DISSERTATION

METABOLIC MANIPULATION OF *TAXUS CANADENSIS* FOR TAXOL PRODUCTION

Submitted by

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In partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 1999

COLORADO STATE UNIVERSITY

January 14, 1999

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY MUENDUEN PHISALAPHONG ENTITLED METABOLIC MANIPULATION OF *TAXUS CANADENSIS* FOR TAXOL PRODUCTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

METABOLIC MANIPULATION OF *TAXUS CANADENSIS* FOR TAXOL PRODUCTION

In order to enhance taxol production in suspension cultures of *Taxus sp.*, the regulation of biosynthetic pathways of the secondary metabolites have been investigated. The studies on elicitation and signal transduction showed interdependence of the ethylene and the methyl jasmonate (MJ) actions in affecting taxol biosynthetic reactions in *Taxus canadensis C93AD*. Reproducible results from independent experiments demonstrated complex changes in taxol and 10-deacetyl taxol, which increased in a manner proportional to MJ and ethylene concentrations. Based on the hypothesis of binding between biotic elicitors and receptor proteins on the plasma membrane, a mathematical model to explain the effects MJ and ethylene on the formation of taxol and other taxanes was developed. The inhibitory effect of MJ on taxol production, especially at concentrations greater than 100 μM, was observed and expressed in mathematical terms in the developed model. Taxol production was enhanced about 30 fold over unelicited conditions using 0.5% CO_2, 15% O_2 and 7 ppm ethylene with 200 μM MJ elicitation eight days after cell culture transfer.

From precursor studies, improved taxol production can be obtained by supplementation of potential taxol side chain precursors and acetyl CoA together with MJ elicitation. The

different profiles observed between taxol-related taxanes and baccatin III-related taxanes during elicitation suggest baccatin III may be either a degradation product of taxol or a product of 10-deacetyl baccatin III. The examination of profiles of taxol and 10-deacetyl taxol with different precursor supplements suggests a direct enzymatic reaction leading from 10-deacetyl taxol to taxol.

A multivariable statistical method, Principal Component Analysis (PCA), was used for quality monitoring and fault detection of the experimental data. A correlation matrix demonstrated a positive relationship between ethylene and taxane concentration, strong positive linear relationships between MJ and taxol, 10-deacetyl taxol and baccatin III, and a negative relationship between MJ and 10-deacetyl baccatin III.

Finally, extension of the stationary phase of the cell cycle in a semi-continuous culture with total cell recycle showed considerable improvement in productivity of taxol and other taxanes relative to batch culture.

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Spring 1999

ACKNOWLEDGEMENTS

The present work could only be completed thanks to help provided by many persons to whom I will be always grateful. I would like to thank the excellent advice, support and friendship given by Dr. Jim Linden, my adviser and Dr. Nazmul Karim, my co-adviser and the interesting comments and questions of my committee members, Dr. Vince Murphy and Dr. Cecil Stushnoff. Thanks to Dr. Phillip Chapman for some advice on the statistical analysis.

Thanks to Dr. Linda Henk, Zoyla Flores and all my friends at CSU for their friendship and support which provides a pleasant work environment. I thank the Thai Government for the financial support for my Master and Ph.D. study and all my Thai friends for making my life more enjoyable during my studies here.

I wish to thank the National Science Foundation Engineering Directorate Project BES-9702582 for support and Dr. M.L. Shuler (Cornell University), Dr. Susan Roberts (University of Massachusetts) and Dr. D.M. Gibson (USDA/ARS at Ithaca, NY) for cell cultures and helpful discussions. Thank to Bill Lauderdale (Bioprocess Center at CSU, CO) for the help for the peak verification.

Finally with all my respect, I am very grateful and thank my family especially my parents, Dr. Prapat Phisalaphong and Wanpen Phisalaphong for their support, inspiration and their faith in me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

More than almost any other commercially useful plant secondary metabolite, the anticancer agent, taxol shows promise for commercial-scale production by plant cell culture. In order to enhance the production yield and productivity, basic research is necessary in concert with technological studies. Improved productivity and yield of taxol and related taxoids can be obtained by understanding the regulation of the biosynthetic pathways of secondary metabolites. The occurrence of metabolic blocks may be overcome by investigating precursors of limiting steps. Elicitation and signal transduction are significant stimulating methods, capable of enhancing levels of enzymes associated with secondary metabolism leading to improved taxol yield.

The studies described in this dissertation will provide understanding of signal-tranduction pathways, precursor feeding strategies, and biosynthetic pathway for taxol production. Based on our interaction and binding modeling of taxol formation in elicited suspension cell cultures of *T. canadensis*, the co-mediation between ethylene and methyl jasmonate to stimulate taxol production can be explained. Finally, the extension of the stationary phase is tested in a semi-continuous culture with total cell recycle for improved productivity.

Statistics associated with Principal Components Analysis (PCA) models is developed for quality monitoring and fault detection of the experimental data. A correlation matrix and a correlation map are developed to elucidate the correlation of MJ and ethylene on taxane production and the relationship among the observed taxanes.

The results from the studies will be useful for the development of fundamental understanding of regulatory processes for taxol production in suspension cultures of *Taxus sp.* and the development of strategies for improved taxol production. Higher plants are suppliers of indispensable raw materials and drugs in the food and pharmaceutical industries. While many academic and industrial research groups around the world are pursuing the plant cell culture route of production, Phyton Catalytic, Inc. (Ithaca, NY) is leading the development of a plant cell culture process for production of taxol. With their German subsidiary, Phyton Gesellschaft fuer Biotechnik GmbH, a large scale (75 m^3) process is being developed under license to Bristol-Meyer Squibb, which recently committed $25 million for commercial production within the next five years. If successful, this process could be the technological foundation for other plant cell culture processes.

1.1 OBJECTIVES

1.1.1 General Objectives.

The overall objective of this research is to understand the regulatory processes for secondary metabolism, leading to improved taxol production from suspension cultures of *Taxus canadensis*, by studying interactions of gas phase composition, elicitors and precursors on taxol formation.

1.1.2 Specific Objectives.

a) Determine effects of ethylene and methyl jasmonate on taxol production.

b) Develop a complete modulation model for the co-mediation of ethylene and methyl jasmonate (MJ) on elicited biosynthetic steps of taxol. Relate the experimental results to the modulation model to explain the interaction of ethylene and MJ on signal transduction.

c) Determine effects of chitin and chitosan oligosaccharides and methyl jasmonate as co-mediators of taxane formation.

d) Determine effects of potential taxol side-chain precursors and acetyl CoA on taxol formation. Investigate the rate-limiting steps of taxol biosynthesis.

e) Obtain information for better understanding of taxol biosynthetic pathway.

f) Perform kinetic studies to understand the interrelationships between cell growth, substrate uptake, taxol and related taxane formation, and the effects of elicitation and precursor supplements on behavior of the system.

g) Investigate effects of scale-up on taxol production and behavior of the system.

h) Examine the possibility to improve taxol production by extension of the stationary phase using a semi-continuous system.

i) Apply statistics associated with Principal Components Analysis (PCA) models for quality monitoring and fault detection of the experimental data. Develop cross correlation matrix to elucidate the relationships between variables in the system and taxane production and correlation among the observed taxanes.

1.2 LITERATURE REVIEW

1.2.1 Secondary Metabolites by Plant Cell Cultures

Plant cell and tissue cultures can be established routinely under sterile conditions from explants, such as tissues of germinating seeds, stems, roots and flowers. Strain improvement, methods for selection of high-producing cell lines, and medium optimization can lead to enhancement in secondary metabolite production. However, most often trials with plant cell cultures fail to produce the desired products. One of the main problems encountered is the lack of basis knowledge of the biosynthetic routes, and the mechanisms responsible for the production of plant metabolites (Dörnenburg and Knorr, 1995). The low yield of secondary metabolites in suspension cell cultures was an important barrier in developing commercial processes. Other barriers included a number of basic biological and technical problems related to the characteristics of plant cells, such as the enormous size compared to microbial cells, growth as aggregates, slow growth rates, and high sensitivity to shear stress. Despite these problems, plant tissues remain a major potential source of medicines, pharmaceuticals, fungicides, pigments, fragrances and flavor compounds. Characteristics of plant cell cultures compared to microbial cultures are shown in Table 1.1 (Dörnenburg and Knorr, 1997).

Thirty years ago, the prospect of the use of plant cells for chemical production was not thought possible. Although almost all higher plants may be cultured and produce some secondary metabolites, low yields of these products limited the economic usefulness of this approach. However, a combined strategy to optimize growth and product formation for yield improvement resulted in the first commercial plant process (Tabata and Fujita,

1985). Shikonin production from cell cultures of *Lithospermum erythrorhizon* in 750 L bioreactors was introduced to the market in Japan by Mitsui Petrochemical Industries in 1984. Shikonin, which can be used as a dye and a medical antiinflammatory compound, was valued in 1983 at approximately \$4,000 kg^{-1}. In the US the development of two processes for commercialization were attempted; the production of vanilla flavor from plant cell cultures by Escagenetics (Knuth and Sahai, 1991) and the production of sanguinarine by *Papaver somniferum* (Park et al., 1990). Presently, Phyton Gesellschaft für Biotechnik GmbH began producing taxol, an anticancer drug, using species of *Taxus* cultures with reactor capacities of up to 75,000 L.

Table 1.1 Characteristics of plant cell and microbial cultures.

Characteristics	Plant Cells	Microbial Cells
Size		
Diameter (μm)	40-200	1-10
Volume (μm^3)	$>10^5$	< 1-50
Inoculum (%)	5-40	≤ 1
Growth	Aggregates	Single cell, pellet, mycel
Cultivation time	2-3 weeks	2-10 days
Cell doubling time (hr)	15-120	0.3-6
Oxygen consumption (mM)O_2/h·g(DW)	≤ 5	60
Shear sensitivity	High	Low; (higher fungi :high)
Water content (%)	>90	~80
Regulation	Highly complex	Complex
Genetic potential	Often variable	Stable
Product accumulation	Mostly intracellular (vacuole)	Often extracellular
Medium cost	Approximately 8 fold compared to microbial cells	

Examples of secondary metabolite production of plant cells and related product accumulation strategies are shown in Table 1.2 (Dörnenburg and Knorr, 1997).

Table1.2. Example of secondary metabolite production of plant cells and related product accumulation.

Cell culture / accumulation	Culture type / strategy	Process design	References
Intracellular Storage of Metabolites			
Mentha piperita	Suspension culture	Continuous cell–recycled, Air-lift reactor	Chung et al. (1994)
Beta vulgaris	Cell aggregates	Fluidized-bed reactor	Klebnikov et al. (1995)
Morinda citrifolla	Cell aggregates	Semi-continuous stirred-tank reactor	Kieran et al. (1993)
Extracellular Production			
Lithospennum erythrorhizon	Hairy roots bubble column	Three-phase reactor	Sim and Chang (1993)
Eschscholtzia californica	Elicitation	Three-phase reactor, Air-lift reactor	Byun and Pedersen (1994)
Release of Intracellular Compounds			
Coleus blumei	Immobilization, Permeabilization	Continuous spray feeding,Column reactor	Martinez and Park (1994)
Morinda citrifolla	Permeabilization	Three-phase system	Bassetti et al. (1995)
Gossypium. arboreum	Immmobilization , Permeabilization	Repeated batch, Semi-continuous process	Choi et al. (1995)
Cruciata glabra	Immobilization	Semi-continuous, Three-phase system	Dörmenberg and Knorr (1997)

Whether plant cell culture processes are economical for secondary metabolites depends on a number of factors, including the market price of the product, market volume, culture growth rate, biomass yield and product yield. The "break-even" point for plant cell culture processes has been quoted to \$1,500 kg^{-1} of compound (Stafford, 1991). A considerable amount of applied research has been conducted to improve productivity to

achieve economic production of valuable compounds from plant cell culture systems as shown in Table 1.3 (Dörnenburg and Knorr, 1995).

Table 1.3. Factors influencing secondary metabolite production in plant cells.

STRAIN IMPROVEMENT	SELECTION SCREENING GENETIC ENGINEERING
MEDIUM VARIATION	NUTRIENTS PLANT HORMONES PRECURSORS ANTIMETABOLITES
CULTURE CONDITIONS	INOCULUM SIZE pH TEMPERATURE LIGHT AGITATION
SPECIALIZED TECHNIQUES	ELICITATION IMMOBILIZATION/ CELL RECYCLE PERMEABILIZATION TWO-PHASE SYSTEMS TWO-STAGE SYSTEMS REACTORS

Isolation and selection may be the first step to obtain high producing cell lines. Due to a problem of inherent genetic and epigenetic instability of plant cells, variability often leads to genetic changes by mutation in the culture, thus screening for a desired cell population from the heterogeneous population is required during cultivation. A number of physical and chemical factors that could influence secondary metabolism of plant cell

culture have been found, for example, the balance of plant hormone concentration, nutrient combination, light, temperature, or pH. Genetic technology based on fundamental understanding of the biosynthetic pathways and related enzymology of a particular product, especially the rate limiting steps of the pathway could be approached to improve plant cell productivity. Where the productivity of the desired metabolites is limited by the lack of the particular precursors, biotransformation using an exogenous supply of biosynthetic precursors may improve the accumulation of compounds (Dörnenburg and Knorr, 1997).

Elicitors, compounds triggering the formation of secondary metabolites, are considered to be one of the effective tools for increasing biosynthesis pathways in plant cell. Process strategies including immobilization, in-situ product recovery, and continuous process operation have potential for large production scale. Specialized techniques such as aqueous two-phase systems, two stage systems and development of specific bioreactor systems are continuously under development to achieve consistent and high levels of production.

1.2.2 Regulatory Pathways in Plant Cells: Signal Transduction of Defense Mechanisms in Plants

1.2.2.1 Plant Hormones

Plant tissue culture is generally dependent on the inclusion of plant hormones and plant growth regulators and/or other growth active substances in the medium (Gamborg et al., 1976). Plant hormones rarely act alone; many of the regulators have interacted in order to produce the final effect.

Ethylene: One of the most important plant hormones is ethylene. Ethylene can specifically affect growth of callus and suspension cultures, stem and root elongation, bud formation, rooting and embryogenesis. The role of ethylene is hard to understand because low concentrations can promote (or sometimes inhibit) a process, whereas higher levels have the opposite effect. Another complication comes from interactions of ethylene with other headspace gases especially O_2 and CO_2. Low oxygen promotes the synthesis of ACC, but inhibits conversion of ACC to ethylene whereas high O_2 stimulates the conversion of ACC to ethylene. Very high CO_2 blocks ethylene action (Gaspar et al., 1996). According to experimental results (Mirjalili and Linden, 1995), the most effective gas mixture composition tested in terms of taxol production with *Taxus cuspidata* was 5ppm ethylene, 10%(v/v) oxygen and 0.5%v/v carbon dioxide.

Ethylene production is associated with plant senescence and had been correlated with jasmonic acid (JA) induction in some instances. According to data of Holbrook et al. (1997) tested with the sunflower cotyledons, the greatest stimulation of ethylene production was by treatment with isomer JA 3 (*3R,7R*) and JA 5 (*3R,7R-CH_3*) esters and acids. For promotion of senescence, JA3 (*3R,7R*) is the most effective using 200 μM concentrations and 10 mM ACC pretreatment. According to the study of Yamane et al. (1981), two chiral centers at C-3 and C-7 are importance for jasmonate activity. The ester was found more effective than the acid. The (3R)-stereo chemistry was critical for the activity although activity was also reduced after substituting the C-7 proton with a methyl group.

