production medium

Sl. No.	Time (days)	L-Tyrosine	L-Tryptophan	L-Phenylalanine	L-Dopa
		(µmoles of phosphate formed/mg protein/min)			
		D	C	E	F
1	0	0.023(±0.006)*	0.021(±0.012)*	0.021(±0.011)*	0.024(±0.002)*
2	4	0.013(±0.002)	0.018(±0.003)	0.014(±0.003)	0.027(±0.008)
3	8	0.018(±0.007)	0.031(±0.010)	0.029(±0.009)	0.035(±0.006)
4	12	0.023(±0.006)	0.049(±0.011)	0.035(±0.004)	0.039(±0.007)
5	16	0.041(±0.005)	0.051(±0.020)	0.073(±0.010)	0.053(±0.004)
6	20	0.081(±0.007)	0.087(±0.013)	0.081(±0.007)	0.132(±0.010)
7	24	0.116(±0.013)	0.092(±0.011)	0.072(±0.004)	0.141(±0.013)
8	28	0.131(±0.011)	0.114(±0.021)	0.080(±0.008)	0.132(±0.040)
9	32	0.138(±0.013)	0.117(±0.016)	0.110(±0.003)	0.129(±0.003)

Each value is the mean ±SEM of five independent determinations

The values were compared with those of group B of Table 7.5. In all cases In all cases $p < 0.05$. *$p > 0.05$.

The variations brought about by the addition of individual amino acids are represented in Fig.7.7 and the values are given in the Table 7.6. As in the earlier cases, here also all amino acids showed an inhibitory effect on GS activity. The suppressive effect of amino acids on GS activity declined gradually towards the end of culture period. This decline was rapid and maximum in the case of L-dopa followed by tyrosine. From the 12th day onwards the suppressive effect of tyrosine on GS activity declined and the activity gradually increased and attained normal level by 28th day. But GS activity, in the medium supplemented with L-dopa increased gradually after 16th day of inoculation and attained highest peak on 28th day while the inhibitory or suppressive effect induced by tryptophan was maintained almost constant till the end of the culture period. The suppressive effect of phenylalanine on GS activity was also similar to that of tryptophan but was less suppressive.

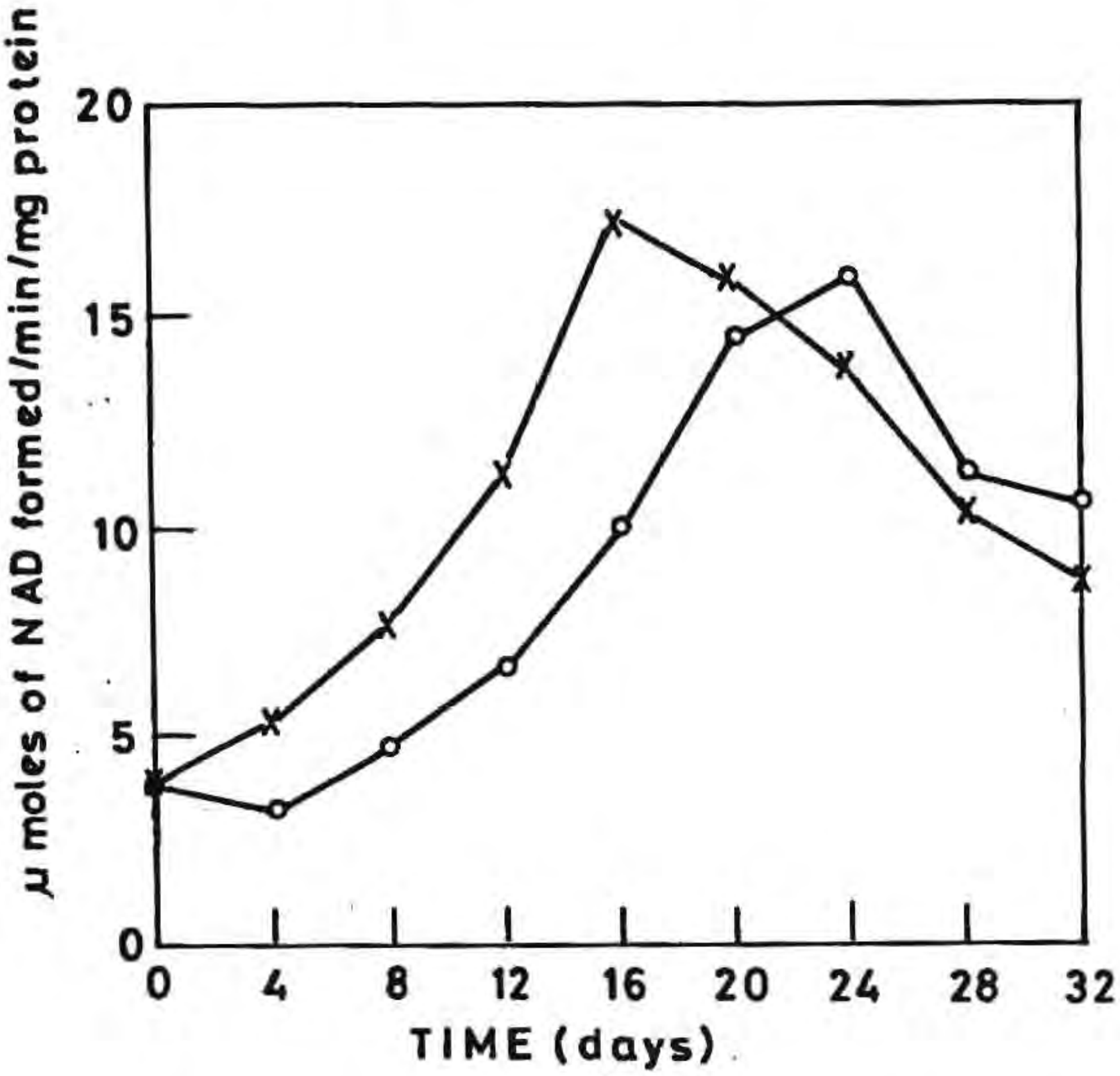
7.3.4. Activity of Glutamate Synthase (GOGAT)

The activity profile of glutamate synthase of cells cultured in growth medium and production medium are represented in Fig.7.8. The pattern of activity in both cultures showed distinct differences. In the cells grown in production medium, the activity increased from the first day itself and reached the maximum on 16th day.

Then it gradually decreased by the end of the culture period. Cells in the growth medium showed an initial decrease in the enzyme activity upto fourth day and then the activity increased gradually and reached maximum by 24th day; again it declined by the end of the culture period. Here also the peak activity of the enzyme was concomittant with the logarithmic phase and active berberine production. The values obtained are also shown in the Table 7.7.

The supplementation of amino acids to the production medium influenced the glutamate synthase activity also. All amino acids when supplemented individually suppressed the enzyme activity, but the pattern was different. In the presence of phenylalanine or tryptophan , the activity declined gradually and reached a minimum by 16th day and after that there was no change till the end of the culture period. The activity pattern of glutamate synthase was also found to be identical both in the presence of L-tyrosine and L-dopa. There was no significant variation in the activity upto 16 days and thereafter a gradual increase was observed in the enzyme activity till the end of culture period but never reached control levels. The variation in the glutamate synthase activity induced by the amino acid supplementation are represented in Fig.7.9 and the values are given in the Table 7.8.

Fig.7.8 : Glutamate synthase activity of cells in suspension cultures of *Coscinium fenestratum*



The cells were grown in production medium/growth medium and the enzyme activity was studied at different periods.

The values plotted are the mean of five independent determinations

Table 7.7 : Glutamate Synthase (GOGAT) activity of *Coscinium fenestratum* cells cultured in growth medium and production medium

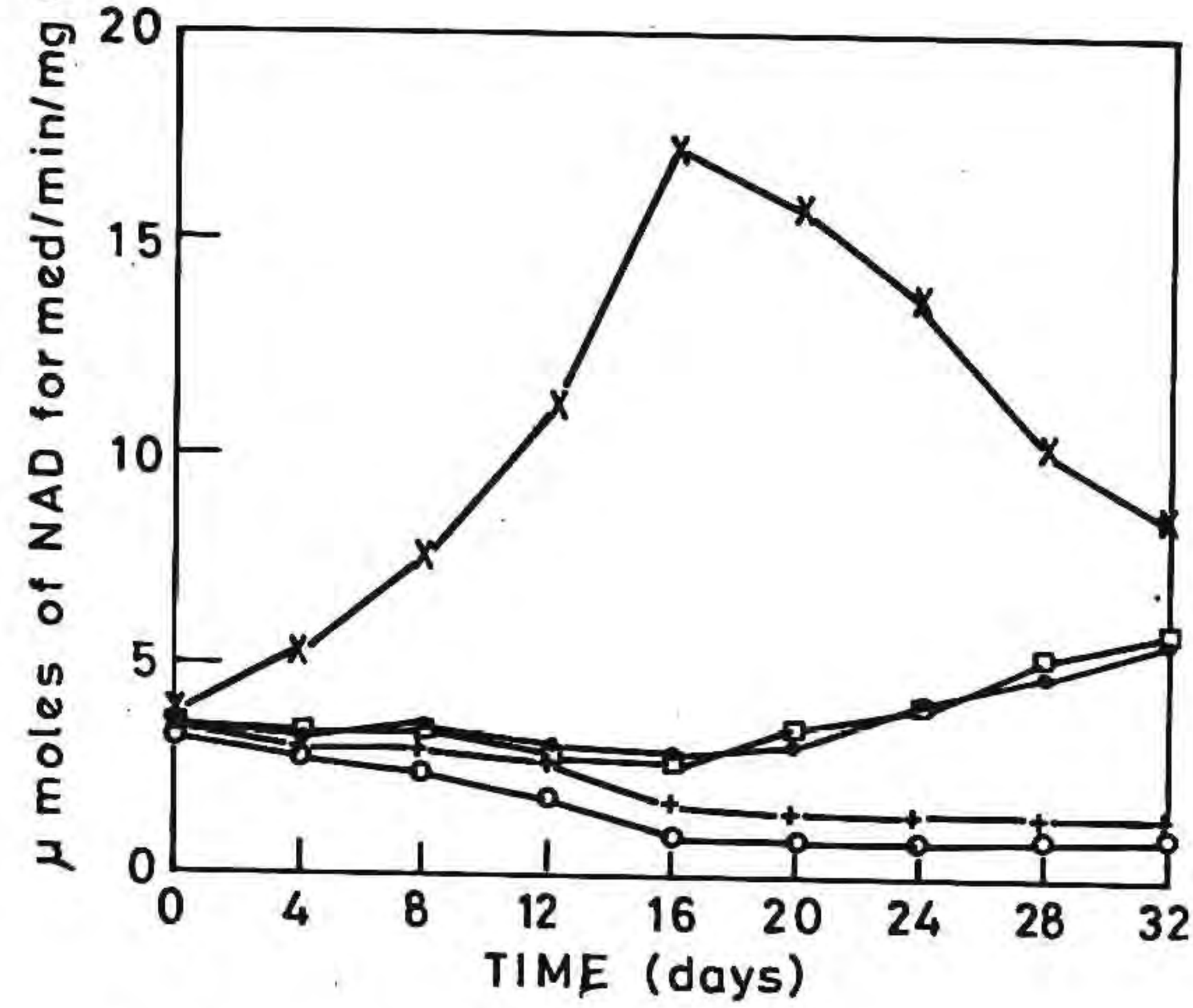
Sl. No.	Time (days)	Growth medium (µmoles of NAD^+ formed/mg protein/min)	Production medium
		A	B
1	0	3.770(±0.081)	3.882(±0.021)*
2	4	3.120(±0.046)	5.265(±0.130)
3	8	4.673(±0.073)	7.513(±0.110)
4	12	6.392(±0.110)	10.770(±0.080)
5	16	9.764(±0.022)	17.249(±0.104)
6	20	14.297(±0.038)	15.873(±0.108)
7	24	15.802(±0.130)	13.663(±0.056)
8	28	11.229(±0.131)	10.160(±0.030)
9	32	10.305(±0.072)	8.434(±0.032)

Each value is the mean ±SEM of five independent determinations

The values of group A and B compared. In all cases $p < 0.05$

*$p > 0.05$

Fig.7.9 : Effect of amino acid supplementation on glutamate synthase activity on the cell cultures of *Coscinium fenestratum*



Enzyme activity of cells cultured in production medium in presence of amino acids (50 µg/l) were determined at different time intervals.

The values plotted are the mean of five independent determinations.