A complementary DNA encoding a mitrogen-activated protein (MAP) kinase homolog has been isolated from tobacco plants. Transcripts of the corresponding gene were not

observed in healthy tobacco leaves but began to accumulate 1 minute after mechanical wounding. Membrane hydrolysis is a part of senescence, thus methyl jasmonate (MJ) and JA may promote their own synthesis through the release of linolenic acid from membranes. The membrane breakdown may also alter the accessibility of the ethylene precursors ACC to ACC oxidase, leading to the synergism of these two growth regulators (Holbrook et al., 1997).

As ethylene is produced from all plant cultures, it accumulates in the headspace in sealed culture vessels. Cultured tissues can be affected by this gas, the generation of which depends upon the type and weight of tissue being grown, volume of the culture vessel, the manner in which it is sealed and culture conditions (Kevers et al., 1992).

The interaction between plant hormones and elicitors to stimulate secondary metabolite production has been reported in many plant systems. The work by Xu et al. (1994) concluded that some plant defense genes are synergistically induced by ethylene and methyl jasmonate. Their experiments with tobacco showed osmotin promoter was not responsive to MJ alone, but in presence of ethylene, galacturanase (GUS) activity was greatest at 0.05 mM and less at greater concentrations of MJ. Their hypothesis was the binding of ethylene to receptors on the plasma membrane might sensitize MJ receptors on the membrane. Hyperactivation raises the possibility that the functional specificity of defense genes in general may be related to particular combinations of signal molecules; such cross interactive signal transduction systems could also explain the common occurrence of "cross-talk" between signaling molecules because cross-activation to produce synergistic response would need to evolve through common biochemical recognition domains (Xu et al., 1994)

Abscisic acid: Abscisic acid (ABA) plays a key role in closing of stomatal apertures (reducing transpiration), control of water and ion uptake by roots (in part by increasing hydraulic conductivity) and with other phytohormones, promoting leaf abscission and senescence. Abscisic acid along with ethylene and jasmonic acid aided in defense of plants against insect wounding. With ethylene, abscisic acid is intimately involved with plant responses to a wide range of environmental stresses (Gaspar et al., 1996). Ethylene or abscisic acid acting together with JA were found to be involved in wound-induced gene activation to regulate a protease inhibitor Pin2 gene expression in tomato and potato (Rickauer et al., 1997 and Dammann et al., 1997).

1.2.2.2 Elicitors

Elicitors have been well established for signal transduction in many secondary metabolite pathways in plant cell cultures. Elicitors are compounds that cause a plant cell respond as if being attacked by pests. Elicitors may form inside or outside plant cells, and are distinguished as endogenous or exogenous inducers. Depending on their origin, they are classified as biotic or abiotic. Biotic elicitors include polysaccharides derived from fractions of fungal or plant cell walls (e.g., pectin or cellulose) and microorganisms (e.g., chitin, chitosan or β-glucans), glycoproteins, and low-molecular-weight organic acids. Elicitors could be intermediates in a signal pathway that transduce detection of such fragments into activation of genes that help the plant defend itself from pests (Dörnenburg and Knorr, 1995). Abiotic elicitors include ultraviolet irradiation, salt of heavy metals, and chemicals that disturb membrane integrity (Dörnenburg and Knorr, 1995). Plants have evolved a common signal transduction mechanism for the expression

of resistance to a wide variety of unrelated pathogens. An elicitor acts as an external stress, which initiates a series of biochemical reactions for plant defense response, leading to excretion of some secondary metabolites (Figure 1.1).

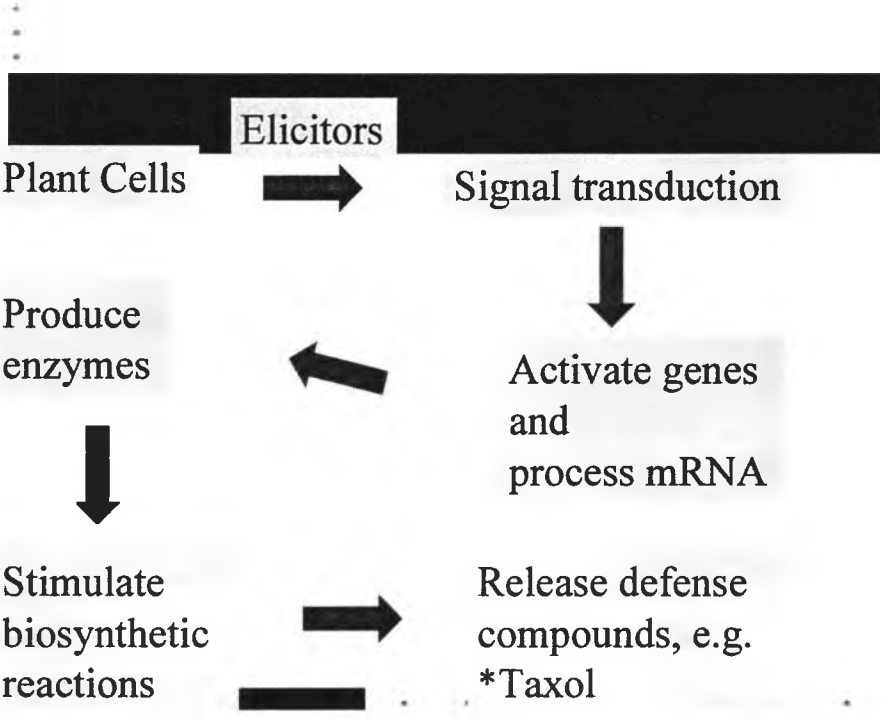


Figure 1.1 Elicitation and signal transduction.

The hypothesis of the interaction between elicitors and cell membrane was proposed by Dörnenburg and Knorr (1995). They suggested that the first reaction of elicitation with a biotic elicitor is binding to specific receptor protein on the plasma membrane; the second step is inhibition of plasma membrane ATPase to reduce proton electrochemical gradient. Gundlach et al. (1992) demonstrated the first step using methyl jasmonate and its derivatives, which results ultimately in the accumulation of secondary compounds. In many defense signal transduction systems, induction of various cellular responses occurs,

including early membrane responses such as the changes in membrane potential, ion flux, oxidative burst, protein phosphorylation.

The possibility of mechanical elicitation by hydrostatic pressures was believed to cause an adjustable mechanical stress on the plasma membrane. This stress then probably stimulates the generation of cell wall-degrading enzymes that release pectin fragments, which serve as signals for the stress response in pressure-treated plant cells (Dörnenburg and Knorr, 1997).

The defense against microbial pathogens and herbivores relies heavily on the induction of defense proteins and low molecular weight antibiotics. The signals between perception of the aggression, gene activation, and the subsequent biosynthesis of secondary compounds are represented by pentacylic oxylipin derivatives. Such fatty acids released upon elicitation plays an important role of signal transduction in plants, animal and microorganism system (Farmer, 1994). In animal systems, activation of a cytosolic PLA_2 is receptor mediated and generates arachidonic acid and lysophospholipids as well-established lipid second messengers (Scherer and Arnold, 1997). Cytosolic Ca^{+2}-independent PLA_2 (group V) functions solely in signal transduction by generating the arachidonic acid modified by lipoxygenase and cyclooxygenase to leucotriens and prostoglandins. An additional pathway for arachiconic acid release is provided by Ca^{+2}-dependent (group II) secreted PLA_2 enzymes. In plants, a pathway leading from linolenic acid to jasmonate is present but arachidonate, the precursor fatty acid to prostaglandins in animal cells, is absent (Scherer and Arnold, 1997).

Jasmonic acid and its derivatives: In plant development jasmonic acid (JA), which acts as mediator of cellular responses arises from action of lipase on membrane-associated

linolenic acid upon wounding, by insect attack or by microbial elicitors. This effect is highly specific and not caused by a number of environmental stresses such as light, heavy metals, or cold or heat shock (Blechert et al., 1995).

The studies of Gundlach et al. (1992) showed that jasmonic acid and its methyl ester accumulate rapidly and transiently after treatment of plant cell suspension cultures of *Rauvolfia canescens* and *Eschscoltzia californica* with a yeast elicitor. Thirty-six plant species tested in cell suspension culture could be elicited with respect to the accumulation of secondary metabolites by exogenously supplied methyl jasmonate. Addition of methyl jasmonate initiates de novo transcription of genes, such as phenylalanine ammonia lyase, that are known to be involved in the chemical defense mechanisms of plants (Gundlach et al., 1992). The results demonstrate the integral role of jasmonic acid and its derivatives in the intracellular signal cascade that begins with interaction of an elicitor molecule with the plant cell surface and results in the accumulation of secondary compounds. Treatment of potato plants by methyl jasmonate created local and systemic protection against *Phytophthora infestans*; the gene hmg1 is wound-inducible and powerfully up-regulated by jasmonic acid (Choi et al., 1994). Preincubation of cells with linolenic acid resulted in JA formation when treated by fungal elicitor. Since linoleic acid did not induce proteinase inhibitor, the JA biosynthesis pathway is deemed necessary in signaling the defense response. Coordinated transcription of the proteinase inhibitor, β-1,3-glucanase, chitinase and hydroxyproline (glycine)-rich proteins were found using the fungal elicitor and MJ (Rickauer et al., 1997). A glycopeptide elicitor prepared from germ tubes of the rust fungus, *Puccinia graminis,* (Ptg) as well as chitin oligosaccharides, chitosan, and MJ stimulated lipoxygensase (LOX) activity in wheat leaves. Immunoblot analysis using

anti-LOX antibodies revealed the induction of 92- and 103-kD LOX species after Pgt elicitor treatment. In contrast MJ elicitation led to significant increase of a 100-kD LOX species. The effects of chitin oligomers and chitosan resembled those caused by MJ (Bohland et al., 1997).

The spike of JA turnover 2-3 hours after fungal elicitor treatment is very similar to a previously reported induction of ethylene by the same elicitor (Rickauer et al., 1997). Since the defense response of the fungal elicitor expends over a longer period than JA accumulation, lipoxygenase (LOX) is thought to give rise to other products that exert a signaling effect on the plant. Treatment with an elicitor from *Phytophthora parasitica* var. *nicotianae* induced a rapid and transient increase in jasmonic acid levels, which was abolished when cells were preincubated with eicosatetraynoic acid (ETYA), an inhibitor of LOX. Pretreatment with ETYA also inhibited the induction of proteinase inhibitors by the fungal elicitor, but not by MJ. Expression of defense-related genes encoding proteinase inhibitor II, hydroxyproline-rich or glycine-rich glycoproteins, glucanase and chitinase, were induced in a basically similar manner by fungal elicitor or MJ. The results indicate that synthesis of jasmonate via the LOX pathway is only part of a complex regulatory mechanism for the onset of many, but not all, defense reactions.

The signal transduction from the interaction of more than one compound sometimes was found more effective. Defense responses usually appear to be regulated by a complex, organ-specific network of signal transduction pathways interacting with each other; when one pathway is blocked, the signal may be shunted into another. Experimental results by Kauss et al. (1992) showed that in some plant defense responses, treatment with only MJ is not effective unless elicitor is also applied and most striking effect requires pre-

incubation with low MJ concentrations followed by treatment with a fungal elicitor. Also some interactions between plant hormones and elicitors had been seen for example, ethylene and salicylic do not induce proteinase inhibitors, but are known to regulate other defense markers. PR-1 and PR-2 are induced by MJ and salicylic or MJ and ethylene (Rickauer et al., 1997). When the plant hormones, ethylene or abscisic acid acted together with JA, wound-induced gene activation was found to regulate pin2 gene expression in tomato and potato (Rickauer et al., 1997 and Dammann et al., 1997).

To assess in *Arabidopsis thaliana* the role of these plant hormones in regulating wound-induced gene expression, wound- and JA-inducible genes were isolated by the differential mRNA display technique. The patterns of expression on mechanical wounding and hormonal treatments revealed differences in the spatial distribution of the transcripts and in the responsiveness of the analyzed genes to abscisic acid and JA. A correlation can be established between sensitivity to JA and the accumulation of the transcripts in systemic tissues upon wounding (Titarenko et al., 1997). A comparative study of the wound indicated that in *A. thaliana* wound signals are transmitted via at least two different pathways. One of them does not involve JA as a mediator and is preferentially responsible for gene activation in the vicinity of the wound site, whereas the other requires JA perception and activates gene expression throughout the aerial part of the plant.

Oligosaccharides: Oligosaccharide elicitors derived from the cell surface of pathogenic microbes, as well as host plants are found to be an effective inducer for various defense responses in plants. Several oligosaccharides that were isolated from plants or microorganisms or that were produced extracellularly by microorganisms have been

selected and screened for their elicitor effects (Dörnenburg and Knorr, 1995). Addition of oligosaccharides to plant cell cultures mimics the effects of elicitation from some pathogenic microbes. However, molecular basis of the specific perception and transduction of these signals largely remain unsolved.

Experimental work by Shibuya et al. (1995), showed that N-acetylchitooligosaccharides of specific size, derived from the cell walls of pathogenic fungi, and also specific fragments obtained by the partial acid or enzymatic hydrolysis of β-glucan from rice blast fungus, *Pyricularia oryzae*, induced defense responses in suspension cultures of rice cells. Binding experiments with an ^{125}I-labeled tyramine derivative of N-acetylchitooctaose showed the presence of a specific, high-affinity binding site for this elicitor on the microsomal as well as plasma membrane of the cultured cells (Shibuya et al., 1995). N-acetylchitooctaose at concentration of 200nM range was also found effective in induction of 1,3 beta-glucanase endohydrolase isoenzyme GII gene in suspension-cultured cells derived from immature barley embryos (Shibuya et al., 1995). A glycopeptide elicitor prepared from germ tubes of the rust fungus, *Puccinia gramintis*, (Pgt) as well as chitin oligosaccharides, chitosan and MeJA stimulated lipoxygenase (LOX) activity in wheat leaves (Bohland et al., 1997). Anthraquininone biosynthesis was also induced in *Morinda citrifolia* by pectic acid, chitin and chitosan. The degree of acetylation was found to be important in increasing permeabilization of plasmalemma, which induced defense responses (PR) (Dörnenburg and Knorr, 1995). Chitin oligosaccharides (dp=6-8) released from fungal pathogens induce plant defense reactions in rice. Lipo-chitin oligosaccharides (dp=4-5) induce the development of a new plant organ, the nodule, in legumes during infection by rhizobia. Dörnenburg and Knorr

hypothesize plants may possess members of an evolutionarily conserved family of chitin binding proteins, which play an important role in chitin binding reception and subsequent signal transduction.

The degree of acetylation, as well as size and structure of the oligosaccharides was found to be important in inducing defense metabolism in the plant cell cultures. Induction of phytoalexin formation in suspension-cultured rice cell by a series of N-acetylchitooligosaccharides and deacetyl chitooligosaccharides was studied (Yamada et al., 1993). N-acetylchitooligosaccharides larger than hexaose induced at very low concentration (10^{-9}-10^{-6}M) the formation of momilactones A and B as well as oryzalexins A, B, and D. GlcNAc oligomers smaller than trimers had almost no activity and a series of deacetylated chitooligosaccharides were also inactive. The data showed the strict requirement for the size and structure of GlcNAc oligomers as well as the sensitivity to them, thus indicating the presence of recognition systems specific for these compounds in plant cells. N-acetylchitooligosaccharides with a degree of polymerization greater than 5 were found active for signal transduction, whereas deacetylated chitosan oligomers caused no effect. Once again, the data showed that strict structural requirements for the induction of ion fluxes were similar to those of specific binding to the putative plasma membrane receptor. The series of signaling events specifically induced by the oligosaccharides, suggested the involvement of transient changes in cytoplasmic ion concentration in oligosaccharide signaling for defense reponses.

A high-affinity binding protein for the N-acetylchitooligosaccharide elicitor of phytoalexin biosynthesis was identified by photoaffinity label and affinity cross-linking in the plasma membrane of suspension-cultured rice cells (Shibuya et al., 1995). The two

conjugates of N-acetylchitooctaose were synthesized and were separately incubated with the plasma membrane prepared by aqueous two-phase partitioning. The covalently cross-linked to elicitor binding site was by irradiation with UV light or by treatment with the cross-linking agent glutaraldehyde. Autoradiography of the SDS-PAGE gel of the solubilized membrane proteins revealed the labeling of a single 75 kDa band in both cases. The incorporation of the radiolabeled ligands into the 75 kDa protein showed a saturable mode of binding. The results showed the sensitivity and the specificity of the 'high-affinity binding site' previously identified by binding assays, as well as with the activities of these oligosaccharides in the induction of phytoalexin biosynthesis and other cellular responses. These data suggested that the 75 kDa protein identified by the affinity labeling represents a functional receptor for this elicitor. The results imply that the site of perception for these elicitors is generally regarded to be the plasma membrane and recognition at cell surface triggers a chain of events, as known as " signal transduction".

1.2.3 Bioreactor Studies, Scale Up and Some Specialized Techniques

The development of large-scale fermentation processes for plant cells are complex due to many problems including the slow growth of plant cells, the low shear resistance and the tendency for cell aggregation. Plant suspension cultures often consist of small aggregates with sizes ranging from fine suspensions, as small as 100-500 μm to aggregates, as big as several millimeters in diameter (Fowler, 1983). As a result of the differences in growth and accumulation characteristics of plant cell cultures compared to microbial growth and metabolite production, the well-established technology of large-scale fermentation of microorganisms can rarely be transferred directly to biomass or

metabolite production from plant cell cultures. A comparison of operating conditions of microbial and plant cell suspension fermentation is shown in Table 1.4 (Singh, 1997).

Table 1.4 Operating conditions of microbial and plant cell suspension fermentation.

	Microbial Cell Suspension	*Plant Cell Suspension*
Cell Concentration (g DW/L)	10-50	10-50
Power Input / Volume (W/L)	1-10	0.1-0.5
Variation in Viscosity (cP)	1-1,000	1-50
Specific Growth Rate (per day)	2-25	0.1-0.5
Biological Oxygen Demand (mg/L hr)	1,000	100
Mass Transfer Coefficient (per hr)	100	20

Reproducing optimum conditions in bioreactors can be difficult with respect to physical factors of mixing and shear. Keeping a reactor well mixed and transferring sufficient oxygen from gas to liquid phase can be managed by pneumatically agitated (e.g., in air lift or bubble column), mechanically agitated (e.g., in stirred tank) or by a combination of both methods (e.g., in stirred tank with air diffuser). The power input to the reactor also results in some detrimental effects on the plant cells, such as shear stress. Because of the shear sensitivity of plant cells, reactor design has to focus on methods that reduce the power input without affecting the mixing and mass transfer abilities in the reactor. It has been suggested that power inputs in the range of 0.1-0.5 W/L are sufficient for good mixing and oxygen mass transfer in plant cell suspension bioreactors (Singh, 1997).

The summarization of the advantages and disadvantages of each reactor type was shown in Table 1.5 (Fowler, 1983). In the airlift reactor, mechanical agitators can be

eliminated and mixing is achieved by the fluid flow obtained from sparking air into a draft tube. Though the airlift reactors have been successfully used for culturing plant cell suspensions at volumes as high as a few hundred liters, it becomes increasingly difficult to obtain good mixing as cell concentration and reactor volume increase. Thus, much of the current work still has concentrated on suspension cell reactors with mechanical agitators. To provide good mixing, sufficient oxygen mass transfer together with reducing shear stress, special impellers with low Power number are suggested to replace the conventional impellers. An alternative method to achieve this requirement is by modifications of low speed impeller and fine bubble diffusers.

Table 1.5 Comparison of the performance of various standard bioreactor systems

Bioreactor Performance	Reactor Type		
	Stirred Tank	Bubble Column	Airlift
Oxygen Transfer	+++	++	+
Low Shear	+	++	+++
Mixing	+++	+	++
Scale-up	Difficult	Easy	Easy
Limitations	Cell death, contamination due to moving part	Death zones, setting of cells due to poor mixing	Dead zones at high cell densities

Immobilization of plant cell cultures has been developed to be an alternative method to improve the metabolite production. Immobilization has a number of advantages, for instance: immobilization provides continuous process operation, reuse of biocatalysts, separation of growth and production phases, and simplified separation of biocatalysts

from the culture medium. The choice of a suitable immobilization system depends on several factors such as density of cells and the arresting potential of the matrices. Gel entrapment has been the most widely used immobilization method because it is cheap, simple and reproducible using mild conditions during the immobilization (Dörnenburg and Knorr, 1995). Some natural elicitors include polysaccharides such as pectin and chitosan have permeabilizing activities and at the same time could be applied as effective elicitors. These are also used in the immobilization of plant cells in gel producing systems (Vorlop and Klein, 1987).

To avoid the effect of feedback inhibition, two-phase systems have been introduced. The removal and sequestering of the product in an artificial compartment may consequently increase total metabolite production. However, in some systems, organic solvents may decrease growth and productivity. Continuous removal of products by addition of a second solid phase as adsorbent then may be suitable for stimulation of the biosynthetic capacity, for example, in production of anthraquinones. Besides, recovery and purification in this system are generally simplified, thus reducing production costs (Dörnenburg and Knorr, 1995 and 1997).

Many secondary metabolites produced by cultures were found to be non-growth associated or mixed-growth-associated product kinetics. Either the conditions favoring high growth levels do not favor production, or conditions favoring high production are detrimental for growth. A single reactor with continuous monitoring of the process to strategically change the operating conditions or a two stage fermentation have been suggested. This contradiction was exemplified in the industrial production of shikonin from cell culture of *Lithospermum erythrorhizon.* The process was carried out in two

stages (Takahashi and Fujita, 1991). The first stage was operated under conditions and medium that favor biomass production. In the second stage, the conditions and medium were changed to initiate secondary metabolite production.

1.2.4 Production of Paclitaxel (Taxol ®) and Related Taxoids in Cell Cultures of *Taxus sp.*

1.2.4.1 Paclitaxel (Taxol ®) and *Taxus sp.*:General Features

Taxol (Paclitaxel), a complex diterpenoid alkaloid, originally isolated from the bark of the Pacific Yew tree (*Taxus brevifolia Nutt.*) has been the subject of intensive research owing to its unique anti-tumour activities (Theodoridis and Verpoorte, 1996). Interest in this compound increased in the late 1970s, when Schiff and his group (1979) described the unique mechanism of its cytotoxic action as a promotor of microtubule assembly. Unlike the Vinca alkaloids, colchicine, and other antimitotic agents that act by preventing the polymerization of tubulin into microtubules, taxol promotes microtubule assembly and suppresses depolymerization (Hezari and Croteau, 1997). Recently numerous articles have reported effects of taxol on a variety of cancers. Taxol has been approved by FDA for the treatment of ovarian and breast cancer. This compound also shows clinical activity against advanced squamous cell carcinoma of the head and neck, malignant melanoma, advanced small/non-small cell lung cancer, germ cell cancer, urothelial cancer, oesophageal cancer, non-Hodgkins lymphoma, multiple myeloma, and gastrointestinal and bladder cancer (Misawa and Goodbody, 1996).

Taxol was first isolated from the dried bark of the pacific yew, *Taxus brevifolia* (about 0.01 % of the dry weight of bark (Fett-Neto and DiCosmo, 1996), which is native to the

Pacific Northwest of the U.S. and Canada. Leaves (needles) of *Taxus sp.* have also been shown to contain taxol in equivalent or greater amounts than found in bark. The occurrence of taxol on needles is obviously interesting considering the faster renewal rate of needles as compared to bark. Removal of bark causes death of tree due to phloem stripping. Taxol also occurs in roots of *Taxus sp.*, but this is a difficult source to exploit in intact plants (Fett-Neto and DiCosmo, 1996). Over 100 different taxoids, including some xyloside conjugates, have been identified in *Taxus sp.* and an endophytic fungus of a particular individual of *Taxus brevifolia* is reputed to produce trace amounts of taxol (24-50 ng $\cdot l^{-1}$ of culture) (Strobel et al., 1996).

Taxol is now being manufactured commercially by extraction from the bark and by semi-synthesis using the precursor (10-deacetylbaccatin III) derived from *T. baccata* needles at yields of at least 0.1% of dry weight (Fett-Neto and DiCosmo, 1996). However, the supply of drug from bark or needles is limited because yew trees grow very slowly and contain only a very small amount of taxol. Approximately 10,000 kg of dried weight of bark are required to yield 1 kg of taxol (this amounts to 3000 trees and sufficient taxol to treat only 500 patients (Kingston, 1994). Moreover, the extraction of these compounds in plants generally requires labor-intensive procedures, and is dependent on seasonal and geographical factors. Therefore, in the long run, it would be highly desirable to be able to produce taxol by a process under entirely controllable conditions to meet extensive demands for the drug.

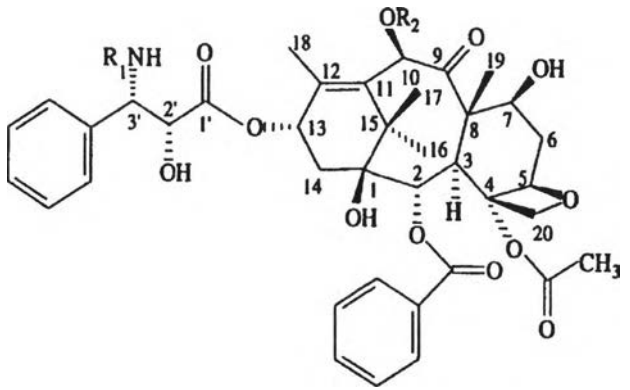
Alternative methods are being developed for increasing taxol production. Recently total synthesis of taxol has been achieved however it required numerous steps, with resulting low yields. Therefore, total synthesis is considering very expensive and not commercially

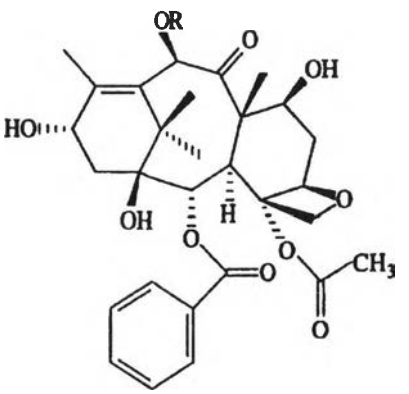
viable. Plant cell culture is a potential alternative source of taxol and related taxoids. Cell cultures are easily renewable. Because of much lower number of contaminates in this process, the cell culture method provides easier and less expensive extraction and purification processes. Moreover, cell cultures offer a very good system for studying the biosynthetic steps leading to secondary products, isolating enzymes and the cloning of their respective genes. Research on the production of taxol and related taxoids has been conducted using species such as *T. brevifolia* (Pacific yew), *T. baccata*, *T. cuspidata* (Japanese yew), *T. chinesis* (Canadian yew), and *T. media* (a cross of *T. baccata* and *T. cuspidata*). So far, the highest productivity of taxol has been reported (153 mg/L in 6 weeks) with *T. chinesis* cell culture (Yukimune et al., 1996). This technology is under development by Phyton Catalytic Inc. under license from Bristol-Meyer Squibb. Commercial production is anticipates within five years in 75 m^3 stirred tank reactor (Dörnenburg and Knorr, 1995)

1.2.4.2 Biosynthesis Pathway and Control

To improve taxol yield in plant cell cultures, it is important to understand the biosynthesis of taxol, especially the rate limiting steps of the pathway. Thus, recent research has focused on determining the control of this secondary metabolite by trying to identify the rate-limiting steps in the biosynthesis pathway. The pathway to taxol is still unknown, but significant progress has been made.

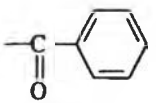
The structure of taxol is quite complex with an unusual diterpene carbon skeleton, eight oxo-functional groups, and an assortment of appended side-chains, for a total of eleven steriocenters (Hezari and Croteau, 1997) as shown in Figure 1.2. Based on the



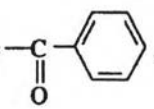


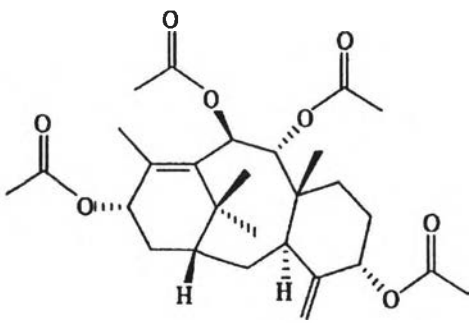
Baccatin-III (8): R=CH_3CO —

10-Deacetylbaccatin-III (9): R=H

Taxol (1)[a]: R_1=  , R_2=CH_3CO —

Cephalomannine (6): R_1= —C(=O)—C(CH$_3$)=CHCH$_3$ (cis) , R_2=CH_3CO —

10-Deacetyltaxol (7): R_1=  , R_2=H



Taxusin

Figure 1.2 Taxol and other taxanes' structure (Floss and Mocek, 1995).

metabolism of other terpenoid natural products, taxol and related taxanes are derivative diterpenes derived from the universal diterpenoid precursor, geranylgeranyl diphosphate (GGPP). The first three steps of taxol biosynthesis have been defined and the responsible enzymes described: (1) cyclization of GGPP to taxa-4(5),11(12)-diene, (2) cytochrome P450-catalyzed hydroxylation of this olefin to taxa-4(20),11(12)-dien-5-α-ol, and (3) acetyl CoA-dependent conversion of the alcohol to the corresponding acetate ester (Hezari et al., 1995, 1996 and 1997). Demonstration of these early steps of taxol biosynthesis suggested that the complete pathway could be defined by a systematic, stepwise approach at the cell-free enzyme level. Combined with in vitro studies to determine contribution to pathway flux, slow steps could be targeted for gene isolation and subsequent overexpression in *Taxus* to improve the yield of taxol and related compounds (Hezari and Croteau, 1997).

The results of Hezari et al. (1995, 1996, and 1997) were extended by Hefner et al. (1996). Microsomal enzymes from *Taxus* stem and cultured cell were used to define the first hydroxylation of taxadiene. The structure of taxa-4(5),11(12)-diene and the reaction product (taxa-4(20),11(12)-dien-5α-ol were confirmed by synthesizing these compounds (Rubenstein and Williams, 1995). The responsible biological catalyst was characterized as a cytochrome P450 (heme thiolate protein). Seven remaining oxygenation steps might involve similar catalysts.

The report by Croteau et al. (1995) indicated the cyclase [taxadiene synthase] and the P450 hydroxylase were both rate limiting with respect to carbon flux to taxol. In vitro studies confirmed that taxadienol was a biosynthetic intermediate and indicated that the hydroxylation step that produces this product was slow relative to subsequent metabolic

transformations (Hefner et al., 1996). From this report, it may be possible to speed taxol biosynthesis by isolating and manipulating the genes for the taxadiene-5-hydroxylase that catalyzes this reaction and putting them on high-expression vectors in cell culture.

On the terpenoid part of the molecule, incorporation of acetate and mevalonate has been shown in *T. canadensis* (Fett-Neto et al., 1994); acetate incorporation in *T. brevifolia* and *T. floridana* was also reported. Incorporation of phenylalanine in the side chain of taxol was reported in *T. canadensis* and *T. brevifolia* (Strobel et al., 1993 and Fett-Neto and DiCosmo, 1996). Phenylalanine had actually been shown to be involved in the biosynthesis of the side chain of some taxoids closely related to taxol (as a precursor to 3-dimethylamino-3-phenyl-propanic acid) in *T. baccata*; the N-benzoylphenylisoserine side chain of taxol was suggested to arise from phenylalanine via β-phenylalanine. According to the work by Floss et al. (1995), a phenylalanine aminomutase reaction shifted the nitrogen one carbon over to generate β-phenylalanine. Additional studies on the mode of attachment of the side chain showed that the side chain was not attached as an intact unit, but most likely as phenylisoserine (Fleming et al., 1994).