Table 7.8 : Effect of amino acid supplementation on Glutamate Synthase (GOGAT) activity of *Coscinium fenestratum* cells cultured in production medium

Sl. No.	Time (days)	L-Tyrosine (µmoles of NAD^+ formed/min/mg protein)	L-Tryptophan	L-Phenylalanine	L-Dopa
		C	D	E	F
1	0	3.760(±0.018)*	3.810(±0.120)*	3.790(±0.110)*	3.710(±0.126)*
2	4	3.237(±0.081)	2.957(±0.110)	2.824(±0.130)	3.353(±0.021)
3	8	3.471(±0.260)	2.853(±0.124)	2.568(±0.135)	3.329(±0.037)
4	12	3.149(±0.142)	2.738(±0.130)	1.806(±0.142)	2.757(±0.091)
5	16	2.980(±0.110)	1.583(±0.122)	0.973(±0.141)	2.614(±0.131)
6	20	3.114(±0.116)	1.447(±0.126)	0.935(±0.027)	3.406(±0.133)
7	24	4.203(±0.108)	1.290(±0.137)	0.883(±0.050)	3.959(±0.150)
8	28	4.765(±0.123)	1.258(±0.110)	0.955(±0.129)	5.118(±0.123)
9	32	5.622(±0.131)	1.313(±0.171)	0.981(±0.121)	5.830(±0.117)

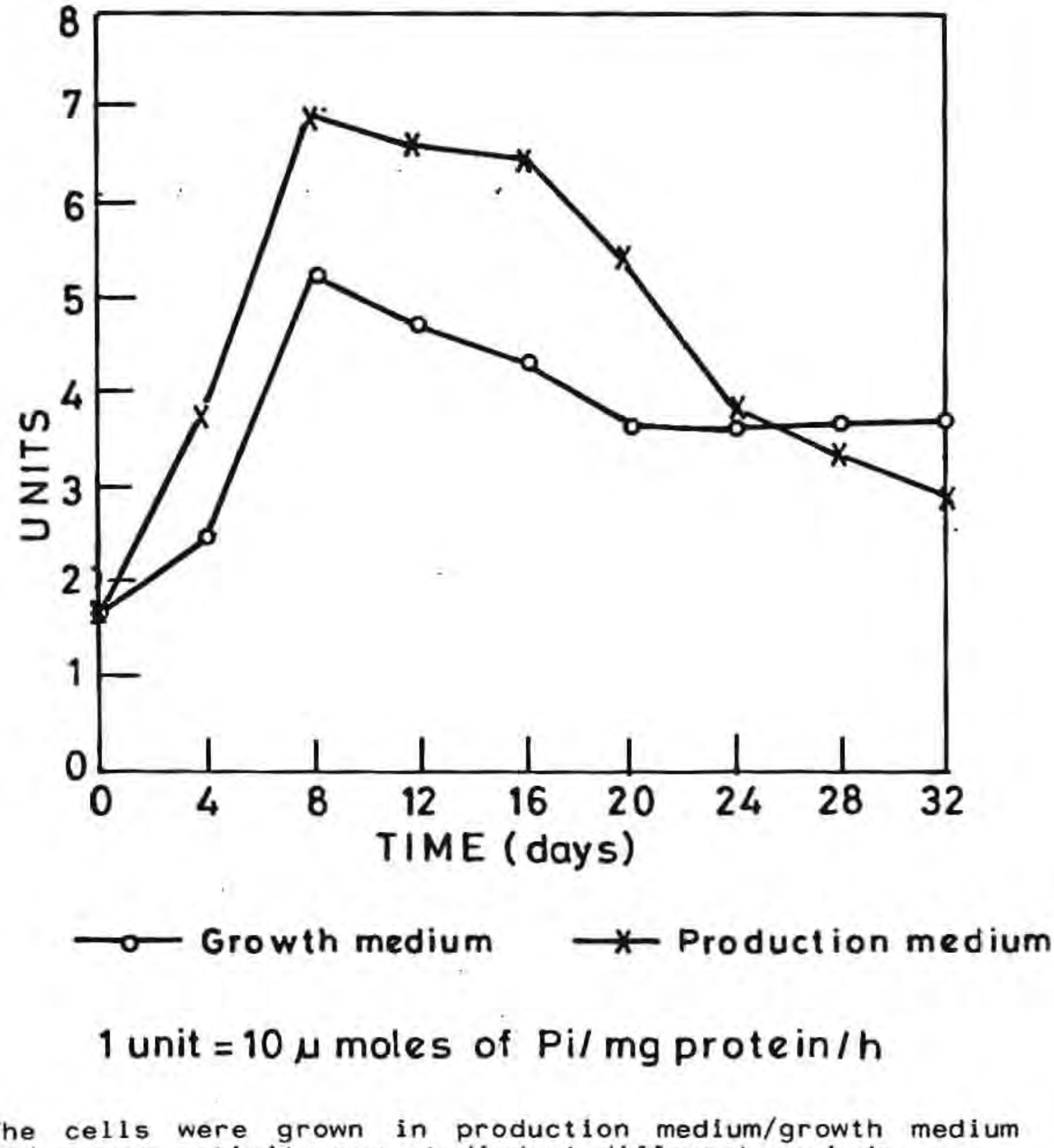
Each value is the mean ±SEM of five independent determinations
The values were compared with those of Group B in Table 7.7. In all cases $p < 0.05$
*$p > 0.05$

7.3.5. Assay of ATPase activity

The ATPase activity of the cells cultured in growth medium and production medium were determined and results are presented in Fig.7.10. In both cultures ATPase activity increased rapidly and attained a peak by eighth day of incubation. This higher activity lasted for 16th to 20th days in production medium and 12th to 16th days in growth medium and thereafter in both cultures ATPase activity declined and attained a steady state. The results obtained indicate that ATPase activity was considérably higher in the cells cultured in production medium compared to that of the growth medium. The results are also given in the Table 7.9.

Fig.7.11 shows the variations in the ATPase activity in the cells cultured in production medium on amino acid supplementation. The addition of amino acids into the medium caused a rapid increase in the ATPase activity compared to the control. But this rapid rise of ATPase activity was only for a short time. After 8th to 12th day there was a decline in the enzyme activity. In the medium supplemented with tryptophan, the ATPase activity attained a peak on eighth day and then declined gradually; attained a steady state by 20th day and thereafter no variation in the enzyme activity was noticed. L-phenylalanine supplementation also showed the same pattern of ATPase

Fig.7.10 : ATPase activity of cells in suspension cultures of *Coscinium fenestratum*



1 unit = 10 μ moles of Pi/ mg protein/ h

The cells were grown in production medium/growth medium and enzyme activity was studied at different periods.

The values plotted are the mean of five independent determinations.

Table 7.9 : ATPase activity of *Coscinium fenestratum* cells cultured in growth medium and production medium

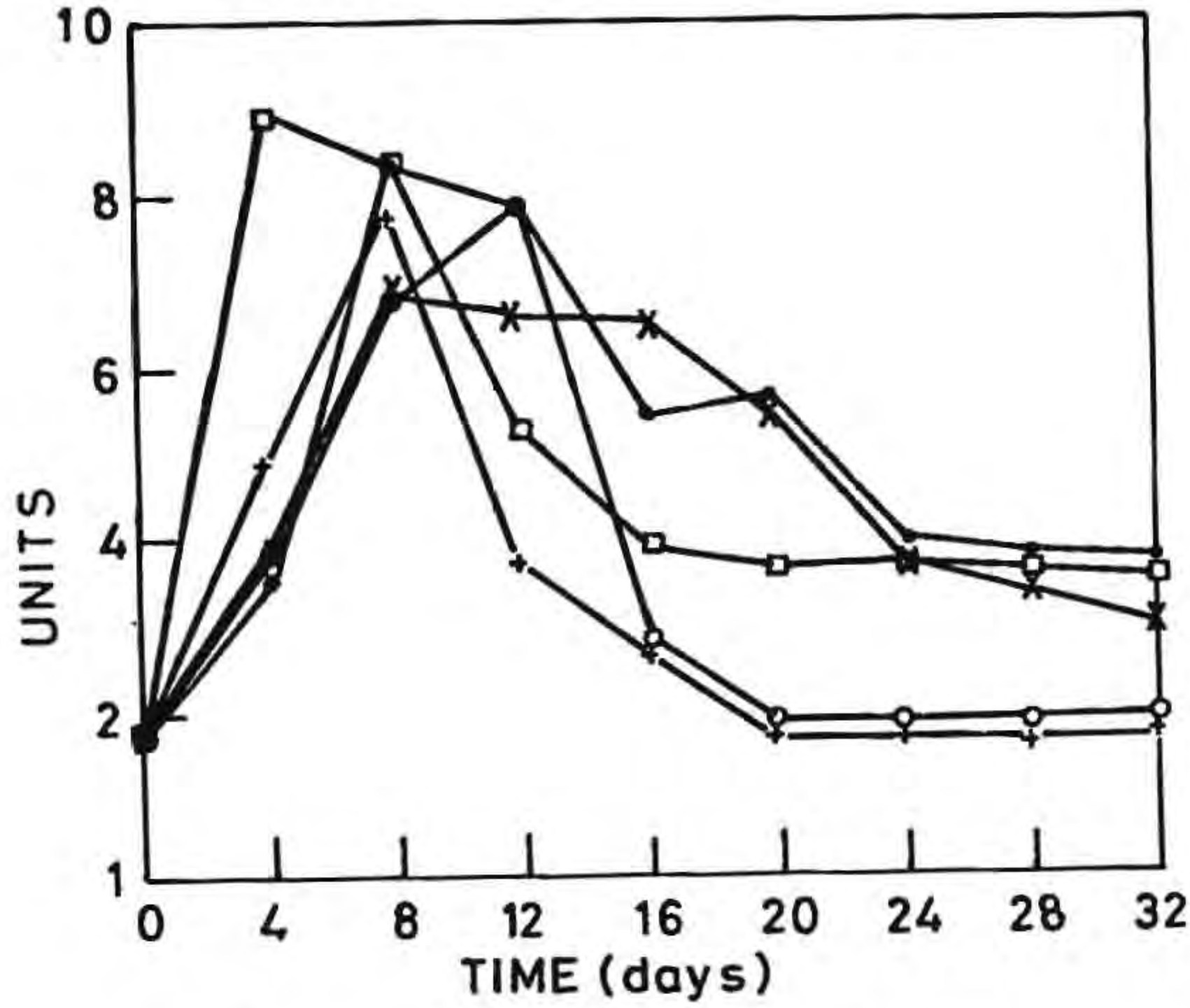
Sl. No.	Time (days)	Growth medium (µmoles Pi/mg protein/hr)	Production medium (µmoles Pi/mg protein/hr)
		A	B
1	0	17.300(±0.017)	17.100(±0.190)*
2	4	24.291(±0.014)	37.695(±0.016)
3	8	52.134(±0.081)	69.281(±0.123)
4	12	46.796(±0.251)	65.763(±0.091)
5	16	42.713(±0.172)	64.788(±0.118)
6	20	36.337(±0.314)	53.662(±0.213)
7	24	35.921(±0.252)	37.685(±0.130)
8	28	36.204(±0.118)	32.853(±0.119)
9	32	36.611(±0.331)	29.130(±0.154)

Each value is the mean ±SEM of five independent determinations

The values of group A and B compared. In all cases $p < 0.05$.

*$p > 0.05$

Fig.7.11 : Effect of amino acid supplementation on ATPase activity in the cell suspension cultures of *Coscinium fenestratum*



1 unit = 10 µ moles of Pi/mg protein/h

The cells were grown in production medium supplemented with different amino acids and the cells were harvested at differrent periods and studied the enzyme activity.

The values plotted are the mean of five independent determinations.

Table 7.10 : Effect of amino acid supplementation on ATPase activity of *Coscinium fenestratum* cells cultured in production medium

Sl. No.	Time (days)	L-Tyrosine (µ moles	Tryptophan Pi /mg protein/hrs)	L-Phenylalanine	L-Dopa
		C	D	E	F
1	0	16.821(±0.104)*	17.473(±0.241)*	17.337(±0.212)*	17.217(±0.181)*
2	4	34.376(±0.221)	47.638(±0.160)	38.181(±0.110)	89.726(±0.348)
3	8	83.341(±0.190)	77.137(±0.166)	66.809(±0.257)	82.827(±0.220)
4	12	79.143(±0.290)	36.428(±0.369)	77.421(±0.410)	51.321(±0.250)
5	16	53.731(±0.321)	26.130(±0.181)	27.853(±0.329)	38.309(±0.114)
6	20	56.124(±0.271)	15.851(±0.347)	17.871(±0.387)	36.211(±0.260)
7	24	39.431(±0.181)	15.836(±0.143)	18.093(±0.235)	36.937(±0.207)
8	28	37.343(±0.135)	15.673(±0.291)	18.359(±0.278)	35.272(±0.235)
9	32	36.692(±0.511)	16.108(±0.126)	18.643(±0.171)	34.543(±0.197)

Each value is the mean ±SEM of five independent determinations

The values were compared with those of group B in Table 7.9. In all cases $p < 0.05$.
*$p > 0.05$.

activity but the peak value was attained on 12th day. The pattern of ATPase activity of the cells cultured in L-tyrosine supplemented medium showed a slight variation compared to that of L-tryptophan and L-phenylalanine. The enzyme activity rapidly increased and attained maximum levels on 4th and 8th day of inoculation respectively and then it declined, but the decline was very slow. By 24th day ATPase activity attained the normal level when compared to that of the control. The presence of L-dopa in the medium increased the ATPase activty of the cells initially and the peak was attained by fourth day and thereafter it declined. The decline was gradual upto the 12th day and by 16th day it attained the normal state similar to that of tyrosine supplemented media. From these results it was seen that the supplementation of amino acids increased the ATPase activity in the initial period and then declined suddenly in the case of tryptophan and L-phenhylalanine. The decrease was gradual in the case of tyrosine and L-dopa. The results ae also given in Table 7.10.

7.4. DISCUSSION

The cells cultured in "Production medium" showed higher levels of NR activity throughout the culture period compared to the cells grown in "growth medium". This indicates that the rate of nitrate metabolism is higher in cells cultured in the production medium than that

in growth medium. Both media are supplemented with nitrate and NH_4^+ as nitrogen sources but the concentration of NH_4^+ is very low compared to NO_3^-. Higher concentration of NH_4^+ in the medium will be very toxic to the cells but the cells prefer NH_4 as nitrogen source since it is in the reduced form. It can be directly utilized for reductive amination if organic acids are available in the cells for the synthesis of amino acids and this may be the reason for the decrease in the NR activity during the early stages of growth. The berberine biosynthesis is at a higher rate in the cells cultured in production medium and there will be more utilization of nitrogen source for the synthesis of precursor amino acid tyrosine for berberine synthesis and other amino acids required for the synthesis of enzymes to meet the increased synthesis of the alkaloid, in addition to the normal nitrogen requirement to meet the usual housekeeping and growth activities of the cells. In growth medium, cells are characterized by low berberine production rate with high cell growth rate compared to the cultures in production medium.