The taxol biosynthetic pathway beyond taxa-4(20),11(12)-dien-5-α-ol is still unknown. Taxol production was found to be primarily plastidic and that baccatin III need not to be a direct precursor. Baccatin III appears to have cytoplasmic and plastidic biosynthetic components, while taxol production is essentially plastidic (Srinivasan et al., 1996). Cycloheximide(10 μM) completely inhibited baccatin III production, but had no effect on taxol production. Subsequently, the kinetic observation by the same group showed that baccatin III and taxol accumulated in parallel until day 15, after which baccatin III ceased to accumulate and the level remained constant until day 25. Taxol continued to

accumulate until day 20, after which the level remained constant. This observation was contradictory to the previously believe notion that baccatin III might be a precursor to taxol (Fett-Neto and DiCosmo, 1996). Srinivasan et al. (1996) suggested that baccatin III may be synthesized from a common intermediate to taxol, but along a separate branched pathway. The observed accumulation of baccatin III continued even as the cells were dying suggested that baccatin III could be a result from taxol degradation (Ketchum et al., 1998).

The activity of acetyl-transferases converting 10-deacetylbaccatin III to baccatin III (Fett-Neto and DiCosmo, 1996 and Zocher et al.,1996) and 10-deacetyltaxol to taxol (Fett-Neto and DiCosmo, 1996) were reported. Both pathways could be processed using acetyl-CoA as acetyl donor, in cell-free protein extracts of needles and cell suspensions.

1.2.4.3 Precursor Feeding Studies

Semi-empirical mathematical models by Shuler's group (Srinivasan et al.,1996) suggested that, taxol production in *T. chinensis* (PRO1-95) cultures is limited by the ability of the cells to convert phenylalanine to phenylisoserine rather than by the branch-point acyl transferase. The examination of taxane profiles in some cell lines indicated a larger amount of baccatin III and 10-deacetylbaccatin III as compared to taxol and cephalomannine showed the occurrence of metabolic blocks between baccatin III and taxol (Fett-Neto and DiCosmo, 1996). Improved taxol yields in callus and cell suspension culture of *T. cuspidata* could be obtained from feeding phenylalanine and other potential taxol side-chain precursors such as benzoic acid, N-benzoylglycine, L-serine, and glycine.

Phenylalanine feeding to *T. cuspidata* callus cultures results in significant increase in taxol yield without detrimental effects on growth. This is probably related to the involvement of phenylalanine as a precursor in the biosynthesis of the C13 N-benzoylphenylisoserine side chain of taxol (Fett-Neto and DiCosmo, 1996).

Benzoic acid supplementation was recorded in improved taxol yield. The metabolism of benzoic acids in higher plants may include the formation of esters and glucosides. Benzoic acid may be also incorporated in the side chain providing the benzoyl moiety acylated to the nitrogen of the phenylisoserine portion of the C13 side chain. The incorporation of labeled benzoic acid into taxol by cut stems of *T. brevifolia* was reported (Lansing et al., 1991).

N-benzoylglycine, serine and glycine are derived from Calvin cycle products. Catabolites from these compounds could enter the shikimic acid pathway, leading to phenylalanine and/or benzoic acid synthesis.

Acetate and mevalonate have been shown to be precursors for the taxoid moiety of taxol. The supplementation of acetate or mevalonate in cell suspensions of *T. cuspidata* did not increase the taxol yield. Darkening of the suspension was observed at 20mM acetate supplementation.

The above studies by Fett-Neto and DiCosmo (1996) suggested that for *T. cuspidata* cell line, the taxoid moiety for taxol biosynthesis is not as limiting as the side chain. The presence of an enzyme in *T. brevifolia* tissue which is capable of linking a phenylpropanoid side chain to C-13 of baccatin-III in the system, suggested that side chain attachment could be a late step in the biosynthesis of taxol.

1.2.4.4 Regulation of Paclitaxel (Taxol®) Production

Taxol is also thought to be one of many defense compounds produced by plants. As a result from Ciddi et al. (1995), suspensions prepared from *Cytospora abietis* and *Penicillium minioluteum* induced the production of taxol from *Taxus brevifolia* cell suspension cultures. The addition of cell extracts and cultures filtrate of *Penicillium minoluteum, Botrytis cinerea, Verticillium dahliae,* and *Gilocladium deliquescens* improved the production of taxol and total taxanes.

Jasmonic acid (JA) and methyl jasmonate (MJ) are involved in signal transduction in many plants. Both jasmonic acid and methyl jasmonate induce proteinase inhibitor synthesis; a model for the signaling pathway has been proposed (Yukimune et al., 1996). Methyl jasmonate stimulation of taxol was first reported by Mirjalili and Linden (1996). Methyl jasmonate promoted the accumulation of taxol and baccatin III to a greater extent than the accumulation of caphalomannine. The increase in taxol, baccatin III, and caphalomannine with 100 μM MJ in cultures was about 5.1-fold, 20.2-fold, and 2.4-fold, respective to the respective values in control cells (Yukimune et al., 1996).

Arachidonic acid (AA) and eicosapentaenoic acid (EPA) and some other polyunsaturated fatty acids have been found to be effective inducers of secondary metabolism. Arachidonic acid is one of the fatty acids in the lipids of plant pathogenic *Oomycete* fungi. Addition of AA mimics the effects of some fungal elicitors. AA (1 mg/L) addition at the time of inoculation increased taxol production by 150 % (Ciddi et al., 1995)

The interaction of MJ and AA on isogene expression has been tested with the conclusion that the AA and MJ pathways are distinct in relation to HMGR (3-hydroxy-methylglutanyl-coenzyme reductase) gene expression and isoprenoid product accumulation (Choi et al., 1994). Addition of AA increases levels of HMGR-specific mRNA and primary phytoalexins (PHY). The compartmentation of HMGRs and other pathway enzymes was thought to be a key factor in regulating the flux toward specific end products. Choi et al. (1994) reported that at low to moderate levels of MJ the presence of MJ enhanced PHY (phytoalexins) accumulation up to 3-fold while high concentrations of MJ inhibited AA-induced accumulation of PHY.

Ethylene as a plant hormone was found important for signal transduction pathway. Taxol productivity increased about 19-fold after elicitation with 5ppm ethylene and 10 μM methyl jasmonate, and 15-fold with 0 ppm ethylene and 100 μM methyl jasmonate (Mirjalili and Linden, 1996). The results showed the interaction of MJ and ethylene might play important roles in signal transduction in taxol production. However, the mechanism of co-mediation is still unclear.

Shear stress could be one of the factors that alter secondary metabolism of plant cells. Shear stress may affect plant cell growth and cellular metabolism negatively or positively depending on the level of applied shear stress, the properties of the cell line and its physiological state. Under applied shear stress, many cell characteristics change including aggregate size, cell growth, cell lysis, respiration rate and secondary metabolism. The shear stress effects on *Taxus cuspidata* and *Taxus brevifolia* cell suspension cultures were studied in the Rotating Wall Vessel (RWV) bioreactor (Sun and Linden, 1999). The results showed shear stress can affect cell growth kinetics, carbon metabolism, nutrient

up take, oxygen consumption and taxol production. Shear stress at certain level ($2.1s^{-1}$) is positive on taxol production which might correspond to the change of cell morphology and cell aggregate size. However, shear stress at high level can damage cell, cause cell lysis and eventually lead to cell death.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Line and Maintenance

The cell line used was *Taxus canadensis* C93AD, kindly provided by the laboratory of Prof. M.L. Shuler (School of Chemical Engineering, Cornell University, Ithaca, NY) and Dr. D.M. Gibson (USDA, ARS, Ithaca, NY). The growth medium was formulated according to Gibson et al. (1993).

Table 2.1 Medium preparation for 1 L medium.

Components	concentration
add before autoclaving	
Gamborg B5 (with 2% sucrose, without auxins and cytokinins) (Gibco BRL, Gaithersburg, MD)	1 pack/ 1L
α-naphthaleneacetic acid (NAA)	2.7μM
6-benzylaminopurine (BA)	0.01μM
make up volume = 1000 mL and adjust pH=5.5 ; put 40 mL medium in each 125 mL flask and sterilize in autoclave for 15 minutes	
add after autoclaving	
Glutamine	2.5 mM
citric acid	62.5 mg/L
ascorbic acid	62.5 mg/L
make up 50 mL stock solution with 0.73 g glutamine, 0.125 g citric acid and 0.125 g ascorbic acid and add 1 mL of this filter-sterilized stock to each 40 mL fresh medium	

The maintenance of cultures was carried out as follows: 10-mL of 14 day old suspensions with about 2.5 mL of settled cell volume were transferred by pipetting into 40 mL of fresh medium in 125-mL Erlenmeyer flasks. Color and size of cell cultures can indicate cell condition. White and non-aggregate cells are healthy cells, which are preferred to use as inoculum for subculture than brown and aggregate cells. Pink or red color is indicative of stress caused by contamination or some poor conditions, such as temperature and deletion of a medium component, which may eventually lead to cell death. The culture flasks were capped with 28-mm. id. Bellco silicone closures (Vineland, NJ) and kept in a New Brunswick Scientific (Edison, NJ) Model G-25 shaking incubator operated in the dark at 130 rpm and 25°C.

2.2 The Gas Mixing Apparatus

The apparatus used for independently controlling the gas flow into each experimental set (Figure 2.1) was adapted from that described by Mirijalili (1995). The oxygen, carbon dioxide and nitrogen flows from the cylinders that were regulated at 20, 20 and 15 psi, respectively through separate flowmeters into corresponding manifolds. The flow rates of oxygen, carbon dioxide and nitrogen through the flowmeters were set at 65, 15 and 65 mL/min respectively. Nitrogen from the flowmeter was humidified before it flowed into the nitrogen manifold. Ethylene flowed from the cylinder that was regulated at 10 psi and was diluted twice with the compressed air before flowed into the ethylene manifold. The outlet pressure of the first and second ethylene dilution device was set at 14 psi and 4 psi, respectively. The gas mixture of oxygen, carbon dioxide from the first line was then mixed with the gas mixture of nitrogen and diluted ethylene from the second line before

flowed into the headspace of the experimental set. The total flowrate of gas mixture in each line was about 30 mL/min. The flows from the oxygen, carbon dioxide and ethylene manifolds were finely adjusted by using the corresponding metering values according to the experimental design. To control gas composition, gas mixture of each line was sampled before and after connection to the experimental set and analyzed using gas chromatography.

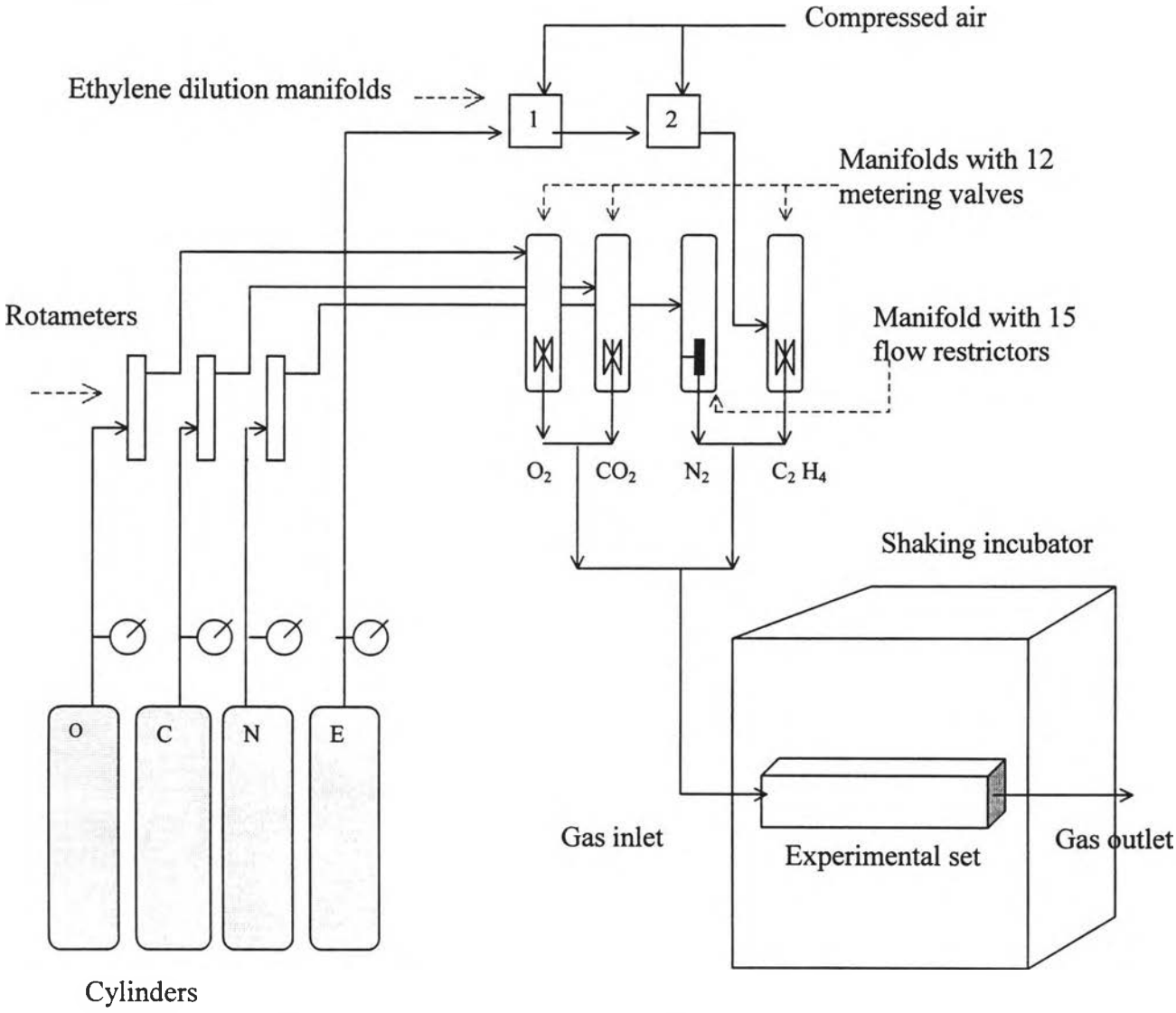


Figure 2.1 Schematic diagram of the gas mixing apparatus.

2.3 Oxygen and Carbon Dioxide Measurements

Oxygen concentration in the gas-flow streams were measured on a Gow Mac model 550 gas chromatograph equipped with a thermal conductivity detector using a molecular sieve column (Alltech 5A, 0.085in. id, 4 ft, SS). The mobile phase was helium at 25 mL/min. The column and detector were kept at 35°C. Gas samples of 1.0 mL were injected and quantified against gas mixtures of standard samples. Carbon dioxide was measured on the same gas chromatography system under the same conditions using an Alltech Porapak N column (0.085-in. id, 6 ft, SS).

2.4 Ethylene Measurement

Ethylene concentration in the gas-flow streams was measured on a Hewlett Packard model 5840A gas chromatography equipped with a flame ionization detector (FID) using an Alltech Porapak R column (0.2 mm id., 6 ft, SS). The mobile phase was helium at 20 mL/min. The column, injection port and detector temperatures were 75°C, 90°C and 180°C respectively. Ethylene in 1.0 mL gas samples were quantitated using standard curve.