Plant cell suspension cultures are usually grown in media containing a mixture of nitrate and ammonia[186,290-293]. Here both "growth media" and "production medium" contain both nitrate and ammonia. But the ratio of nitrate to ammonia is higher in growth medium compared to production medium. The addition of mixture of amino acids or a

protein hydrolysate enhances the growth in many cases[186,290-295]. But it was observed in our own studies, discussed in the previous chapter, and in many other cases that the addition of a single amino acid, inhibits growth[292-294,297].

In the present investigation it was observed that the addition of a single amino acid reduced the NR activity considerably, particularly in the early phases of culture period. The inhibitory effect of amino acids on NR activity decreased after 12th day of culture and it was more clear in the case of tyrosine supplemented medium. At the early stages, NR activity was considerably reduced and in the course of time, the concentration of supplemented amino acid decreased due to its consumption by the cells for its different activities. When the concentration of the added amino acid was decreased the NR activity increased gradually. It was more evident in the case of L-tyrosine which is the precursor of berberine biosynthesis. This amino acid must have rapidly utilized by the cells both for primary metabolism like protein synthesis and berberine biosynthesis. This may be the reason for sudden increase in the NR activity after 12th day of incubation. In other cases, i.e. tryptophan and phenylalanine supplementation, the NR did not show complete activation as in the case of tyrosine probably because these amino acids may not be completely utilized. In the case of L-dopa, its inhibitory effect on NR activity is not severe as observed

in the other amino acids. It also followed a pattern similar to the others.

The phenomenon of inhibition of growth by single amino acids in plant cell cultures has been shown by Filnel[296] in tobacco cell culture and by Gamborge[297] in soybean culture. Heimer and Filner[298,297] showed that single amino acid inhibits not only growth of tobacco cell culture but also uptake of nitrate and the nitrate reductase activity in this culture. They concluded that single amino acid supplementation specifically inhibited nitrate assimilation and consequently the growth too. Berhrend et al[300] have shown that inhibition of growth by single amino acid occurs also in tomato and carrot plant cell cultures as well as in tobacco and soyabean cell cultures. They also found that the development of nitrate reductase activity is inhibited by single amino acids.

In the case of *C. fenestratum* cell cultures also, supplementation of single amino acid reduced the NR activity in early stages and during the later stages the inhibitory effect was reduced. In the previous chapter, it was demonstrated that the supplementation of amino acids reduced growth and berberine production. This can be attributed to the reduction in NR activity and thereby nonavailability of ammonia for the synthesis of amino acids. Berberine synthesis was not reduced in the presence of

tyrosine and L-dopa because they are the precursors and intermediates of berberine biosynthesis. Growth inhibition by amino acids could be explained by specific inhibition of one or more of the steps of nitrate assimilation leading to nitrogen starvation of cells. The amino acids supplemented inhibit the assimilation of intracellular ammonium into amino acids and that leads to the inhibition of nitrate uptake and nitrate reductase[300].

Glutamate dehydrogenase is one of the important enzymes catalyzing the reductive amination of α-ketoglutamate to form glutamic acid. Thus it is one of the key enzymes participating in the assimilation of ammonia. A distinct difference in the activity profile of GDH in the cells cultured in growth medium and production medium was observed. GDH activity was higher in the production medium and can result in increased incorporation of NH_3 into organic acids to synthesize amino acids necessary for the synthesis of primary metabolites like protein and amino acids,which are required for the synthesis of berberine and maintenance of normal activities in the cells.

The activity profile of Glutamine synthetase and glutamate synthase were almost identical both in production medium and growth medium. The higher activity of glutamine synthetase and glutamate synthase in the

production medium shows that ammonium assimilation was higher in the cells cultured in production medium compared with the other. The identical activity profile of GS and GOGAT was very significant because normally two ammonia assimilating enzymes participate as a cyclic pathway for the transformation of other organic acids leading to the formation of new amino acids and regeneration of glutamic acid from glutamine which is synthesized by the reductive amination of glutamic acid. Thus the activities of all these enzymes of ammonia assimilations- glutamate dehydrogenase, glutamine synthetase and glutamate synthase are coordinately regulated. In the production media, the cells show more activity for all these enzymes than in the growth medium indicating a higher ammonia incorporation in the former which can be related to the increased rate of berberine biosynthesis.

The addition of individual amino acids reduced the activity of all these three enzymes eventhough it was not completely inhibited. Thus ammonia assimilation was prevented or reduced. This ammonia accumulation may be the reason for the inhibition of nitrate reductase and reduction in growth rate and berberine biosynthesis. The inhibitory action was more towards the beginning of the culture period and in almost all cases, the enzymes

were released from inhibition during the later stages of culture period, when the added amino acid concentration was reduced due to the consumption by the cultured cells for different metabolic activities. It was more evident when the amino acid supplemented was L-tyrosine or L-dopa, both of which participate in the biosynthesis of berberine. The supplemented L-tyrosine and L-dopa might have been completely utilized for berberine biosynthesis and other metabolic functions and that could be the reason for rapid increase in the activity of enzymes in these cultures after 12th or 16th day of incubation, whereas in the other cultures supplemented with tryptophan or phenylalanine this increase was relatively slow or not significant.

The major route for nitrogen assimilation has long been considered to operate through the reductive amination of α-ketoglutarate catalyzed by GDH. Now it has been shown that an alternate route of nitrogen assimilation comprising of the enzymes glutamate synthase and glutamine synthetase operate in many organisms including bacteria and higher plants as a cyclic pathway. It was observed that this pathway operates under conditions of ammonia limitation[301-304] and also when nitrate or nitrite forms the nitrogen sources[305]. The data presented here reveals a similarity in the activity of all the four nitrogen

assimilating enzymes. This indicates that the activity of one of these enzymes is influenced by other enzymes since the products of one form the substrate of the next and thus are coordinately regulated. Other factors such as ATP, reductants (NADH), α-ketoglutarate, glutamine and the presence of other organic acids etc also determine the activity of all these nitrogen assimilating enzymes. From these investigations it became obvious that cells of *C. fenestratum* when cultured in production medium showed high level of nitrogen assimilation compared to growth medium.

Another enzyme which can indicate the metabolic status of a cell culture is ATPase which is involved in the turn over of ATP within a cell. The results indicated that the activity profile of ATPase was identical in both cell cultures with higher activity in the cells cultured in production medium. An increased activity of ATPase during the lag phase of cell growth cycle has been reported by Poole[30] Jacoby et al[307] and Nissen[308]. Ravisankar and Grewal[309] have used the ATPase activity as an index of active cell growth in Dioscorea and Physehchlaina because of the positive correlation between cell growth and ATPase activity. ATPase activity of the cell cultures in the production medium was higher. It may result in the

availability of more energy for berberine biosynthesis followed by the absorption of more nutrients from the medium for this purpose.

The changes in the ATPase activity brought about by the addition of individual amino acids is also interesting. The supplementation of amino acid to the production medium caused a sudden rise in ATPase activity.

These results indicate that one of the reasons for increased production of berberine in the production medium is an enhanced rate of nitrogen metabolism as evidenced by higher activities of four major enzymes involved in nitrogen metabolism. A modulation of the activity of these enzymes as in the case of amino acid supplementation causes an alteration in the rate of berberine production by cells in culture. However it is not clear from these experiments how the activity of these nitrogen metabolising enzymes of the cells in culture are regulated.

CHAPTER VIII

CHAPTER VIII

PARTIAL PURIFICATION AND CHARACTERIZATION OF TETRAHYDROBERBERINE OXIDASE AND ITS MODULATION

8.1. INTRODUCTION

Berberine is the first alkaloid whose biosynthetic pathway has been completely elucidated[81]. This has been discussed in detail in a previous chapter.

The berberine biosynthetic pathway at the enzyme level has been worked out mainly using cell cultures of different species of berberise. The biosynthesis of berberine in most plant systems proceeds through the intermediate tetrahydrocolumbamine formed by methylation of (S)-scoulerine at C-9 position which is oxidised to columbamine by an enzyme (S)-tetrahydroprotoberberine oxidase (STOX)[282]. Subsequently it is converted to berberine with the formation of methylenedioxy group by an enzyme, berberine synthase (Fig.8.1). This pathway of berberine synthesis is realized in the families, Berberidaceae, Papaveraceae and Manispermaceae[82] and seems to be the main pathway in plant kingdom for berberine biosynthesis. Based on experiments, using radioactive precursors in intact plant - *Hydrastis canadensis*, Barton et al[279] suggested the possibility of an intermediate, tetrahydroberberine (S-Canadin) and its enzymatic conversion into

berberine by dehydrogenation. Yamada and Okada[310] reported the biotransformation of tetrahydroberberine to berberine by an enzyme preparation from the cultured cells of *Coptis japonica*. These reports differ from the established biosynthetic pathway of berberine described above. Galneder et al[82] compared the pathway of berberine formation in *Coptis japonica* with the established pathway elucidated in berberise species. This led to the discovery of an alternate pathway in *Coptis japonica* cell cultures. In this alternate final step (Fig.8.1) tetrahydrocolumbamine is converted to (S)-canadin [(S)-tetrahydroberberine] by the enzyme (S)-Canadine synthase which is followed by the oxidation of (S)-Canadine to berberine by the enzyme THB oxidase (Tetrahydroberberine oxidase).

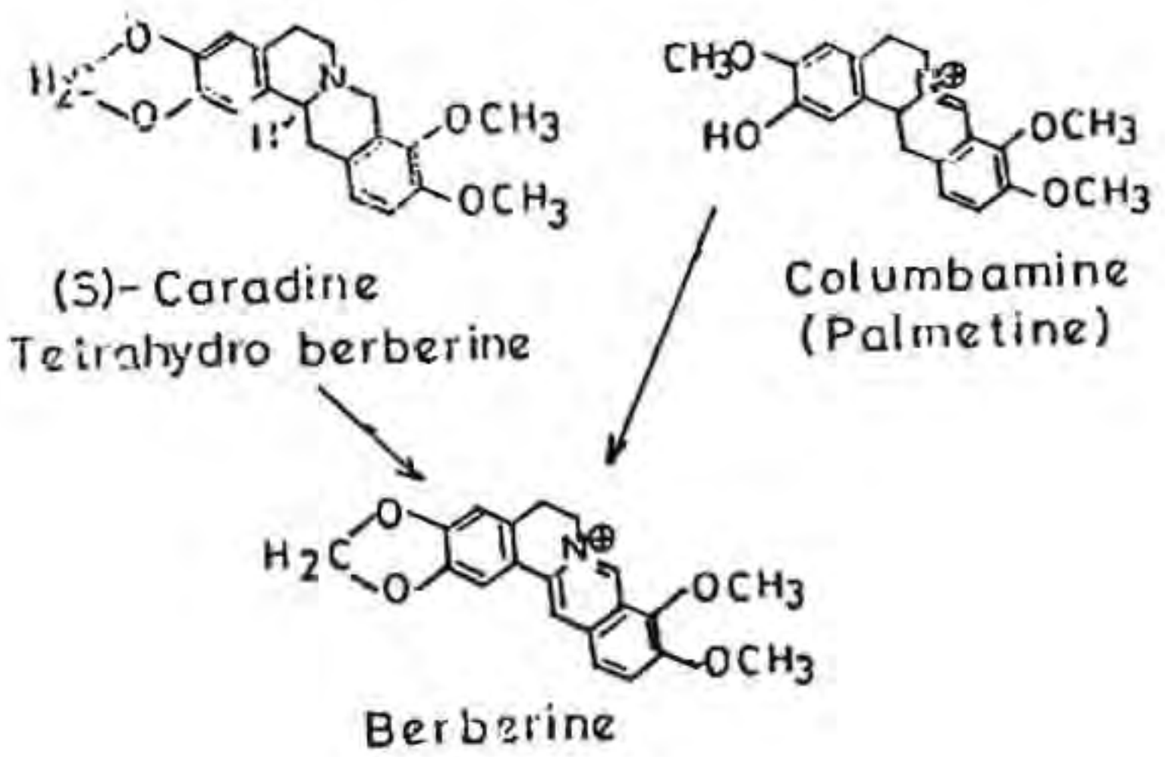


Fig. 8.1

The operation of this final step can be proved by demonstrating the activity of this enzyme in the system. THB oxidase has been purified and characterized by Okada et al[202] from the cell cultures of *Coptis japonica*. But it is not known through which of the pathways the berberine is predominantly produced in *Coscinium fenestratum*. One of the methods to study this is to look into the activity of the key enzyme THB oxidase and to examine the kinetic property of the enzyme. Experiments have therefore been carried out to demonstrate the activity of THB oxidase in whole plant as well as in cultured cells of *C. fenestratum* and thereby to show the conversion of THB to berberine. The enzyme has been partially purified and its kinetics were studied with particular reference to the modulation of its activity so that the rate of berberine production could be suitably influenced. These results are presented and discussed in this chapter.

8.2 MATERIALS AND METHOD

8.2.1. Plant tissue and cultured cells

The fresh plant and tissue (*Coscinium fenestratum*) consisting of leaf petiole and bark was used for enzyme extraction. Enzyme was also prepared from the cultured cells grown in production medium and growth medium.