2.5 Sample Assays

2.5.1 Sugar Assay

For sugar analysis, 200 μL medium free from cells was filtered through a 13 mm, 0.45 μm Gelman (Ann Arbor, MI) PVDF filter. Sucrose, glucose, and fructose were measured on a Waters and Associates (Milford, CT) Model 6000 high-pressure liquid chromatography (HPLC) system equipped with a refractive index detector using a Bio-

Rad (Richmond, CA) HPX-87P monosaccharide (300x7.8 mm) column. The mobile phase was distilled water at 0.6mL/min. The column temperature was controlled at 85°C. Sugars in 20 μL samples (Gilson Autosamples, Middleton, WI) were quantitated using standard curves.

2.5.2 Cell Dry Weight

The plant cells in the sample were centrifuged and washed three times with distilled water. The cells were collected into an aluminum weighing pan, which was previously dried and weighed. This aluminum pan with plant cells was dried at a temperature of 70°C for 40 hours and kept in a desiccator until cooled to room temperature. Then weight of loaded aluminum pan was determined and cell concentration was calculated from the difference between the weights of the aluminum pan with cells and the weight of the empty pan per the volume of the sample.

2.5.3 Analysis of Taxol and Other Taxanes

Taxol, and other taxanes were measured on a Waters Associates Model 501 HPLC system equipped with a Waters Associates Model 486 UV detector at 228 nm using a Phenomenex Curosil G (250x4.6mm) column. The mobile phase was acetonitrile-water (45:55) at 1.0 mL/min. Extracellular taxol and other taxanes were measured in 2 mL of a supernatant from each sample that was filtered through a 0.20-μm Gelman nylon filter (Ketchum and Gibson, 1993 and 1995). Then, 150 μL of methanol was passed through the filter to dissolve taxol and other taxanes from the membrane and collected into 0.2-mL vials for HPLC analysis. The analyzed data was qualified and quantified against the taxol and other taxane standards, which were prepared in the same way as the samples.

Verification of taxanes was based on absorption spectra and electron-spray mass spectrometer parent ion analysis. Four taxanes have been identified based on retention time identity with standards obtained from Hauser Chemical Research, Inc. (Boulder, CO): 10-deacetylbaccatin III (10-DAB), baccatin III, 7-xylosyl-10-deacetyltaxol, and taxol. The identification of 7-xylosyl-10-deacetyltaxol peak has not been confirmed at this point (the details are discussed in Appendix C). Since the retention time of this peak from HPLC analysis is in the range of 10-deacetyl taxol, in the studies of this dissertation, we refer this compound as 10-deacetyl taxol (10-DAT).

CHAPTER 3

ETHYLENE AND METHYL JASMONATE INTERACTION AND BINDING MODELS FOR ELICITED BIOSYNTHETIC STEPS OF TAXOL IN SUSPENSION CULTURES OF *TAXUS CANADENSIS*

Elicitors have been well established for signal transduction in many secondary metabolite pathways in plant cell cultures. Addition of methyl jasmonate and its derivative initiates de novo transcription of genes, such as phenylalanine ammonia lyase, that are known to be involved in the chemical defense mechanisms of plants (Gundlach et al., 1992). The results demonstrate the integral role of jasmonic acid and its derivatives in the intracellular signal cascade that begins with interaction of an elicitor molecule with the plant cell surface and results in the accumulation of secondary compounds. The study by Mirjalili and Linden (1996) identified approaches that enhance taxol productivity in cell culture, and established the complex interdependence of ethylene and methyl jasmonate (MJ) in affecting taxol biosynthesis. In this study, concentrations of taxol increased in a manner roughly proportional to MJ concentration (see below for discussion of allosteric relationship). The level of enhancement is dependent on ethylene concentration; optimally taxol production after three weeks of growth was enhanced 30-fold over unelicited conditions using headspace gas concentrations of 0.5% CO_2, 15% O_2

and 7 ppm ethylene with 200 μM MJ elicitation 8 days after culture transfer.

Through experiments described here, the understanding of the ethylene/MJ interaction can be refined. *T. canadensis* cultures were exposed to various ethylene concentrations (from 0 to 20 ppm) in the headspace continuously. MJ concentrations from 0 to 400 μM were added in replicated fashion to each set of cultures, and after two weeks the culture media were analyzed for taxane composition and concentration. Reproducible results from independent experiments demonstrate baccatin III concentrations are relatively unaffected compared to changes in taxol levels, which increase in a manner directly proportional to the ethylene concentration. Insights into taxol biosynthesis were solidified through model validation and possible interpretations were made about ethylene binding to membrane receptors.

Co-mediation of Ethylene and Methyl Jasmonate

The role of ethylene is difficult to understand because effects vary with developmental stage and because low concentrations can promote a process, while higher levels have the opposite effect (Gaspar et al., 1996). This is seen with our data in which continuous presence of 7 ppm headspace ethylene (an estimated 70 nM dissolved concentration in the culture medium (Mirjalili and Linden, 1996) resulted in greater levels of taxol than at higher ethylene concentrations. Ethylene concentrations at or greater than 50 ppm were shown to have inhibitory effects on taxol production (Mirjalili and Linden, 1996).

The statistical model established in this study from the data of Mirjalili and Linden (1995) demonstrates that the optimal headspace gas combinations was 17-15% (v/v) O_2, 0.5-1% (v/v) CO_2 and 6-8 ppm ethylene, as shown in Figure 3.1. The statistical regression analysis and the linear regression equation are shown in Appendix A.

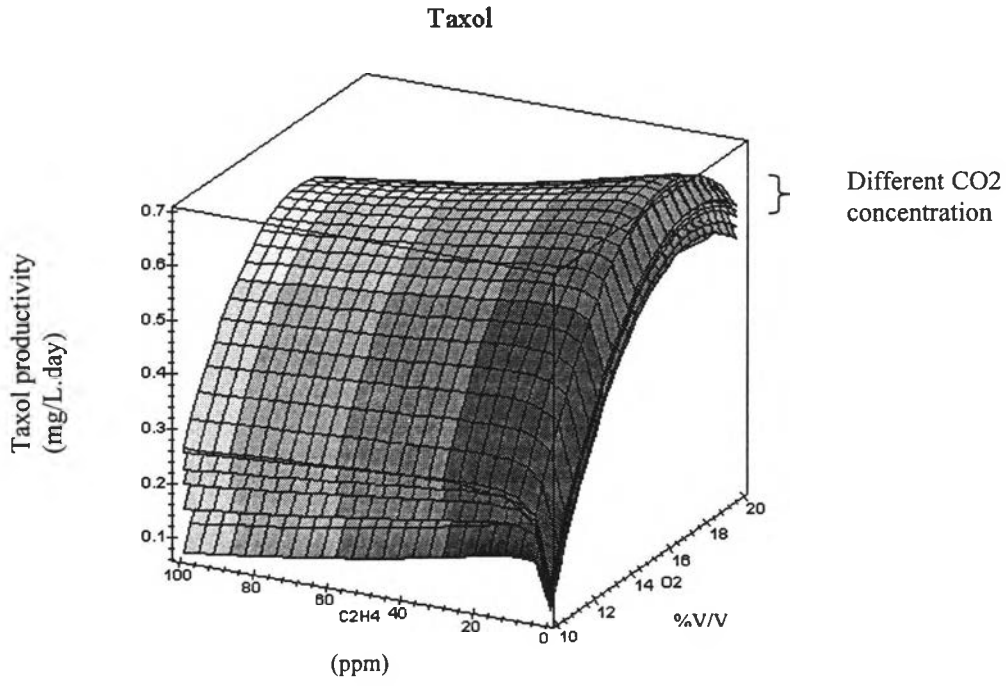


Figure 3.1 Dependence of taxol production on headspace gas concentrations of ethylene, carbon dioxide, and oxygen.

Similarly, MJ-induced rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures is optimal at 0.1mM concentration of MJ; distinct inhibition occurs at higher concentrations (Dörnenburg and Knorr, 1995). Plant pathogen-related (PR) defense genes are synergistically induced by ethylene and methyl jasmonate. Recently, ethylene was found to be involved in wound-induced gene activation in tomato, in which it acts together with MJ to regulate expression of the protease inhibitor *pin2* gene (O'Donnell et al., 1996). A correlation can be established between sensitivity to MJ and the accumulation of the transcripts in systemic tissues upon wounding (Titarenko et al., 1997). Polyphenylalanine lyase activity and coumarin derivative synthesis are regulated

by ethylene and methyl jasmonate (Kauss et al., 1992). Xu et al. (1994) speculated that the binding of ethylene to its receptors on the plasma membrane sensitized hypothetical MJ receptors on the membrane.

Methyl jasmonate is one of several mediators derived from lipids in the cell membrane that represent an important class of elicitors. Examples are described by Farmer et al. (1994), Porat and coworkers (1996), Saniewski et al. (1994) and others (Gaspar et al., 1996 and Holbrook et al., 1997). Methyl jasmonate stimulation of taxol production was first reported by Mirjalili and Linden (1996). Other groups continue to study MJ (Ketchum et al., 1998 and Yukimune et al., 1996); the best reported case caused production of 112 mg/L of taxol. The control cultures, with no MJ, produced about 1 mg/L (Ketchum et al., 1998).

3.1 Experimental Studies

Full factorial design experiments were used for the study of independent action of methyl jasmonate (MJ) at concentrations between 0 to 400 μM and ethylene concentrations between 0 to 30 ppm on production of taxol and other taxanes. For the experimental study, 1.5 mL of 8-day-old suspensions were placed in individual wells of Falcon (Lincoln Park, NJ) twenty-four-well plates. Prepared methyl jasmonate solutions in ethanol (1% of suspension) were then added to each well according to the above experimental design. The multi-well plates were individually sealed back in their original containers. The inlet and outlet gas mixture lines were then inserted into the lids of each plate to control headspace gas mixture composition (combination of N_2, O_2, CO_2, and C_2H_4). The plates were then incubated in an incubator in the dark at 25°C with 130 rpm

shaking. Samples were taken for analyses of taxanes, and sugars after 13 days of elicitation. The experiments were done in triplicate, and the samples for analysis were from the mixtures of 0.6 ml of each triplicate treatment.

3.2 Results

3.2.1 Kinetics of Taxane and Baccatin III Derivatives Formation

Suspension cultures of *Taxus canadensis* (C93AD) were challenged with various concentrations of ethylene and methyl jasmonate (MJ). The concentrations of taxol and other taxanes were determined after 21 days of cell culture transfer. Eight MJ concentrations (0-200 μM) were studied at four constant headspace ethylene concentrations (3.7, 7.4, 10.6 and 27.6 ppm), one oxygen concentration (15% (v/v)), and one carbon dioxide concentration (0.5 %(v/v)). The results of taxol, 10-deacetyl taxol (10-DAT), baccatin III and 10-deacetyl baccatin III production responses to the concentrations of MJ and ethylene are shown in Figure 3.2. The formation of taxol and other taxanes was dependent on the relative concentration of both MJ and ethylene. At the three lower ethylene concentrations, taxol accumulation was roughly linear with MJ concentration; the same is true for 10-DAT at the two lower ethylene treatments. Continuous flow of higher concentrations of ethylene resulted in sigmoidal relationships between MJ concentration and taxol and 10-DAT.

The maximum concentrations of taxol, 10-DAT and baccatin III were obtained using 0.5% CO_2, 15% O_2, and 7 ppm ethylene with 200 μM MJ injection after 8 days of cell culture transfer. In this case, taxol, 10-DAT and baccatin III concentrations were respectively 16.53, 15.92, 1.82 mg/L or 20, 20 and 18 fold over concentration for

unelicited conditions. 10-DAB is the only observed taxane, which was obtained at higher concentrations in non-MJ elicited systems than in the elicited systems.

These data were supported by an additional experiment in which ethylene was held at 6 ppm and MJ concentrations were varied from 0-400 μM. Data in Figure 3.3 show taxol and other taxane concentrations after 21 days of cell culture transfer at various concentrations of MJ. Taxol and 10-DAT accumulations increased with MJ loadings < 200 μM. The inhibition effect of MJ was observed at MJ > 200 μM. The effect of MJ on taxane formation in the second experiment was similar to that in the first one. The optimal MJ concentration for elicitation of taxol production in both experiments was the same (200 μM). However, 10-DAT and taxol concentrations obtained from the later one were lower. The instabilities of this cell line have been observed frequently during our studies and by other research groups (Shuler, personal communication).

From other reports, the optimal concentration of MJ for elicitation of taxol production was 10 μM in *T. cuspidata* (Mirjalili and Linden, 1996), 100μM in *T. media* (Yukimune et al., 1996) and 200 μM in *T. canadensis* (C93AD) (Ketchum et al., 1998).

The ratios between these secondary metabolites are highly dependent on the relative concentrations of ethylene and MJ provided to the suspension cultures. The fractional bar lengths in Figure 3.4 provide a view of the changes in the ratios of the taxanes with MJ between 0 and 200 μM at different ethylene concentrations. Reproducible results at 0-400 μM MJ and 6 ppm ethylene from independent experiments (Figure 3.5) demonstrate 10-DAB and baccatin III concentrations are relatively less affected compared to changes

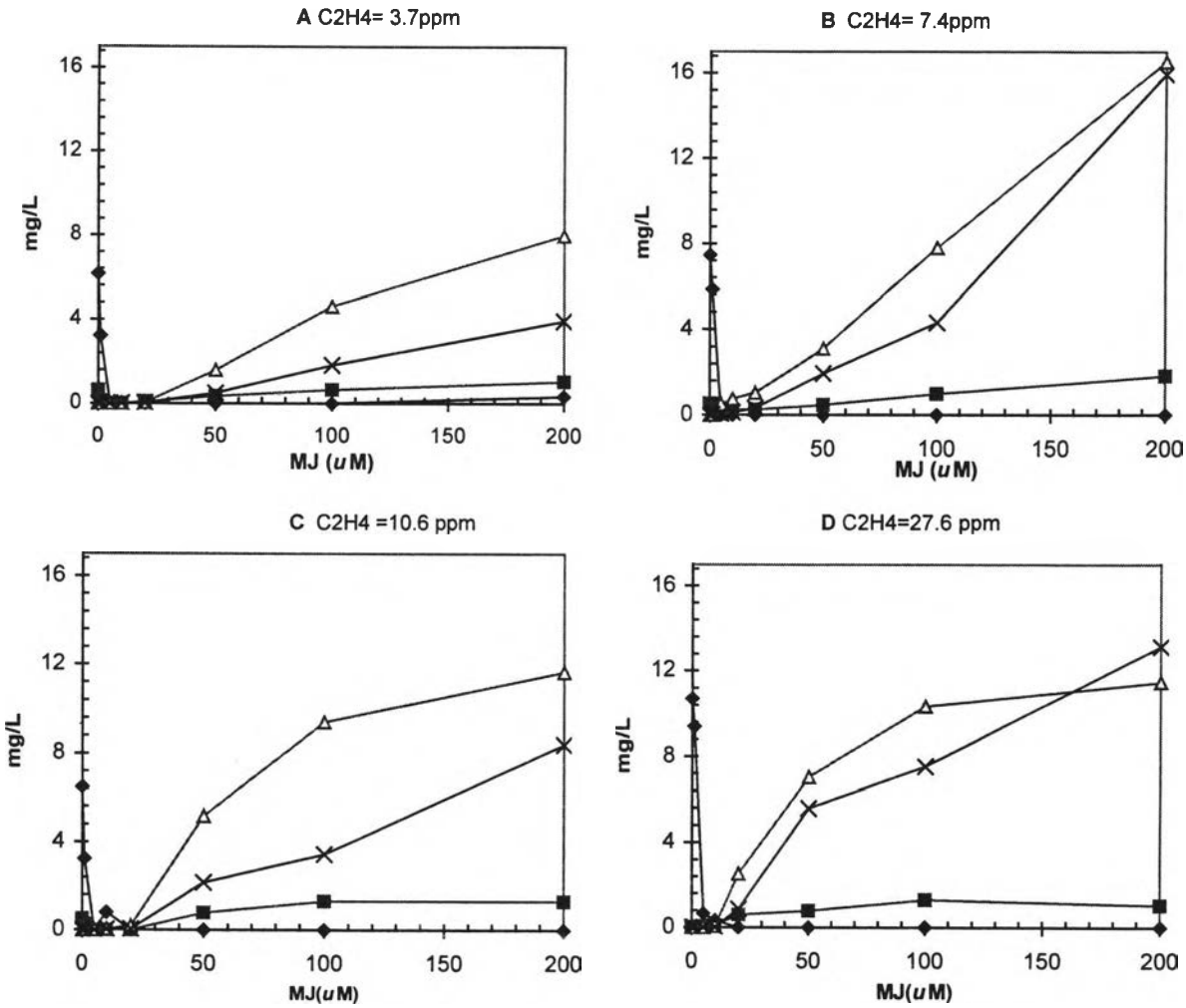


Figure 3.2 Taxanes concentrations at 13 days after addition of MJ and supply gas mixture in the headspace.