8.2.2. Enzyme extraction and partial purification

About 50 gm of the plant tissue (consisting of leaf petiole and bark) was homogenized with 50 ml of phosphate buffer (0.1 M, pH 7.5) containing 5 mM dithiothritole (DTT) in a prechilled mortar along with quartz sand and 10 mg of polyvinylpyrolidone (insoluble) at 1 to 4°C and repeated the extraction three times. The homogenate was filtered through cheese cloth and the filtrate was centrifuged at 4°C at 10000 rpm for 10 minutes. The supernatant was collected and was passed through a column (2x20 cm) packed with neutral Amberlite resin XAD-2. The column was eluted with phosphate buffer (0.1 M,pH 7.5) containing 5 mM DTT. The eluate was again passed through another column (2x20 cm) packed with CM Sephadex equilibrated with the same buffer containing 5 mM DTT. The eluate was free from yellow pigments including berberine. Protein of the eluate was precipitated at 0.9 saturation of ammonum sulphate and was separated after centrifugation at 10000 rpm for 20 minutes. The pelleted protein was resuspended in minimal volume of 60 mM phosphate buffer (0.06 M pH 7.5) containing 2 mM DTT. The protein solution was dialysed against the same buffer and again centrifuged at 4°C at 18000 rpm for 30 minutes. The clear colourless supernatant was used as the enzyme solution for various assays and kinetic studies.

The same extraction and purification procedure was applied in the case of cultured cells also.

8.2.3. Enzyme assay

The activity of THB oxidase was measured by using tetrahydroberberine as substrate with appropriate blank as described in Chapter II.

8.2.4. Enzyme kinetics

The effects of different concentration of substrate, enzyme concentration and time dependence on THB oxidase activity were studied. The details are given in Chapter II.

8.2.5. Effect of different metallic ions and EDTA on enzyme activity

Influence of different metal ions and metal chelators were tried on THB oxidase activity. Solutions of $CuSO_4$, $FeSO_4$, $CaCl_2$, $MgSO_4$, NAD, NADP and metal chelator EDTA were prepared at concentration of 1 mg/ml. The enzyme solution was preincubated with an appropriate amount of these solutions for 10 minutes individually and started the reaction by adding the substrate.

8.2.6. Protein estimation

Protein content of the enzyme preparation was estimated by the method of Lowry et al as described in Chapter II using an aliquot of the extract.

8.3. RESULTS

8.3.1. Partial purification of THB oxidase from leaf tissue of *C. fenestratum*

The ezyme was extracted and purifed from the leaf petiole tissues of *C. fenestratum*. The specific activity of the enzyme in the crude extract was 3.5 units and the extract was yellowish in colour due to the presence of berberine and other pigments. After the first centrifugation the solution was passed through a column packed with neutral Amberlite resin XAD-2 which absorbed all berberine present in the extract. Since the eluate was still yellow in colour, probably due to the presence of some other pigments, it was again passed through another column packed with CM Sephadex which absorbed all the coloured materials and the eluate had a specific activity of 9.0 units. These coloured materials can otherwise interfere with the enzyme assay. Protein was precipitated from the eluate at 0.9 saturation with ammonium sulphate and the protein pellet obtained after centrifugation was dissolved in phosphate buffer. The supernatant obtained after the final centrifugation was clear and colourless which was used for the kinetic studies. This partially purified extract had a specific activity of about 12 units.

The enzyme activity in the crude extract and at the different stages of purification process were monitored and the yield and the degree of purification are given in Table 8.1. The degree of purification after each stage was calculated as the ratio of specific activity of each to that of the initial extract. The yield of the enzyme was calculated by taking the total amount of enzyme in the initial extract as 100 percent. Adsorption chromatography and fractional precipitation yielded about three fold purification with a final specific activity of about 12 units of enzyme. The yield of the enzyme was only about 38 percent at the final stage of purification. The enzyme appeared to be less stable as it could not be stored even at low temperatures for more than four days.

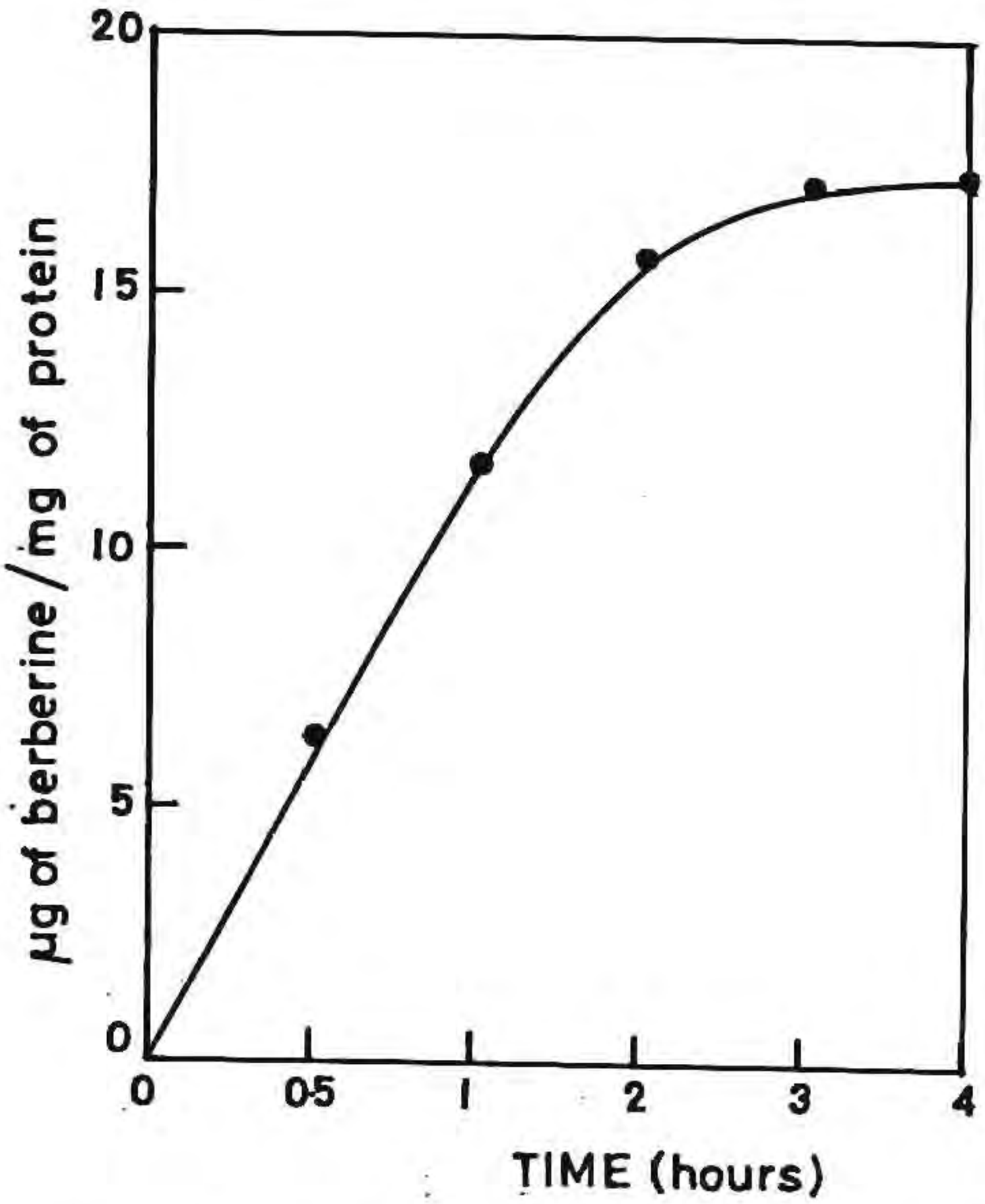
8.3.2. Effect of time

The influence of different incubation periods on the enzyme activity was increased and the results are represented in the Fig.8.2. The incubation period of the reaction mixture was extended from thirty minutes to four hours and the berberine formed in the reaction mixture was measured. There was a linear increase in the velocity of the reaction upto about two hours after which there was no significant change in the velocity of the reaction. It was observed from this time course

Table 8.1 : Partial purification procedure of THB oxidase

Sl. No.	Purification steps	Vol. of extract (ml)	Total protein (mg)	Total activity (µg of berberine)	Specific activity (µg berberine/mg protein/hr)	Yield (%)	Purification (X fold)
1	Crude extract	150	735.0	2604	3.54	100	1.00
2	XAD-2 and CM-Sephadex	72	228.0	2147	9.38	83	2.65
3	Ammonium sulphate precipitation and dialysis	20	85.2	987	11.58	38	3.27

Fig.8.2 : Effect of time on THB oxidase activity



The reaction was carried out at 30°C and pH 7.5 for different time intervals

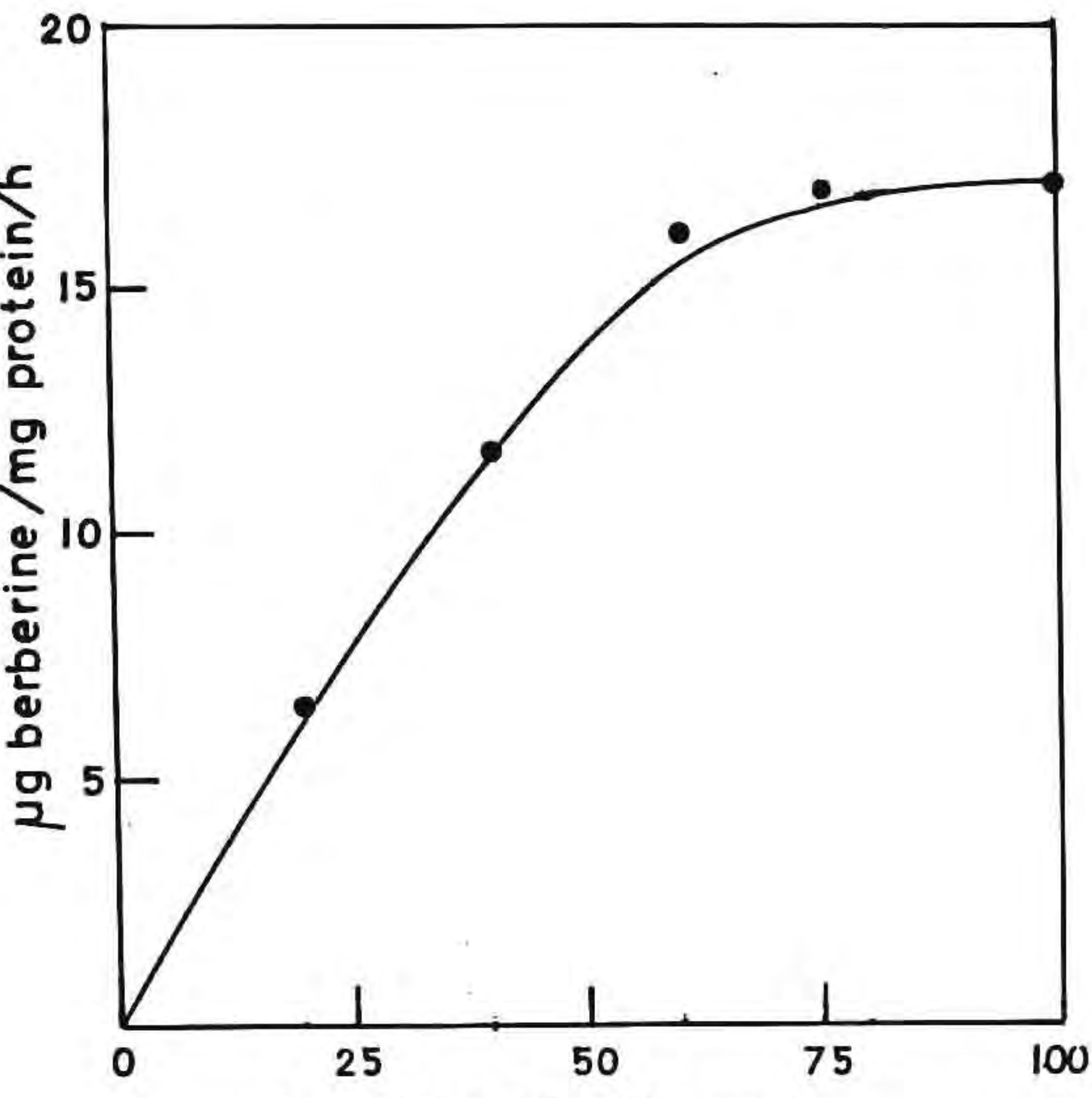
The values plotted are the mean of three independent determinations

study that the complete conversion of the substrate present in the reaction mixture into berberine did not occur even after four hours of incubation. The maximum conversion obtained was approximately 50 percent.

8.3.3. Effect of substrate concentration

Influence of different concentration of substrate (THB) on THB oxidase was studied. The results are presented in the Fig.8.3. The rate of formation of product increased linearly with the increase in the concentration of the substrate only upto about 100 µg/ml. On further increase in the concentration of substrate there was no significant increase in the velocity of the reaction. The plot of the velocity of the reaction against the concentration of substrate gave a portion of a rectangular hyperbola indicating a saturation kinetics. The V_{max} (maximum velocity) and the K_m (the concentration of the substrate required to give half the maximum velocity) were calculated from the double reciprocal plot (Fig.8.4) of THB oxidase. On plotting the reciprocal of the concentration of the substrate against reciprocal of the velocity of the reaction (double reciprocal plot) a straight line graph typical of a monosubstrate reaction was obtained. The K_m was calculated to be 37 µg/ml of tetrahydroberberine.

Fig.8.3 : Effect of substrate concentration on THB oxidase activity



The reaction was carried out at 30°C and pH 7.5 for 1 hr.

The values plotted are the mean of three independent determinations

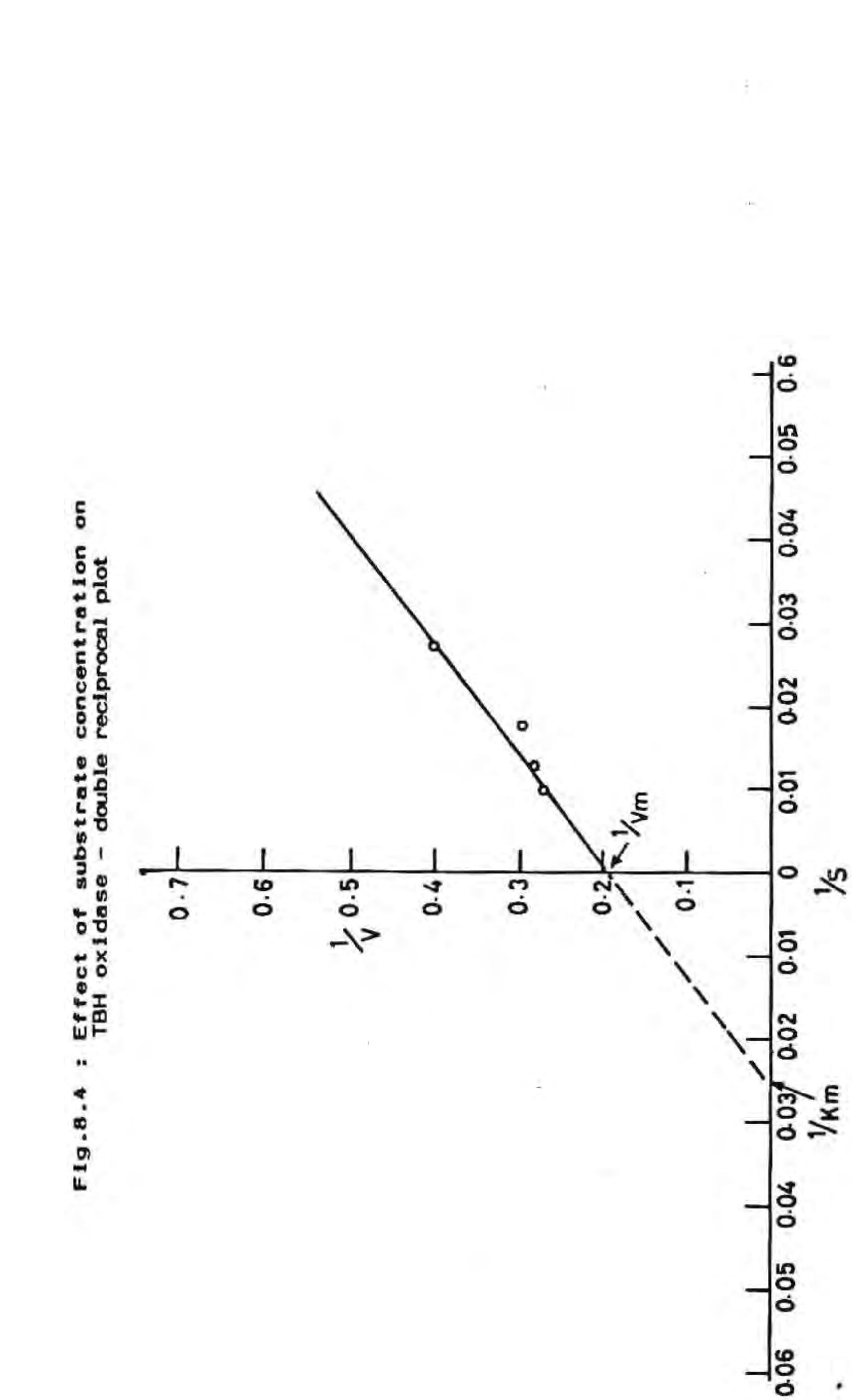


Fig.8.4 : Effect of substrate concentration on TBH oxidase - double reciprocal plot

8.3.4. Effect of enzyme concentration

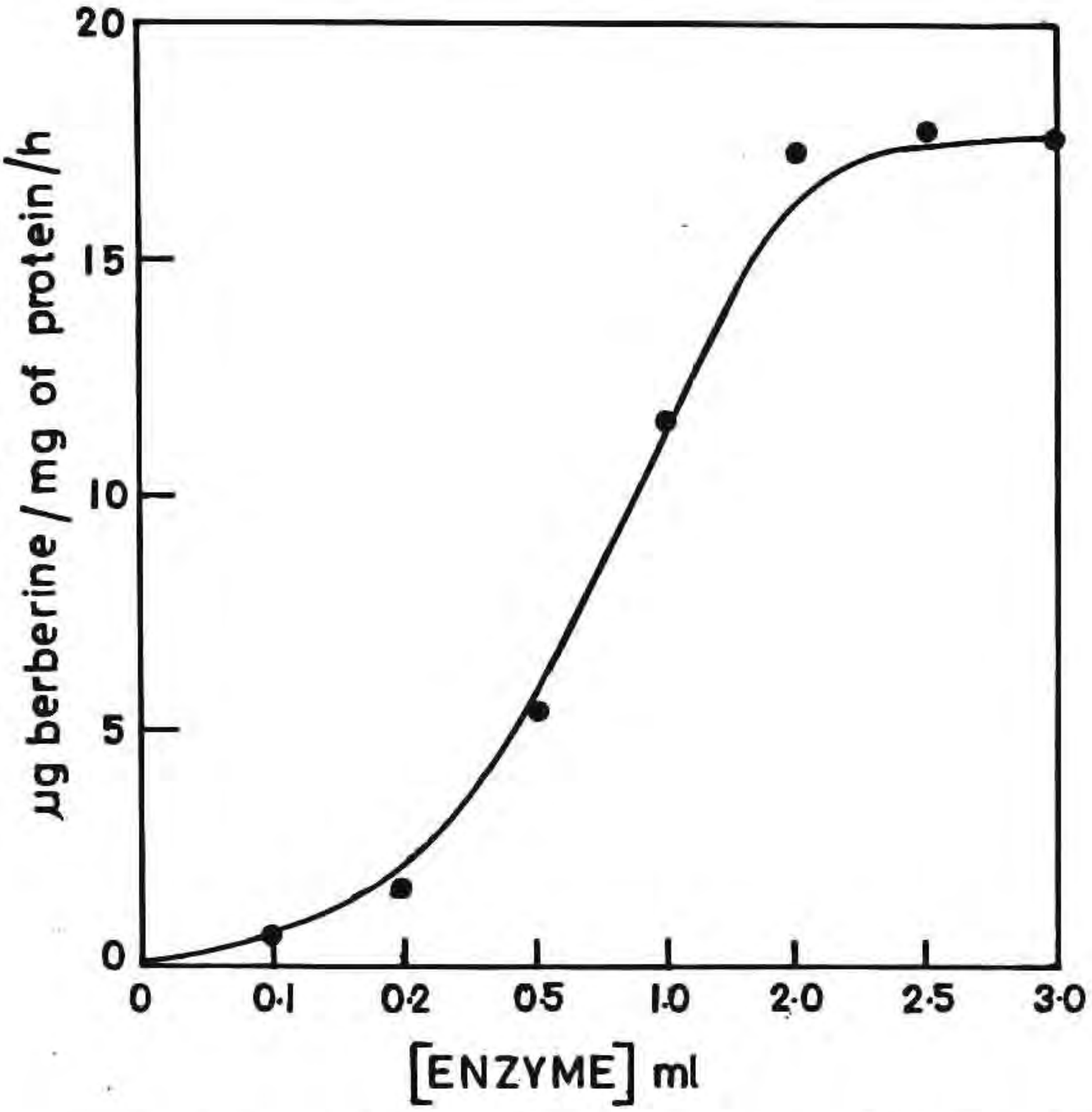
The effect of change in concentration of enzyme on the rate of reaction was also studied and the results are presented in the Fig.8.5. It shows a linear increase in the rate of reaction with the increase in the enzyme concentration upto 4.26 mg protein of partially purified enzyme. Above this the reaction was not increased considerably.

8.3.5. Effect of modulators on enzyme activity

The effect of different metallic ions, co-factors and chelators such as EDTA on the activity of enzyme was studied. There was no remarkable difference in the THB oxidase activity except in the case of copper and EDTA (Fig.8.6). THB oxidase activity was enhanced by 37 percent in the presence of $CuSO_4$ when compared to that of control. But in the case of EDTA, the activity was reduced by 46 percent of the control.

Since copper ions enhanced the activity of THB oxidase different concentrations of $CuSO_4$ were used to find out the optimum concentration to get maximum enzyme activity. Fig.8.7 shows the effect of different concentrations of $CuSO_4$ on THB oxidase activity. The results indicated that $CuSO_4$ at a concentration of 50 µg/ml showed maximum activity. Concentrations above this did not bring any change, instead slightly reduced the enzyme activity.

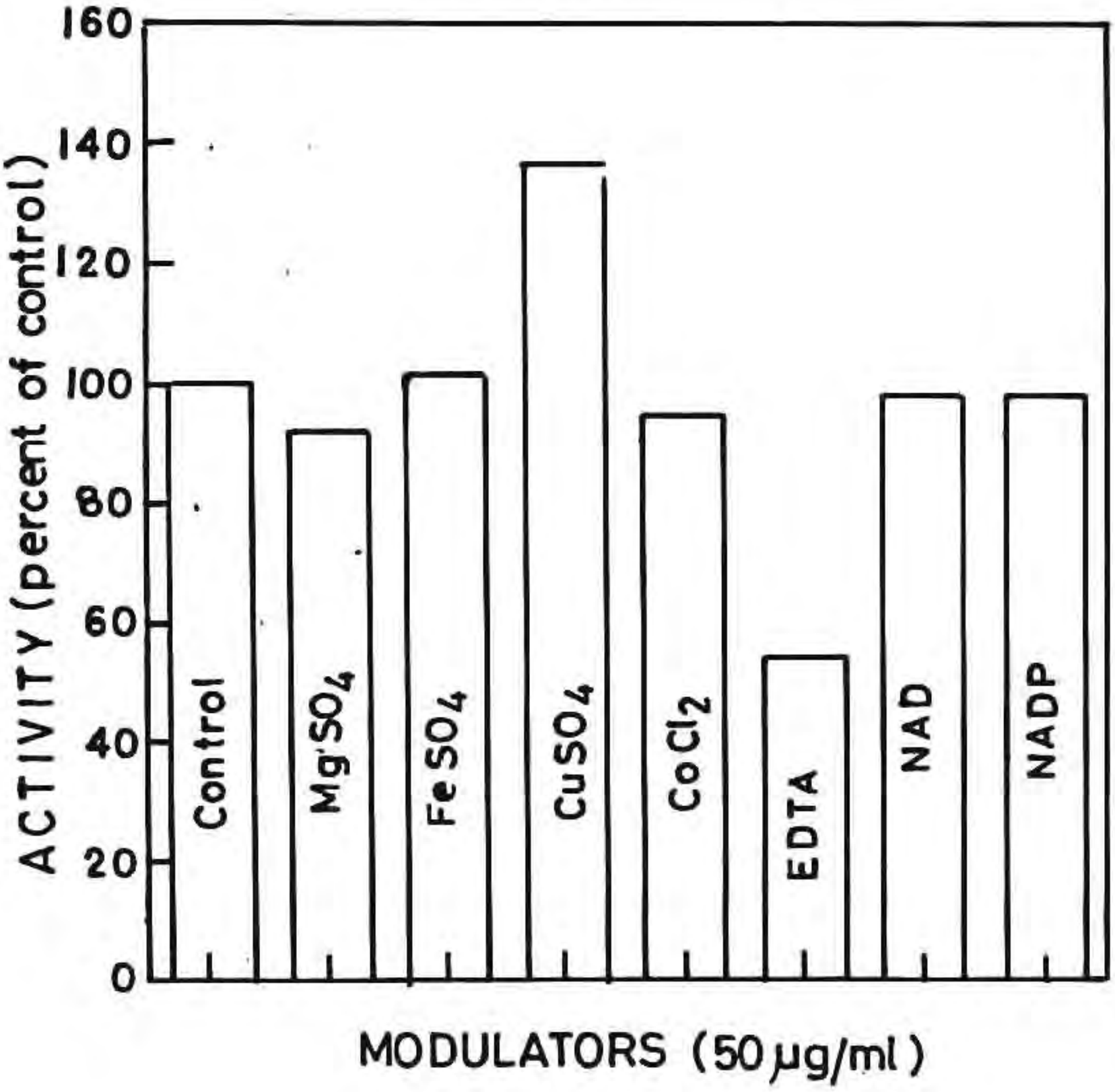
Fig.8.5 : Effect of enzyme concentration on THB oxidase activity



The reaction was carried out at 30°C and pH 7.5 for 1 hr. using different volume of the enzyme made upto a total volume of 0.5 ml with the buffer. The stock enzyme used has 2.26 mg protein/ml.