◆ = 10-DAB ■ =BACCATIN III △ =10-DAT X = TAXOL

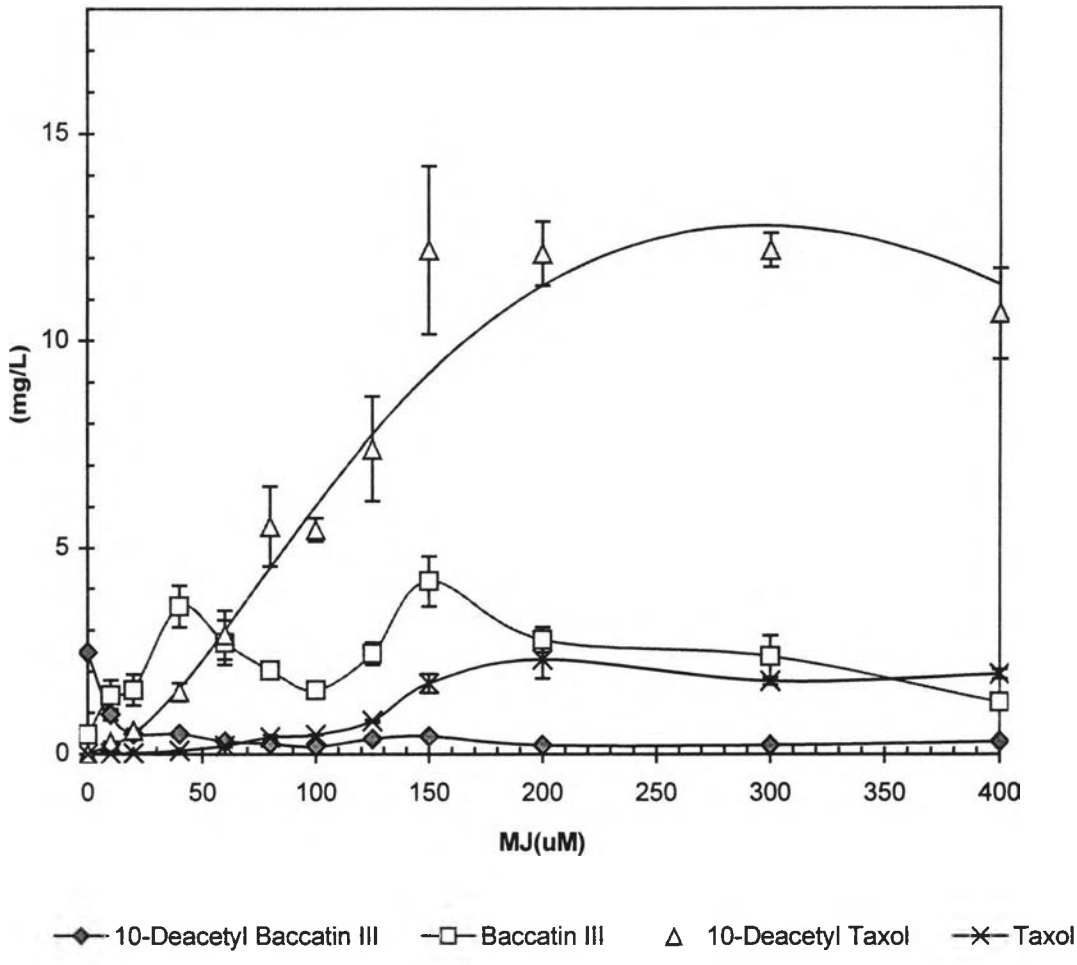


Figure 3.3 Taxanes concentrations at 13 days after applying various methyl jasmonate concentrations (0-400 *u*M) and the supply gas mixture to the head space (6 ppm ethylene, 15% oxygen and 0.5% carbon dioxide).

in taxol and 10-DAT levels, which increase in a manner directly proportional to MJ concentration. 10-deacetyl baccatin III is the only observed taxane that decreased with the increase of MJ concentration. At zero and 1.0 μM MJ, this compound is found at between 3 and 11 mg/L, compared to <2 mg/L at all other MJ loadings.

3.2.2 Allosteric Relationships

Studies on the phenomenon in the binding of ligands to proteins led to observations that are anologous to cooperative rates with enzyme kinetics and the concept of allosteric regulation. Models of binding of substrate molecules to multiple sites of enzymes have been described by Blanch and Clark (1997). This regulation also includes binding of ligands to some proteins without catalytic function, by considering a ligand as a molecule binding to a receptor protein.

The induction of plant defense responses related to the elicitor binding to a specific receptor, probably located in the plant plasma membrane was proposed by Dixon et al. (1990). The binding to the specific receptor leads to changes in the transcriptional activity of genes involved in the production of plant defense compounds. Binding of fusicoccin receptor to one or several phosphorylated proteins could interfere with existing signal transduction pathways, the auxin pathway, or activation of protein kinases or phosphatases (Korthout and DeBoer, 1994). Evidence of elicitors binding to a specific receptor on the plasma membrane in plant cells have been reported. The presence of specific high affinity binding of a synthetic heptaglucoside and fungal glucan phytoalexin elicitors on soybean plasma membrane has been observed by Cosio et al. (1990). A high affinity binding protein for the N-acetylchitooligosaccharide elicitor of phytoalexin

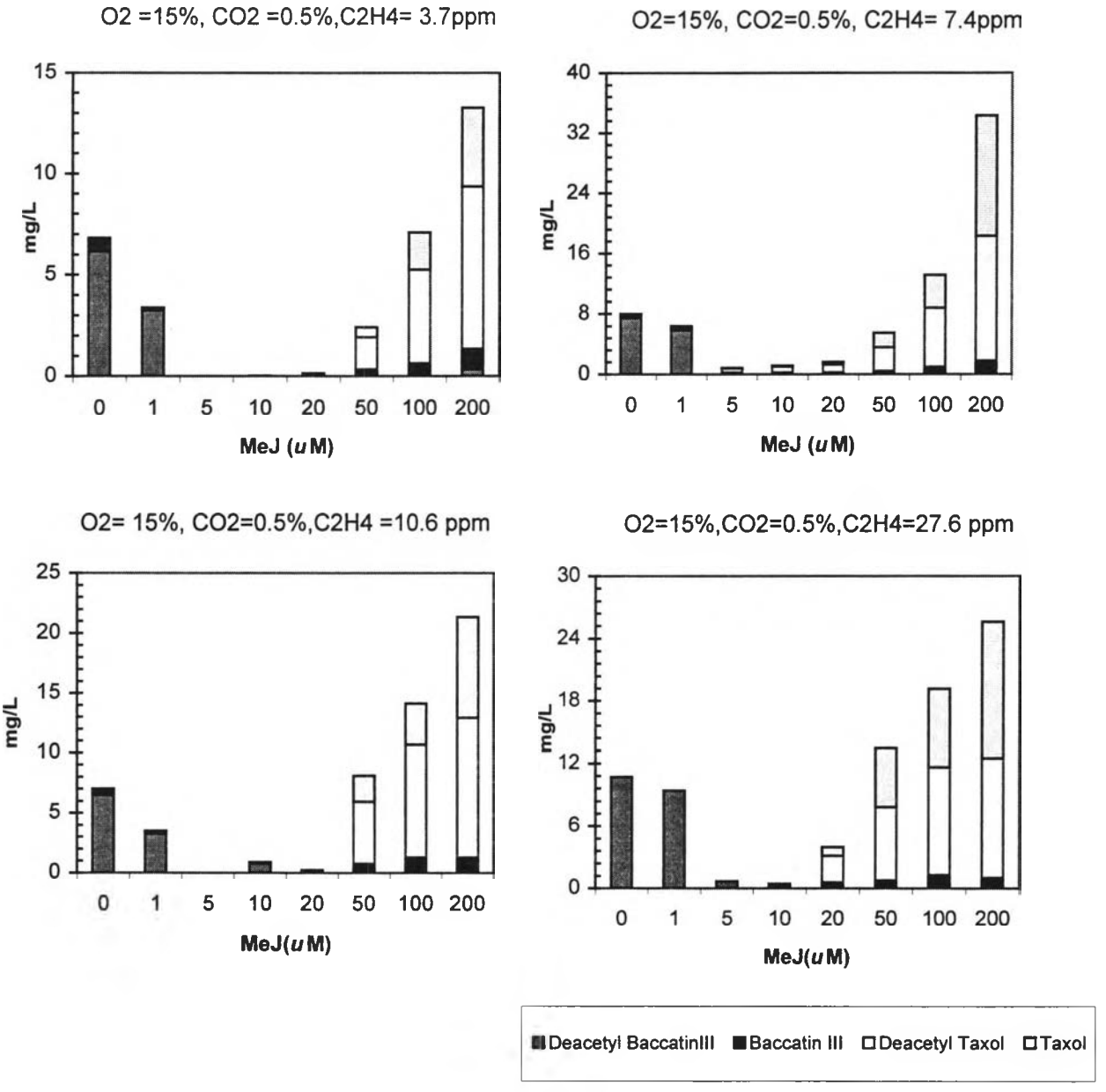


Figure 3.4 The ratios of taxol, 10-deacetyl taxol, baccatin III,and 10-deacetyl baccatin III at 13 days after addition of MJ and supply gas mixture in the head space.

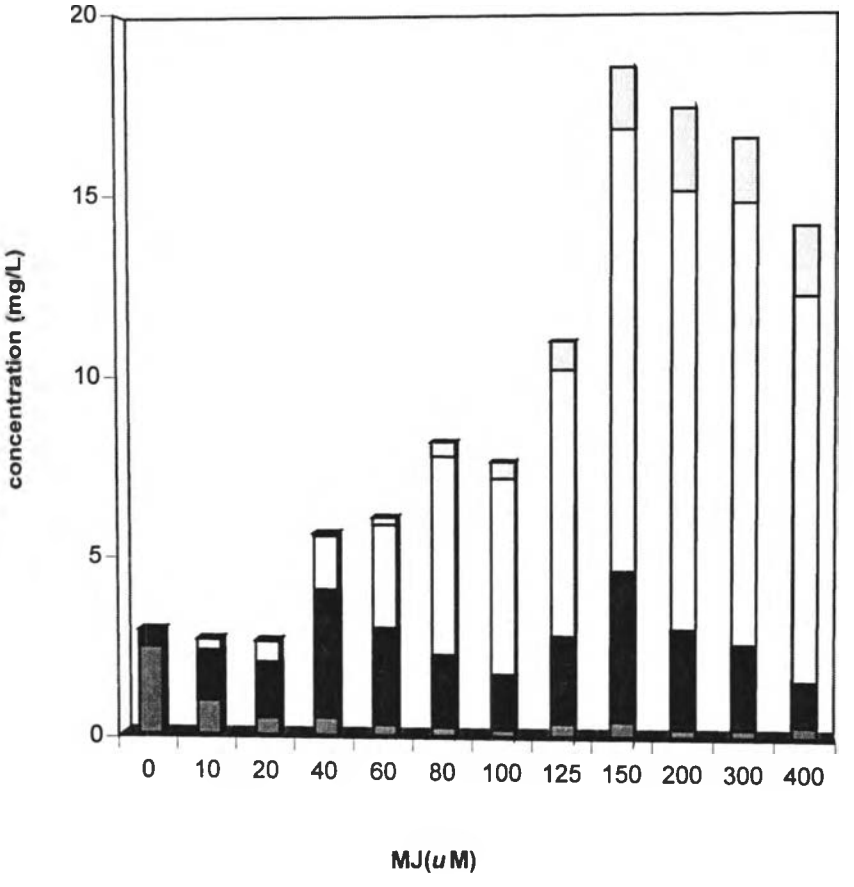


Figure 3.5 The ratios of taxol, 10-deacetyl taxol, baccatin III,and 10-deacetyl baccatin III at 13 days after applying various methyl jasmonate concentration (0-400 uM) and the supply gas mixture to the head space (6 ppm ethylene, 15% oxygen and 0.5% carbondioxide).

▨ = 10-DAB ■ =BACCATIN III □ = 10-DAT □ = TAXOL

biosynthesis was identified by photoaffinity label and affinity cross-linking in the plasma membrane of suspension cultures of rice cells (Ito et al., 1997).

Plant defense genes are synergistically induced by ethylene and methyl jasmonate. The mechanism of co-mediation between MJ and ethylene to stimulate secondary metabolism is still unclear. The hypothetical binding of ethylene to the ETR1 receptors on the plasma membrane might sensitize MJ receptors on the membrane has been proposed (Xu et al., 1994). Another view is just the opposite; MJ altered ethylene binding to ETR1. Dörnenburg and Knorr (1995) suggest that the first reaction of elicitation with a biotic elicitor is binding to a specific receptor protein on the plasma membrane; the second step is inhibition of plasma membrane ATPase reduces the proton electrochemical gradient, which influences the energetic of the cell as well as Ca^{2+} flux and subsequent signal transduction events.

Binding of Ligands to Proteins : Quantitative Consideration

Considering the case of the two identical ligands binding to a protein, where the second ligand binds more rapidly than the first, the situation can be shown as in equation (1).

$$P_2 + L \xrightarrow{K_1} P_2L$$
$$P_2L + L \xrightarrow{K_2} P_2L_2 \qquad (1)$$

Where L is a ligand binding with protein P.

P_2 indicates that the receptor protein contains two identical subunits. The binding constants for the two steps are:

$$K_1 = \frac{[P_2L]}{[P_2][L]} \qquad K_2 = \frac{[P_2L_2]}{[P_2L][L]} \tag{2}$$

If we define Y = the fraction of saturation

$$Y = \frac{concentration\ \ of\ \ receptor\ \ proteins\ \ with\ \ bound\ \ ligand}{total\ \ receptor\ \ protein\ \ concentration} \tag{3}$$

Or we can get Y in terms of P_2 and L as in equation (4).

$$Y = \frac{[P_2L] + 2[P_2L_2]}{2([P_2L] + [P_2L_2] + [P_2])} \tag{4}$$

If the first step is very slow relative to the second, we can combine two steps into one step as in equation (5)

$$P_2 + 2L \xleftrightarrow{K} P_2L_2 \tag{5}$$

And the binding constant (K) can be written as

$$K = \frac{[P_2L_2]}{[P_2][L]^2} \tag{6}$$

Thus, Y can be simplified as in equation (7).

$$Y = \frac{[P_2L_2]}{[P_2L_2] + [P_2]} \tag{7}$$

Therefore, Y can be written in terms of ligand concentration as in equation (8)

$$Y = \frac{K[L]^2}{1 + K[L]^2} \tag{8}$$

Or we can rewrite in a linear form as in equation (9)

$$\log\left(\frac{Y}{1-Y}\right) = 2\log[L] + \log[K] \tag{9}$$

Equation (9) is known as Hill's equation (Blanch and Clark, 1997), which has a general form as in equation (10).

$$\log\left(\frac{Y}{1-Y}\right) = n\log[L] + \log[K] \tag{10}$$

If we relate the rate of biosynthetic reaction to produce taxol and some other taxanes as linearly proportional to the binding of the ligands to receptor proteins, Y can be obtained from the related concentration term of taxol (or taxanes) concentration as in equation

$$Y = \frac{taxol \quad or \quad other \quad taxanes \quad concentration}{\max imum \quad taxol \quad or \quad other \quad taxanes \quad concentration} \tag{11}$$

By assuming that [L] is the concentration of MJ or an intermediate in the signal transduction pathway that is linearly proportional to [MJ], n and K can be obtained from the plot of log [Y/(1-Y)] versus log [MJ] as in equation (12).

$$\log\left(\frac{Y}{1-Y}\right) = n\log[MJ] + \log[K] \tag{12}$$

where $Y = [taxol] / [taxol]_{max}$

n = the number of ligands bound

K = the binding constant.

Binding models for taxol and deacetyl taxol production

The allosteric relationship is seen from the effects of MJ on taxol and 10-DAT production. Kinetic parameters, n and K were obtained from the slope and intercept of log (Y/(1-Y)) versus log [MJ] plot. For the first experimental set, eight MJ concentrations (0- 200 μM)

were studied at four constant headspace ethylene concentrations (3.7, 7.4, 10.6 and 27.6 ppm). The comparisons of experimental data with the binding model at different ethylene concentrations are shown in Figures 3.6 and 3.7.

The allosteric relationship is seen more clearly from the results of the second experiment set (Figure 3.3). Twelve MJ concentrations (0-400 μM) were provided to all of the cultures at one ethylene concentration (6 ppm), one oxygen concentration (15% v/v), and one carbon dioxide concentration (0.5% v/v). Hill plots between MJ and normalized concentrations of both taxol and 10-DAT demonstrate respective linear log-log relationships for all data points but the lowest (10 μM) and the two greatest (300 and 400 μM) where inhibition occurs (Figure 3.8 A for taxol data and Figure3.8 B for 10-DAT data). The comparison of experimental data and the values from the binding model of the second experiment is shown in Figure 3.9.

All data sets from other experiments were similarly analyzed; the number of bound MJ ligands calculated from the slope of the Hill plots in each experiment is between 2 and 3. The number of bound ligands (n) and binding constant (K) from all observed treatments are summarized in Table 3.1. Effects of ethylene on kinetic parameters of the binding model (K and n) are shown in Figure 3.10. The value of n increases in a non-linear manner with ethylene concentration (7.4 < 10.4 < 3.7 < 27.6); recall that at 7.4 ppm ethylene optimum production occurs. We view the bound ligand in this case could represent MJ binding nonspecifically in plasma membrane, as discussed below. The highest value of K (binding constant) and lowest number of bound ligands is observed at the optimum ethylene concentration (7.4 ppm).

3.2.3 Biosynthesis Inhibition by Methyl Jasmonate

As noted above, inhibition by MJ on taxol and 10-DAT production was observed especially at MJ concentrations greater than 200 μM. Inhibition effects of MJ could be expressed in mathematical terms by the following equation.

$$\frac{P_{observed} - P_{predicted}}{P_{predicted}} = \left(\frac{[MJ]}{[MJ]_{\max}} \right)^{\gamma} \qquad (13)$$

where,

P = product concentration (mg/L)

[MJ] = methyl jasmonate concentration (μM)

γ = a power constant that averaged =2.6 for all treatments

The comparisons of experimental data with the binding model and the combined binding and inhibition model are shown in Figures 3.6, 3.7 and 3.9; dashed lines represent the curves developed from only the binding model, and the solid lines represent the binding model combined together with the inhibition effect. Data in Figures 3.6A, 3.6C and 3.6D fit the binding/inhibition model better than the allosteric binding model over the range of MJ concentrations from 0-200 μM. The results in Figure 3.6B demonstrate that no inhibition is observed at 200 μM MJ when the ethylene concentration is 7.4 ppm.

These data are supported by an additional experiment in which ethylene was held at 6 ppm and MJ concentrations were varied from 0-400 μM. Data in Figure 3.9 show a close fit with the simple binding model from 0-200 μM, as also seen in Figure 3.6B. Inhibition is then apparent at MJ concentrations greater than 200 μM. The seemingly anomalous, but oft repeated observation over many years with many *Taxus* cell lines, of optimal taxol productivity at 5-8 ppm ethylene is then perhaps explained by this co-mediation analysis. The kinetic parameters, number of bound ligands (n), binding constant (K) and inhibition factor (γ) from all observed treatments are summarized in Table 3.1.

Another possible significance of these constants may relate MJ to ethylene interaction with receptors in ETR1. The effect of ethylene on the kinetic parameters of the model is shown in Table 3.1 and Figure 3.10. At concentrations of ethylene lower or higher than the optimal point (7.4 ppm in this study), a lower binding constant ($K < 10^{-6}$) is observed than at the optimal ethylene concentration ($K > 10^{-6}$). The number of bound ligands (n) was slightly higher and approached 3 at non-optimal ethylene concentrations.

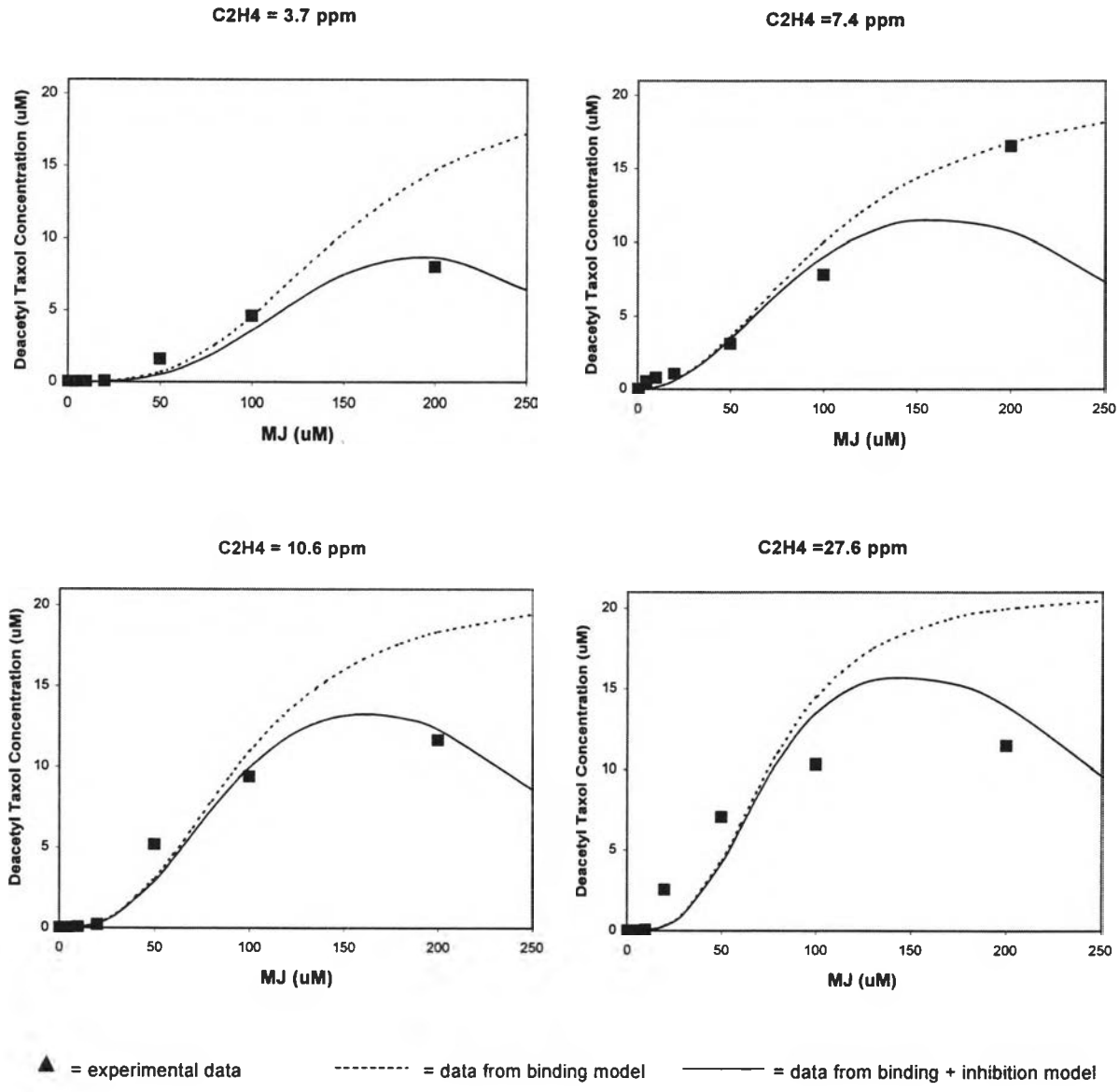


Figure 3.6 The comparison of 10-deacetyl taxol concentrations obtained from experiments with the two hypothetical models at different methyl jasmonate concentrations (0-200 μM) and different ethylene concentrations:
A. 3.7 ppm, B. 7.4 ppm, C. 10.6 ppm, D. 27.6 ppm.

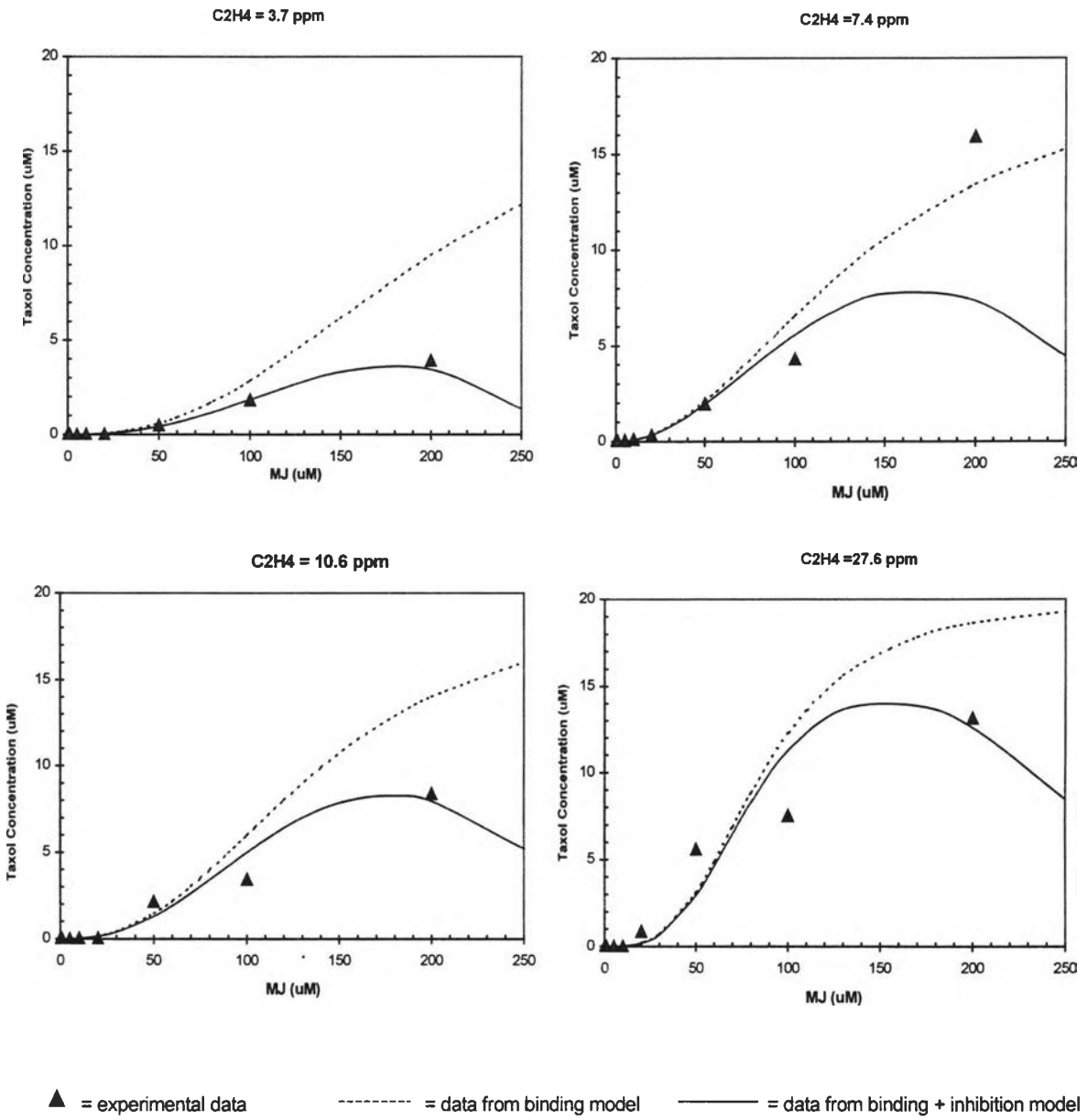


Figure 3.7 The comparison of taxol concentrations obtained from experiments with the two hypothetical models at different methyl jasmonate concentrations (0-200 μM) and different ethylene concentrations: A. 3.7 ppm, B. 7.4 ppm, C. 10.6 ppm, D. 27.6 ppm.