The values plotted are the mean of five independent determinations

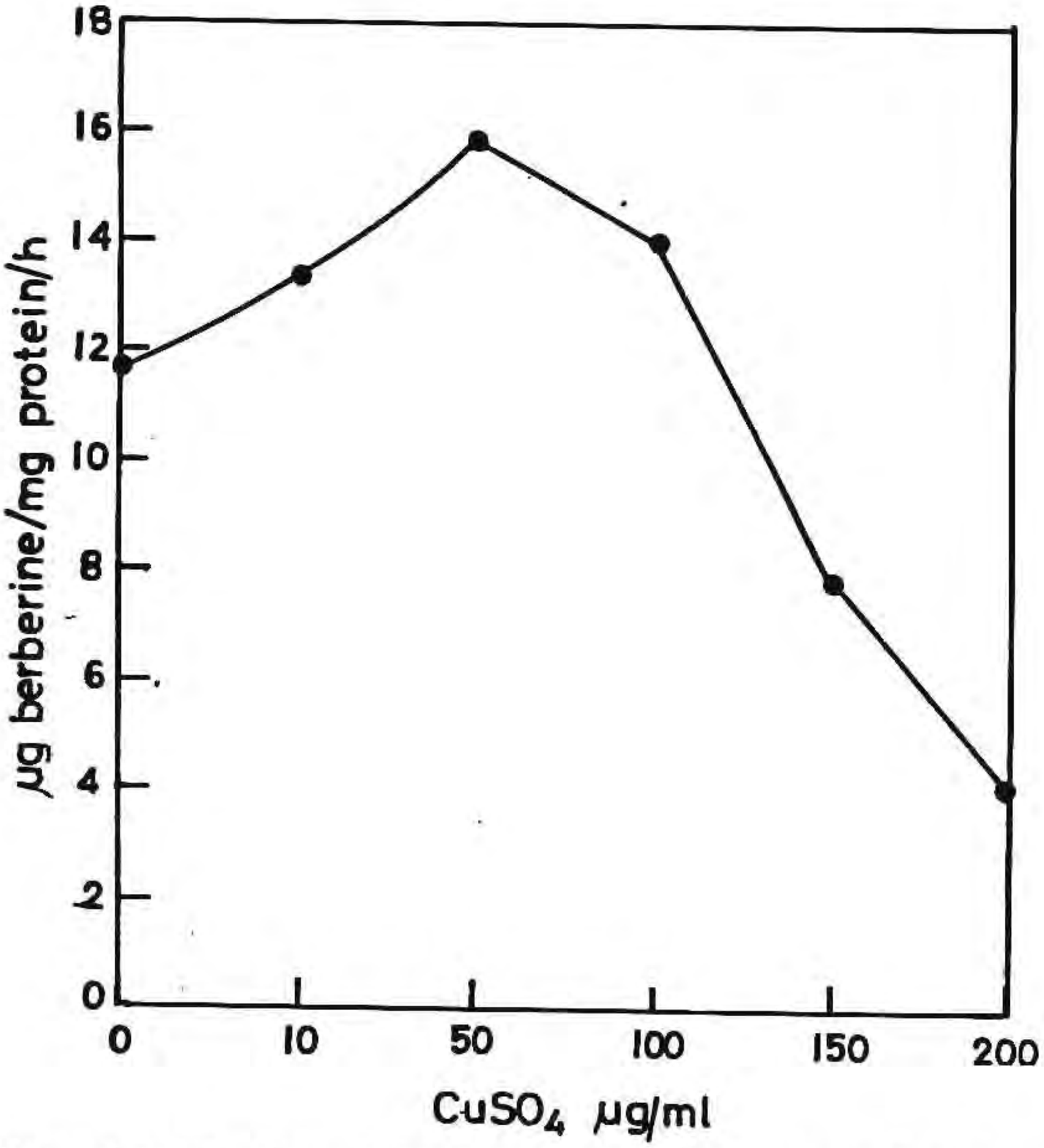
Fig.8.6 : Effect of different modulators of THB oxidase activity



The reaction was carried out at 30°C and pH 7.5 in presence of different substances (50 µg/ml) for 1 hr and the mean values were expressed as percentage of the control.

The values plotted are the mean of five independent determinations

Fig.8.7 : Effect of $CuSO_4$ on THB oxidase activity



The reaction was carried out at 30°C and pH 7.5 in the presence of different concentrations of $CuSO_4$ for 1 hr.

The values plotted are the mean of five independent determinations

There observed a linear increase with the increase in concentration of $CuSO_4$ upto 50 µg/ml.

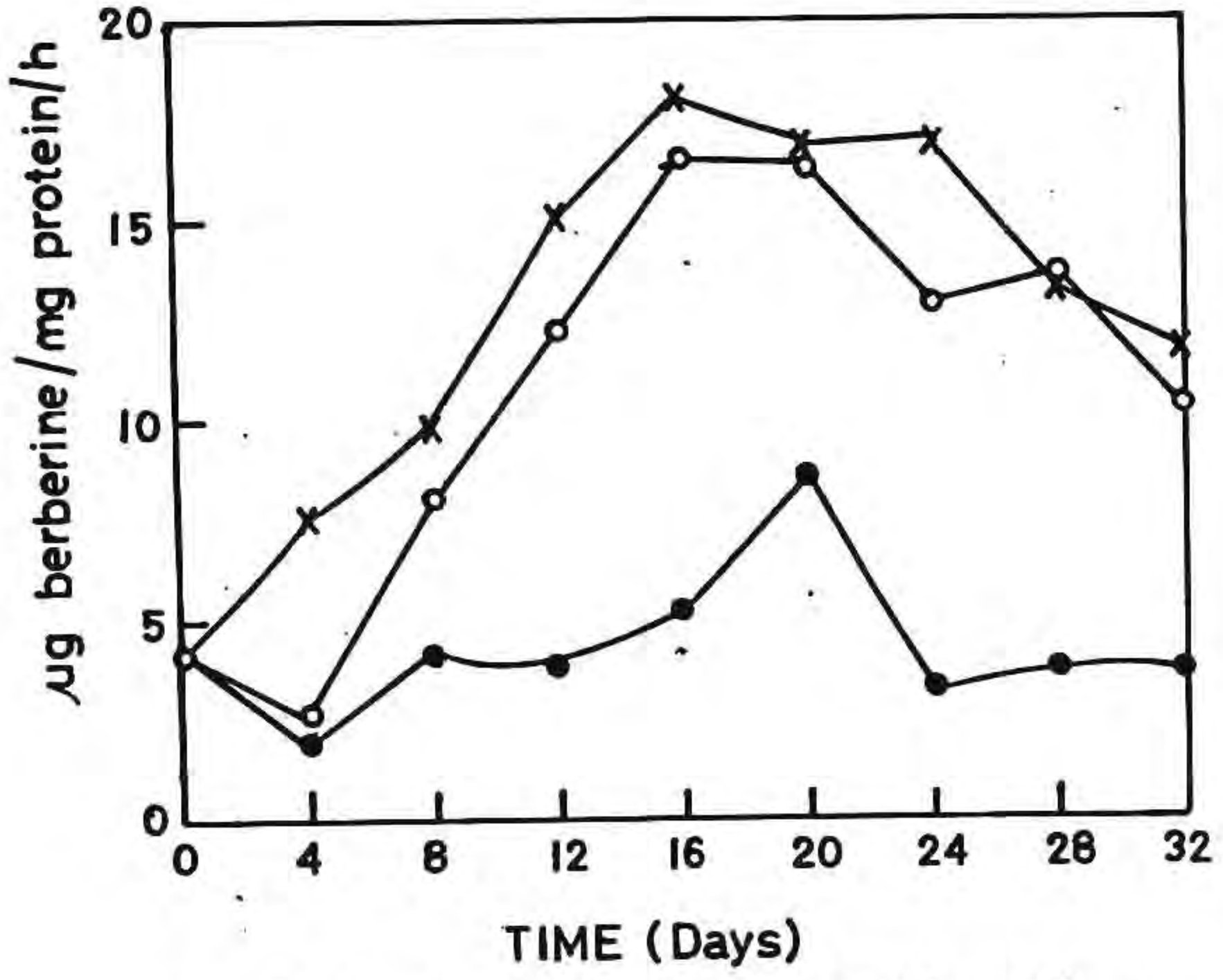
8.3.6. THB oxidase activity of cultured cells

Enzyme preparation was also done from the cultured cells, grown in production medium and growth medium, and the specific activity was compared to each other. Fig.8.8 represents the activity of THB oxidase of the cells cultured in the two media at different periods. The pattern of change in enzyme activity was almost identical in both cultures. But the cells grown in production media showed a significantly higher enzyme activity compared to the other. During the initial stages of the culture period in both the media, the activity was less but in both cultures the enzyme activity reached a peak stage between 16th to 24th day of culture period. In the case of production media, the enzyme activity started to decline gradually after the 24th day whereas in the growth media it declined to a lower level on 24th day and there was no further decrease upto the end of culture period.

8.3.7. Effect of amino acid supplementation on THB oxidase activity

The effect of the single amino acids supplemented to the production medium on THB oxidase activity was studied. There observed some variation in the THB oxidase activity in response to the amino acid supplemented to the culture media. Fig.8.9 shows that there was no decline

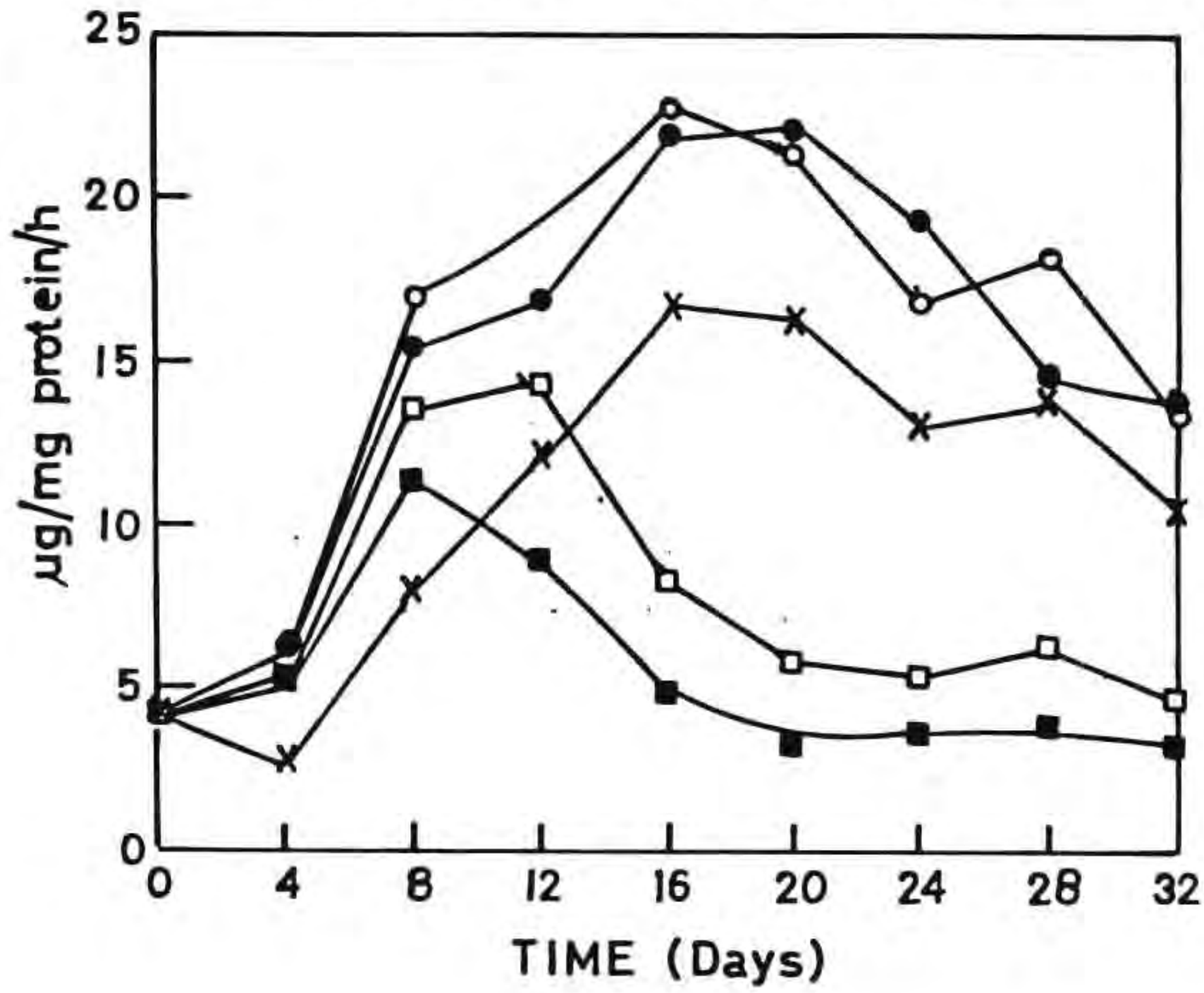
Fig.8.8: THB oxidase activity in the cell suspension cultures of *Coscinium fenestratum* grown in different nutritional conditions



The cells were grown in production medium with or without 100 µg/l of $CuSO_4$ or in growth medium and harvested at different tim intervals, and the THB oxidase activity was studied.

The values plotted are the mean of five independent determinations

Fig.8.9 : Effect of amino acid supplementation on THB oxidase activity in the cell suspension cultures of *Coscinium fenestratum*



The cells were grown in production medium supplemented with amino acids. The cells were harvested at different time intervals and THB oxidase activity was studied.

The values plotted are the mean of five independent determinations

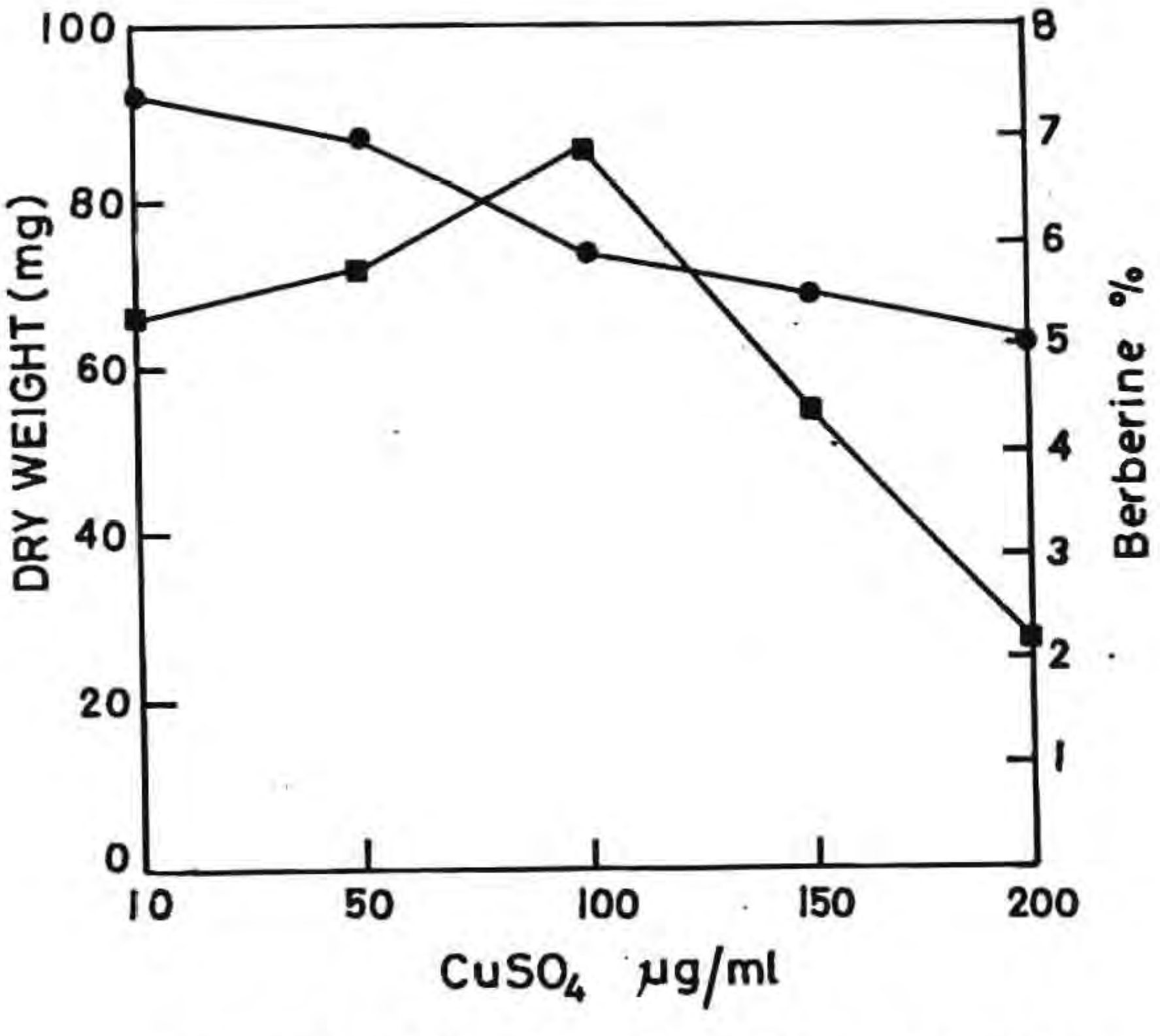
in the THB oxidase activity as was observed in the case of control. But there was an increase during the initial period of culture, and this was maximum in the case of tyrosine and L-dopa supplemented media. Whereas in the case of tryptophan and phenylalanine there observed a decline in the enzyme activity which reaches a minimum during the mid stages of culture period. This type of decline was not seen in the case of L-tyrosine and L-dopa supplementation.

8.3.8. Effect of $CuSO_4$ on THB oxidase activity and berberine production by the cell cultures

Since $CuSO_4$ has got an enhancing effect on THB oxidase activity, different concentrations of $CuSO_4$ were administered in the production medium and the effect on alkaloid production, cell growth and THB oxidase activity was studied. Fig.8.10 gives the influence of different concentrations of $CuSO_4$ on growth and berberine production when supplemented to the production medium. The values are given in the Table 8.2. Increase in the concentration of $CuSO_4$ in the medium reduced the cell biomass formation considerably eventhough the berberine content was increased. The optimum concentration of $CuSO_4$ for the increased production of berberine was found to be 100 µg/l. There was about 40 percent increase over control. Concentrations above this reduced cell biomass and berberine concentration considerably. This may be due to the toxic effect of $CuSO_4$ on cells. The cell biomass and berberine content was calculated on 28th day of incubation.

Fig.8.8 shows the THB oxidase activity of cells at different periods, cultured in production media supple-

Fig.8.10 : Effect of concentration of $CuSO_4$ on growth and berberine production in the cell suspen cultures of *Coscinium fenestratum*



The cells were grown in production medium supplemented with different concentrtions of 10 µg/l $CuSO_4$ was taken as the basal value for 28 days.

The values plotted are the mean of five independent determinations

Table 8.2 : Effect of different concentrations of $CuSO_4$ on growth and berberine production

Sl. No.	$CuSO_4$ ($\mu g/l$)	Fresh wt. (mg/culture)	Dry wt. (mg/culture)	Berberine in medium (mg/50 ml)	Total berberine (mg/culture)	Berberine content (percent of dry weight)
1	0	519.34	106.73	2.18	4.40	4.12
2	10	453.15	92.48	2.11	4.88	5.27
3	50	421.61	86.93	2.19	5.00	5.75
4	100	360.35	73.22	2.70	5.05	6.90
5	150	334.51	68.40	1.83	2.99	4.37
6	200	288.09	63.23	0.72	1.36	2.15

Initial inoculum : Fresh weight : 319.25 mg
Dry weight : 65.72 mg

Values given are the average of duplicate cultures.
The variation was less than 5%.

Table 8.3: $CuSO_4$ supplementation on berberine production, growth and THB oxidase activity of *Coscinium fenestratum* cell cultures

Sl. No.	Time (days)	Dry weight (mg/culture)	Berberine content (percent of dry weight)	Specific activity (µg berberine/mg protein/hr)
1	0	68.45	0.62	4.08
2	4	62.13	0.96	7.52
3	8	59.62	1.88	9.75
4	12	63.50	3.65	15.13
5	16	65.44	5.83	18.06
6	20	67.92	6.77	16.82
7	24	71.28	6.81	16.95
8	28	70.84	6.93	13.22
9	32	68.25	5.30	11.75

Medium : Production medium + 100 µg/1 $CuSO_4$

Protein content : 3.44 mg/ml enzyme solution

The values given are the average of duplicate analysis.

The variation was less than 4%

mented with 100 µg/l of $CuSO_4$. There observed an increased activity of the enzyme when compared with the control (activity in production media). The enzyme activity reached a maximum on the 12th day itself and it remained at that level upto the end of the culture period with a slight decline towards the end. The rise in the enzyme activity was almost concommitant with the rise in berberine content of the cells in culture. The values are given in the Table 8.3.

8.4 DISCUSSION

The biotransformation of tetrahydroberberine using an enzyme preparation from the fresh plant tissue and also from the cultured cells of *C. fenestratum* indicate the presence of the enzyme - tetrahydroberberine oxidase (THB oxidase) in *C. fenestratum*.

The enzyme has been isolated in a partially purified form. There was about three to four fold purification and the yield of the enzyme was about 38 percent. Almost identical degree of purification and yield were obtained for enzyme from cells maintained in culture also. The THB oxidase has been purified to homogenity from *Coptis japonica* by Okada et al[202]. The enzyme has been reported to be made up of two identical subunits with a total molecular weight of 58,000 daltons. We have not attempted to characterise this enzyme in detail as our primary objective

was to show the presence of this enzyme thereby to demonstrate the pathway for berberine synthesis through oxidation of tetrahydroberberine.

The enzyme was partially purified from the plant tissue and its kinetics and influence of certain metallic ions co-factors and chelators were studied.

On studying the effect of time on the velocity of the enzyme catalyzed reaction, it was noticed that only approximately 50 percent of the substrate was transformed into production even after long periods of reaction. It never increased above 50 pecent even when it was incubated for over four hours.

Yamada and Okada[310] also reported that the enzyme preparation from *Coptis japonica* cell cultures transformed only a maximum of 50 percent of the chemically synthesized tetrahydroberberine. Since it was chemically synthesized it is supposed to have the (R, S)-configuration in equal amounts. The 50 percent conversion of ths substrate suggests the possibility that the enzyme is active only on one of the stereoisomers. They optically resolved the (R, S)(±)-tetrahydroberberine and each of the R-(+) and S-(-) forms were individually treated by the enzyme preparation. Only S(-) tetrahydroberberine was completely converted to berberine by the enzyme and its stereoisomer

was not transformed into berberine. Our data also showed that only about 50 percent of the chemically synthesized THB has been transformed into berberine by the enzyme preparation. This suggests a possibility of stereo-specificity of THB oxidase of *C. fenestratum* demonstrated started with the enzyme of *Coptis japonica*. Okada et al[202] demonstrated that the purified THB oxidase catalyzes the conversion of S(-) THB to berberine by the removal of four hydrogen atoms by molecular oxygen which results in the formation of two mole of H_2O_2 per mole of berberine produced.

Study of the effect of change in substrate concentration on the velocity of THB oxidase reaction showed that it follows a saturation kinetics indicating the formation of enzyme substrate complex. The K_m value of the THB oxidase from *C. fenestratum* is $1x10^{-7}$ moles. This shows that the enzyme has very high affinity for the substrate tetrahydroberberine. It further indicates that the intra-cellular concentration of THB is very low at any point of time and suggests that this compound is probably used up at a very high rate. These kinetic data suggest that the conversion of tetrahydroberberine to berberine is a very active process in *C. fenestratum* cells and indicates that the major pathway through which berberine is produced

is this alternate pathway, where THB is oxidased to berberine by THB oxidase. The K_m value repoted for THB oxidase from *Coptis japonica* was 6.5 µ moles[202].

Among the different modulators only copper ions showed enhancement in the enzyme activity. All other metallic ions and co-factors used did not produce any considerable variations in the enzyme activity. $CuSO_4$ at a concentration of 50 µg/ml gave highest enzyme activity when enzyme was preincubated with it. Different concentrations of $CuSO_4$ was supplemented to the production medium and it was observed that highest berberine production was obtained at a concentration of 100 µg/l. A more or less same response of copper on berberine production when supplemented to the medium was reported by Morimoto et al[311] in *Coptis japonica*. The enhancement in the berberine production may not be solely due to the enhanced activation of THB oxidase alone. The decrease in the activity of the enzyme on pretreatment with EDTA and increase in its activity in presence of metallic ions particularly copper ions suggest that THB oxidase is a copper dependent enzyme. It is not known whether these copper ions have any role in the oxidation reaction.

The THB oxidase preparation from the cultured cells grown in production medium showed higher specific activity. Cells grown in production medium supplemented with $CuSO_4$

also showed an increased activity of THB oxidase. All these results suggest that the enzyme plays a crucial role in the biosynthesis of berberine but may not be the only rate determining step of production.

The supplementation of amino acids into the production medium increased the activity of THB oxidase particularly when tyrosine or L-dopa was added to the medium but no significant effect was observed in the case of other amino acids. The increased activity of THB oxidase can be linked to the enhancement in the berberine biosynthesis when tyrosine or L-dopa was added. It is not known whether it is a direct effect of these substances on the enzyme. Thus in these investigations it was observed that the rate of berberine biosynthesis or the ability of the cultured cells to synthesize berberine has a direct relation with the activity of the enzyme, THB oxidase. Therefore this enzyme can be considered as one of the rate determining steps in berberine synthesis in *C. fenestratum*.

From the above information, it was concluded that, the final step in berberine biosynthesis in the cell cultures of *Coscinium fenestratum* is similar to the alternate final step operating in the cell cultures of *Coptis japonica* as reported by Galueder et al[82] and it is different from the established biosynthetic pathway. elucidated using

the cell cultures of berberis species and *Hydrastis canadensis*[81,279]. But the occurrence of other enzyme systems operating in the final step similar to that in berberis cannot be ruled out. The fact that there are two alternate pathways for one and the sáme secondary metabolite clearly indicates that the pathway of biosynthesis of one secondary metabolite elucidated in one plant species cannot be generalized for other plants containing the same compound even if its metabolic route is completely established at the enzyme level for that species.

CHAPTER IX

CHAPTER IX

SUMMARY AND CONCLUSION

A better exploitation of medicinal plants for obtaining commercially valuable compounds is attempted here using tissue culture techniques. Locally available medicinal plants viz. *Sida cordifolia, Lobelia nicotianifolia, Piper nigrum, Tinospora cordifolia, Coscinium fenestratum* and *Rauwolfia tetraphylla* were selected for the *in vitro* studies with a view to produce the respective alkaloids by callus and cell suspension cultures. Eventhough the above mentioned plants contain different types of secondary metabolites including various alkaloids, investigations were aimed at the production of only certain specfic alkaloids such as ephedrine, lobeline, piperine, berberine and reserpine which form the major components of these plants. Experiments were carried out to develop cell cultures of these plants to study the production of these alkaloids and optimize the conditions for production of berberine by manipulating the nutrient and biochemical parameters and the results are summarised below.

Callus cultures of these plants were established using suitable explants in MS basal media supplemented

with suitable combination of 2,4-D/NAA and BAP. Among the auxins tried for callus induction, 2,4-D was the most effective one. An optimum auxin-cytokinin ratio was required for rapid callus induction for a particular explant in each plant.

Kinetic analysis of cell growth showed that the auxin-cytokinin ratio required for maximum biomass formation was different from that required for callus initiation. Therefore the strategy adopted for maximum biomass production was to initiate callus in the medium which favours callus induction and then transfer into medium which favours biomass production.

In *L. nicotianifolia*, the callus cultures showed the regeneration of shoots and therefore plant regeneration from the callus cultures were studied in detail and a rapid method for the successful regeneration of plantlets was standardized.

The callus cultures of *L. nicotianifolia* produced lobeline,that of *Tinospora cordifolia* and *C. fenestratum* produced berberine and *R. tetraphylla* callus cultures were found to contain reserpine while the callus cultures of *Sida cordifolia* and *Piper nigrum* were not able to accumulate their alkaloids ephedrine and piperine respectively. The alkaloid content in the callus cultures of *T. cordifolia* and *C. fenestratum* was much higher compared to that

of the callus cultures of other plants. Therefore callus cultures of *T. cordifolia* and *C. fenestratum* were established in MS medium supplemented with 10 µM 2,4-D and 1 µM BAP. Both cell suspension cultures synthesized berberine and secreted into the nutrient medium. Based on the results obtained on kinetic analysis of cell growth and berberine production, the cell suspension cultures of *Coscinium fenestratum* were selected for further detailed studies to optimize the media and hormonal conditions and also for studying the metabolism of berberine with a view to maximize the berberine production.

The kinetic studies of growth and berberine production by the cell cultures in four different basal media showed that B_5 medium was useful for obtaining high cell biomass and MS medium was ideal for maximum berberine production.

The effect of different growth regulators on cell growth and berberine production was studied in detail. Different concentrations of auxins such as 2,4-Dichlorophenoxy acetic acd (2,4-D), Naphthalene acetic acid (NAA), Indole acetic acid (IAA), Indole butyric acid (IBA) and 2,4,5-Trichlorophenoxy acetic acid (2,4,5-T) in combination with 1 µM BAP and without BAP were used for these studies From the results obtained, it was understood that NAA was the most suitable auxin for obtaining higher rate

of berberine biosynthesis and 2,4-D for higher biomass formation. Studies with various concentrations of NAA revealed that the optimum concentration of NAA for increased berberine production was 35 µM along with 1 µM BAP. Effect of cytokinins such as benzyl amino purine and 6-furfuryl amino purine (Kinetin) on growth and berberine production were compared and BAP was selected as the suitable one for higher rate of berberine production. Different concentrations of BAP in combination with 35 µM NAA was supplemented to the basal medium and the biomass and berberine production was studied. From these investigations it was concluded that the optimum concentrations of NAA was 35 µM and BAP was 8 µM for higher rate of berberine production.

Based on the above studies, two media were formulated- "Growth medium" and "Production medium". Growth medium is B_5 basal medium supplemented with 10 µM 2,4-D and 1 µM BAP which was used for obtaining maximum amount of cell biomass. The production medium consists of MS basal medium supplemented with 35 µM NAA and 8 µM BAP, which was used for obtaining higher rate of berberine production. Thus a two stage culture system was successfully tested for berberine production using the cell cultures of *C. fenestratum*. The cells were grown in the growth medium and after obtaining sufficient amount of biomass it was transferred to the production medium for berberine production

at higher rate. This two stage culture system may be useful for the continuous or semi-continuous production of berberine in an industrial process.

Supplementation of precursor amino acids like tyrosine or similar primary metabolites like L-dopa into the production medium though resulted in an increase in berberine production, the increase did not appear to be commensurate with the amount of precursors supplemented to the medium. Amino acids like L-tryptophan and L-phenylalanine, even-though are not involved directly in the biosynthesis of berberine, have reduced the amount of berberine produced by cells indicating a regulatory effect on the biosynthesis of berberine.

Nitrate reductase (NR), Glutamine synthetase, Glutamate dehydrogenase and Glutamate synthase were selected for monitoring the level of primary metabolism by studying their activity in cells maintained in growth medium and production medium. The activity of ATPase was also determined. All the four nitrogen metabolising enzymes studied here showed higher activity in production medium in which berberine production is maximum compared to that of growth medium. But when amino acids were supplemented to the production medium, the activities of all the four nitrogen metabolising enzymes studied were reduced to different

degrees. It showed the regulatory activity of some amino acids on nitrogen metabolism particularly the assimilation of nitrate and ammonia directly or indirectly. The activity of ATPase was also shown to be higher in cells grown in production medium compared to that of growth medium. The amino acid supplementation to the production medium caused a sudden rise in the ATPase activity. These results indicated that one of the reasons for increased production of berberine in the production medium may be an enhanced rate of nitrogen metabolism as evidenced by higher activities of four major enzymes involved in nitrogen metabolism. A modulation of the activity of these enzymes as in the case of amino acid supplementation caused an alteration in the rate of berberine production by the cells in culture.

An enzyme preparation from the whole plant as well as cell cultures of *C. fenestratum* catalyzed the biotransformation of tetrahydroberberine to berberine. Tetrahydroberberine oxidase (THB oxidase), the enzyme involved in this final step where THB is converted to berberine, was partially purified and its kinetics and effect of certain modulators such as metallic ions, co-factors and chelators were studied. The kinetic studies indicated that the K_m value of the enzyme is $1x10^{-7}$ moles. This shows that the enzyme has a very high affinity for its substrate tetrahydroberberine

and this compound is probably used up at a very high rate. The kinetic studies suggest that the conversion of tetrahydroberberine to berberine is a very active process in *C. fenestratum* and this enzyme catalyzes a rate limiting step in the biosynthesis of berberine. Among the different modulators only copper ions showed enhancement in the enzyme activity. This shows that THB oxidase may be a copper dependent enzyme. These results indicated that berberine biosynthesis takes place in *C. fenestratum* through an alternate pathway where THB is converted into berberine unlike the normal pathway proposed for the berberine biosynthesis in berberis where palmetin is converted to berberine. It may be similar to that occurring in *Coptis japonicam*.

The THB oxidase activity was shown to be higher in cells cultured in "Production medium" compared to that in "Growth medium". Supplementation of copper sulphate to the production medium also showed increased activity of the enzyme along with increase in berberine production compared to that of control. All these indicate that THB oxidase plays a crucial role in the biosynthesis of berberine but may not be the only rate determining step in the biosynthetic pathway of berberine. The supplementation of amino acids into the production medium increased the activity of THB oxidase particularly when L-tyrosine

or L-dopa was added but no significant effect was observed in the case of other amino acids. The increase in the THB oxidase activity can be linked to the increased production of berberine in the presence of L-tyrosine or L-dopa. These results indicate that the ability of the cultured cells to synthesise berberine has a direct relation to the activity of the enzyme THB oxidase. Therefore this enzyme can be considered as one of the rate determining steps of berberine biosynthesis in *C. fenestratum*.

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APPENDIX

APPENDIX-A

Major groups of compounds	Source plant	References
A. Quinones		
1. Anthraquinones	*Cinchona ledgeriana*	27
	C. pubescens	28
	Morinda cetrifolia	313
	M. lucida	313
	Rubia cordifolia	315,314
	R. fruticosa	30
	Galium mollugo	316,317
	Rubia sp.	316,317
	Sherardia sp.	316,317
	Asperula sp.	316,317
	Rumex alpinus	318,319
	Rhamnus fragnula	318,319
	R. purshiana	318,319
2. Naphthaquinones	*LiLithospermum erythrorhizon*	32
	Echium lycopsis	320,31
	Catalpa ovata	321
B. Phenylpropanoids		
1. Anthocyanine	*Daucus carota*	322,323,324
	Bupleurum falcatum	41
	Euphorbia millii	43,44
	E. tirucalli	43 44
	Strobilanthes dyeriana	45

Major groups of compounds	Source plant	References
	Matthiola incana	325
	Vitis vinifera	46
	Petunia hybrida	326
	Ipomoea batataa	327
	Ipomoea batataa	328
2. Tannins and Proanthocyanidins	*Sangaisorba officinalis*	329
	Rose cv Paul's scarlet	330
	Cryptomeria japonica	331,332
	Pseudotsuga menziesii	333
	Ginkoiloba	334
	Ribes sanguineum	334
	Lotus corniculatus	335
	Onobrychis viciifolia	335
3. Flavanoids and Isoflavonoids	*Eucalyptus teretcornis*	47
	Prunus avium	48
	Anethum graveolens	49
	Scutellaria baicalensis	336
	Paganum harmala	337
	Andrographis paniculata	50
	Podophyllum versipelle	338
	Glycyrrhiza echinata	51
	G. uralensis	339
	Glycine max	340,341, 163
	Phaseolus vulgaris	340,341, 163

Major groups of compounds	Source plant	References
	Canavalia sp.	343
	Medcago	343
	Trifolium	343
	Vigna angularis	164
	Pueraria lobata	165
	Derris elliptica	344
	Crotalaria burhia	345
4. Stilbenes and Dihydrostilbenes	*Picea excelsa*	346
	Arachis hypogaea	347
	Marchantia polymorpha	348
	Jungermannia subulata	349,350
5. Phenalenones	*Daphocohea*	351
6. Lignans	*Podophyllum peltatum*	352,353
	Plagiorhegma dubium	354
	Linum flavun	355
	Vigna angularis	164
7. Coumarins	*Nicotiana tabacum*	356,357
	Petroselinum hortense	356,357
8. L-dopa and betalains	*Stizolobium hassjoo*	358
	Mucuna pruriens	359
	Portulaca grandiflora	142,360, 361
	Beta vulgaris	362
	Chenopodium rubrum	363

Major groups of compounds	Source plant	References
	Phytolacca americana	363
	Gomphrena globosa	149,364
9. Hydroxycinnamic acid Derivatives	*Nicotiana tabacum*	365
	N. glauca	52
	Coleus blumei	52
	Anchusa officinalis	366,367
	Rosmarinus officinalis	57
	Saliva officianalis	57
	Saliva triloba	57
	Perilla frutescens	368,369
	Lavandula vera	370
	L. angustifolia	371
	Coleus spicatus	372
	Syringa vulgaris	373
	Forsythia sp.	374
	Rehmannia glutinosa	375
	Chenopodium rubrum	363
	Matricaria chamomilla	376
C. Isoprenoids		
1. Monoterpenes	*Thuja occidentalis*	377
	Valeriana wallichii	378
	Perilla frutescens	379
	Pinus radiata	380
	Jasminum officinale	60

Major groups of compounds	Source plant	References
	Rosa damascena	60
	Pelargonium fragrans	381
	Chrysanthomum cineriaefolium	382-384
	Gardenia jasminoides	385
2. Sesquiterpenes	*Solanum tuberosum*	61
	Papaver somniferum	63
	N. tabacum	63
	Trichoderma viride	386,387
	Capsicum annuum	388
	Gossypium hirsutum	64
	Ipomoea batatas	389
	Ruta graveolens	390
	Calypogeia granulata	391
	Artemisia annua	391
	Parthenium argentatum	392
	Matricaria chamomilla	393
	Pimpinella anisum	394
3. Diterpenes	*Thujopsis dolabrata*	395
	Chamaecyparis obtusa	395
	C. pisifera	395
	Platycladus orientalis	395
	Salvia miltiorrhiza	396
	Tripterygium wilfordii	399

Major groups of compounds	Source plant	References
	Euphorbia lathyrus	400
4. Triterpenes	*Cucurbita maxima*	401
	Digitalis sps.	256-261 402
	Thevetia	403
	Akebia quinata	405
	Euphorbia tirucalli	406
	Tripetrygium wilfordii	399
	Maytenus buchananii	407
	Solanum dulcamara	69
	Delphinium ajacis	409
	Isodon japonicus	409
	Solanum mammosun	410
	S. aviculare	411
	S. nigrum	412
	S. laciniatum	414,70, 413
	Dioscorea deltoidea	415,416
	D. floribunda	66
	D. tokoro	66
	Trigonella foenum-graecun	69
	Agave wightii	417
	Tribulus terrestris	71
	Panax ginseng	418,419
	Trianthema portulacastrum	73
	Physalis minima	74,75

Major groups of compounds	Source plant	References
	Picrasma quassioides	76
5.Carotenoids	*Lycpersicon esculentum*	77
D. Alkaloids		
1. Indole alkaloids	*Catharanthus roseus*	420-431
	Rauwolfia serpentina	432-434
	Tabernaemontana divaricata	94,95
	T. iboga	94,95
	T. elegans	94,95
	Stemmadenia tomentosa	96
	Voacanga africana	96,89,435
	Ochrosia elliptica	97,436
	Picralima nitida	98
	Ailanthus altissima	99,100
	Peganum harmala	101
	Cinhona ledgeriana	102
	C. succirubra	103-105,29
2. Isoquinoline alkaloids	*Papaver bracteatum*	437,107, 108
	P. somniferum	438-441
	Corydalis ophiocarpa	438-441
	Dioscoreophyllum cumminssii	109
	Plagiorhegma dubium	442
	Berberis stolonifera	111

Major groups of compounds	Source plant	References
	Coptis japonica	112,113
	Thalictrum minus	114,115
	Nandina domestica	117
	Fumaria capreolata	118
	Eschscholtzia californica	443
	Macleaya microcarpa	120
	Ephedra gerardiana	444
	Stizolobium hassjoo	445
3. Quinolizidine alkaloids	*Lúpinus sp.*	446,122
	Cytisus sp.	446,122
	Laburnum alpinum	446,122
	Baptisia australis	446,122
	Sarothamnus scoparius	446,122
	Lupinus polyphyllus	119
	Heimia salicifolia	173
4. Tropane Alkaloids Scopolamine	*Datura innoxia*	129
	Hyoscyamus niger	125
	Duboisia leichhardtii	126,127
	D. myoporoides	127,128
	D. hopwoodii	126
	H. muticus	129
	H. albus	130
	Atropa belladonna	131

Major groups of compounds	Source plant	References
5. Pyridine Alkaloids	*Nicotiana tabacum*	447,142,144
	Duboisia leichhardtii	447,142,144
	D. myoporoides	127
	D. hopwoodii	126
	N. rustica	175,176
	Phaseolus aureus	134
	Trigonella foenum-graecum	135
	Lobelia inflata	177,224,448
6. Purine Alkaloids	*Coffea arabica*	136,137
	Camellia sinensis	137
E.Other compounds		
1. Aldoximie derivatives	*Eschschdtzia californica*	449
	Descurainia sophia	450 ,451
	Alyssum minimum	450,451
2. Amino acid derivatives	*Helianthus annuus*	452,453
	Putterlickia verrucosa	93
	Nicotiana tabacum	454
3. Volatiles	*Ruta graveolens*	455
	Solanum tuberosum	456
	Lycopersicon esculentum	456
	Ricinus communis	457

Major groups of compounds	Source plant	References
	Allium sativum	458
	Apium graveolens	171
Polyacetylenes and thiopenes	*Bidens pilosa*	157
	Carthamus tinctorius	158
	Alternaria carthami	158
	B. alba	160
	Tagetes patula	161
Organic acids	*Amaranthus tricolar*	459
Polyketides	*Cassia torosa*	160
	Daucus carota	162