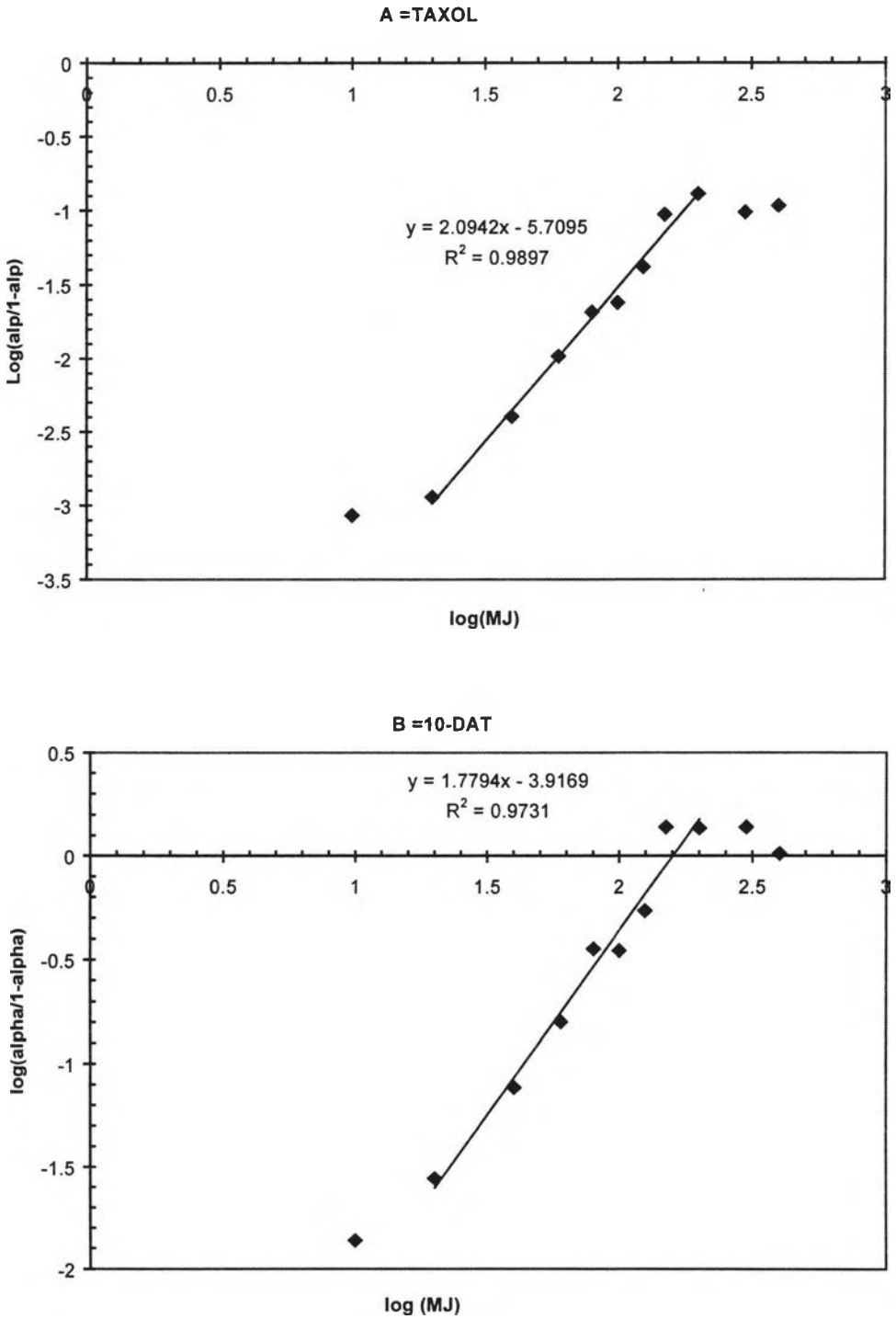


Figure 3.8 The Hill's plots illustrating the case for MJ binding to receptor protein at plasma membrane inducing A. taxol and B.10-deacetyl taxol formation (at 0-400 uM MJ and 6ppm ethylene)

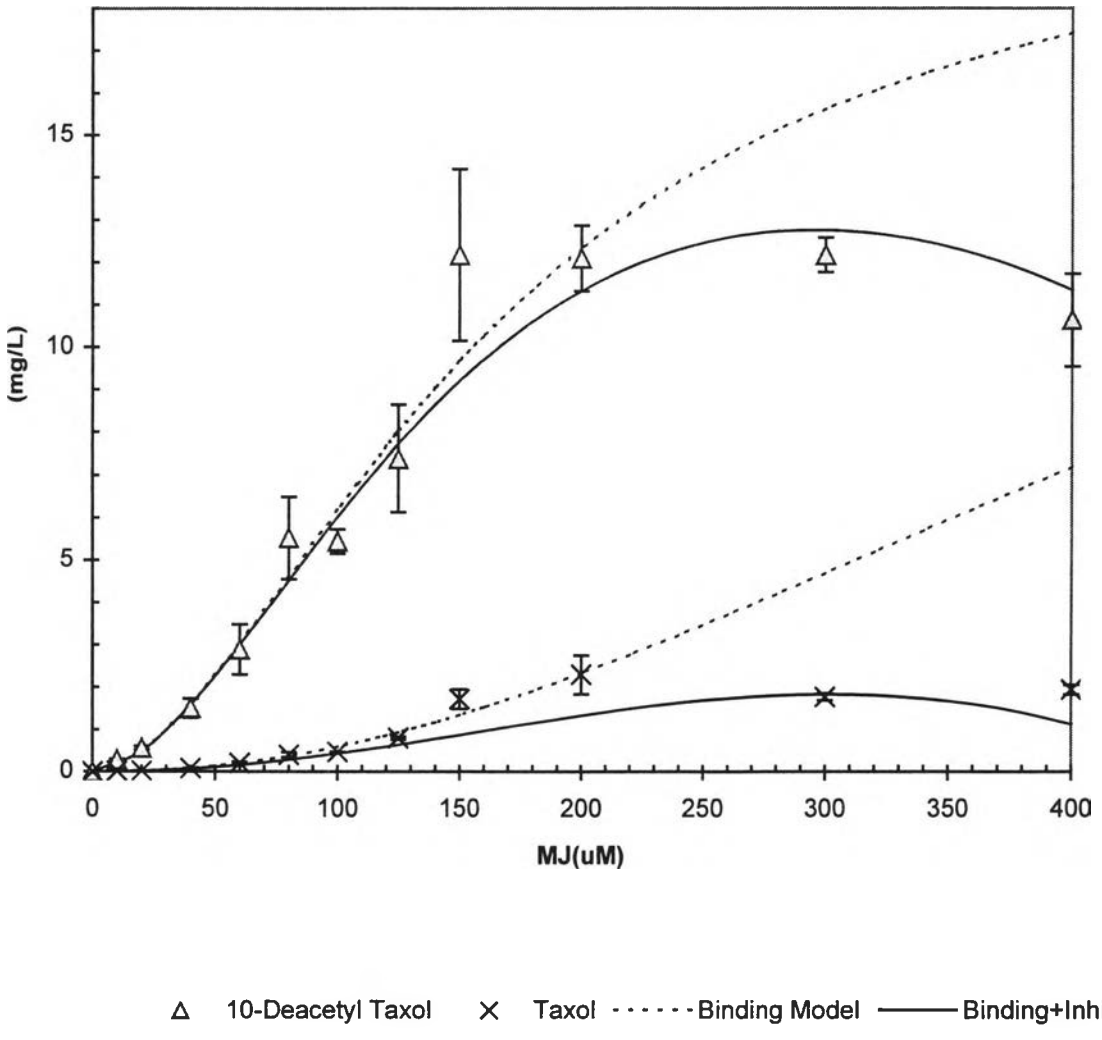


Figure 3.9 The comparison of experimental data and those obtained from two hypothetical models.

Table 3.1 The number of bound ligands (n), binding constant (K) and inhibition factor (γ) from all observed treatments.

1 a) Analysis from taxol data

Experiment set I

Ethylene (ppm)	Bound ligand (n)	Binding const (K)	Inhibition factor (γ)
3.7	2.45	2.09×10^{-6}	2.6
7.4	2.10	3.72×10^{-5}	2.6
10.6	2.45	5.37×10^{-6}	2.6
27.6	3.10	1.00×10^{-6}	2.6

Experiment set II

Ethylene (ppm)	Bound ligand (n)	Binding const (K)	Inhibition factor (γ)
6.0	2.09	1.20×10^{-6}	2.6

1 b) Analysis from 10-deacetyl-taxol data

Experiment set I

Ethylene (ppm)	Bound ligand (n)	Binding const (K)	Inhibition factor (γ)
3.7	3.05	2.24×10^{-7}	2.6
7.4	2.13	5.01×10^{-5}	2.6
10.6	2.67	4.90×10^{-6}	2.6
27.6	3.10	1.41×10^{-6}	2.6

Experiment set II

Ethylene (ppm)	Bound ligand (n)	Binding const (K)	Inhibition factor (γ)
6.0	1.78	1.20×10^{-4}	2.6

The physical model of ETR1 from the characterization work of Chang et al. (1993), Chen and Bleecker (1995), Wilkinson et al. (1995), and Woltering et al. (1997) coincidentally places two ethylene molecules per homodimer two-component receiver. The allosteric model was developed assuming homodimeric receptors.

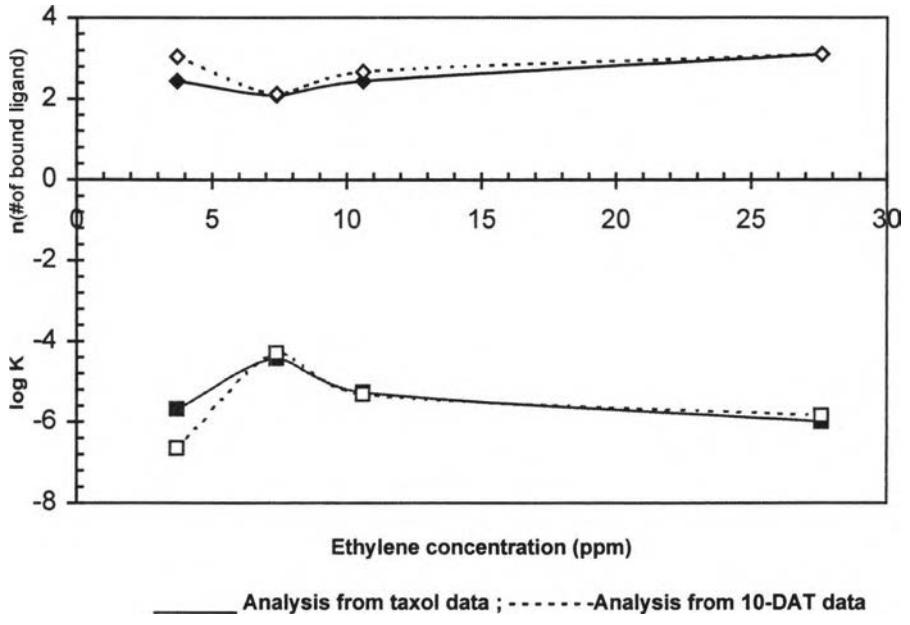


Figure 3.10 The effect of ethylene on the kinetic parameters (number of bound ligands (n)and binding constant (K)) of the binding model.

3.3 Discussion

Three possible rate-limiting processes could be envisioned for the dual substrate pathway for taxol biosynthesis. Ethylene and MJ interaction could be affecting a limitation due to enzyme activity, precursor availability or substrate delivery to specific organelles (Ciddi et al., 1995). Direct effects on any one or more of these three possibilities seem unlikely; indirect effects through signal transduction pathways are consistent with our hypothesis based on binding modeling and allosteric effects. Our literature review has uncovered nothing about MJ receptors or MJ binding constants. On the other hand, binding sites for ethylene on cellular membranes from many plant genera

have been established for years with kinetic evidence for two polypeptides with different binding affinities (Harpham et al., 1996), and recently the transmembrane ethylene receptor protein, ETR1, has been characterized (Clark et al., 1998). In attempting to model this system, we view a two-step process: MJ absorption in the membrane is directly related to MJ concentration, but its interaction with the ethylene binding site, ETR1, is effective only at higher concentrations. Hence, at low to high MJ concentrations, the modulated ethylene binding initiates signaling steps toward induction of enzymes that synthesize the N-benzoyl-3-phenylisoserine or add the side chain to the baccatin III ring. The ethylene effect is dependent on the MJ concentration, as sigmoidal plots of taxol and deacetyltaxol productivity versus MJ concentration are found, as described above. This allosteric behavior indicates modulation of ethylene binding by MJ. At medium to high MJ concentrations, the modulation site is saturated, and no greater productivities are seen.

The initial steps of the signal pathway for ethylene are at least known to have similarity to two-component regulators of prokaryotes (Schaller, 1997). Each component contains a conserved domain and a variable domain. Most sensor proteins consist of a variable amino-terminal domain (typically located in the periplasmic space flanked by two transmembrane domains) and a conserved carboxyl-terminal histidine kinase domain located in the cytoplasm. Signal perception on the N-terminal domain results in autokinase activity by the transfer of the phosphate from the histidine to a certain aspartate residue in the cognate cytoplasmic response regulator (Gaspar et al., 1996 and Holland et al., 1996).

MJ may act as an "ethylene sensitivity factor" in modulation of ethylene binding to

ETR1, which initiates the intracellular signal cascade that results, ultimately, in the accumulation of secondary compounds. The mode of action may involve non-specific dissolution of MJ in the membranes, decrease in the order of regions of the phospholipid bilayer, and direct alteration of ethylene binding to ETR1. This sort of scenario has been suggested as lipid activation of enzymes resulting from interactions with annulus phospholipids that surround the membrane-embedded portion of many proteins (Dewitt et al., 1996; Fett Neto and DiCosmo, 1996; Gamble et al., 1998; Saniewski and Wegrzynowicz, 1994; and Schaller et al., 1992).

3.4 Conclusions

Ethylene and methyl jasmonate (MJ) act as co-mediators of cellular responses in many plant systems. We postulate that specific biosynthetic steps for taxane production in plant cell culture are regulated by allosteric regulation of ethylene binding, based on our interaction and binding modeling of taxol formation in elicited suspension cell cultures of *T. canadensis*. This allosteric behavior could, but not necessarily, indicate modulation of ethylene binding by MJ.

These observations provide hints about the order by which these compounds appear in the final assembly of taxol. Synthesis or attachment of the N-benzoyl-3-phenylisoserine ester to C-13 of 10-deacetylbaccatin III to form taxoids appears under the control of MJ. Since the deacetyltaxol accumulates to high concentrations in the culture medium, its conversion to taxol may be a rate-limiting step. Glycosylation may be important for export of this intermediate from the cytoplasm, and hydrolysis of the xylosyl residue may occur extracellularly under some culture conditions. However,

acetylation of the C-10 position should occur intracellularly, probably analogous to acetylation of 10-deacetylbaccatin III, as demonstrated by experiments using acetyl-CoA by crude cell homogenates of *T. cuspidata* (Zocher et al., 1996). Similarly, since the accumulation of baccatin III is linked to MJ, as is 10-deacetylbaccatin III, these also may have an acetyl-CoA transesterification relationship.

CHAPTER 4

CHITIN- AND CHITOSAN- OLIGOSACCHARIDES WITH METHYL JASMONATE AS CO-MEDIATORS OF TAXANE FORMATION FROM *T. CANADENSIS* SUSPENSION CULTURES.

Simple carbohydrates and lipids are proving important as signal induction mediators for regulation of plant growth and development. Fungal cell wall-dervied oligosaccharides are one group of the former. Methyl jasmonate (MJ) is a lipid-derived elicitor. Both classes of elicitors activate signal transduction pathways and regulate expression of genes for production of phytoalexins and other secondary metabolites. Taxol® (generic name paclitaxel; hereafter called taxol) and other taxanes are elicited by these materials in *Taxus canadensis* suspension cultures.

Two types of oligosaccharides, both potentially derived from the chitin cell walls of pathogenic fungi, act as potent elicitors in suspension-cultured plant cells. The first of these, *N*-acetylchitooligosaccharides induce phytoalexin (momilactones and oryzalexins) formation in the rice cells even at nM ranges (Yamada et al., 1993). Inhibition studies with various other oligosaccharides show specificity of the binding site for oligosaccharides of DP greater than *N*-acetylchitohexaose (Ito et al., 1997). Using alkalanization of extracellular medium, Felix et al. investigated a time and concentration-dependent saturation of chitin

oligosaccharide binding sites in tomato suspension-culture cells (Felix et al., 1998).

Chitosan, the deacetylated form of chitin, did not induce phytoalexin formation in the rice system (Yamada et al., 1993), but is an active elicitor in other plant cultures systems. Chitosan elicitors induce formation of phytoalexins in legumes (soybean, chickpea, bean, alfalfa, pea) and *bolanaceous* sp. (potato, sweet pepper) (Cote and Hahn, 1994). However, anthraquininone biosynthesis was stimulated in *Morinda citrifolia* by chitin and chitosan (Dörnenburg and Knorr, 1995). In actuality, the difference between chitin and chitosan is a continuum of the degree of N-acetylation of the glucosamine residues in the polymer (Brine et al., 1992). The degree of acetylation was found to be important in inducing defense responses. During the first few days of incubation after adding elicitor, production of chitinase increased and then declined when anthraquinone biosynthetic enzymes became active (Dörnenburg and Knorr, 1995).

Fatty acid signalling in plants has been reviewed recently by Farmer (Farmer, 1994). Jasmonic acid arises in plants from linolenic acid via the octodecanoic pathway. Rapid, but transient, synthesis of cis-jasmonic acid has been demonstrated in whole plants and in suspension cultures (Blechert et al., 1995). Many plant species tested in cell suspension culture were elicited by exogenously supplied MJ with respect to the accumulation of secondary metabolites (Gundlach et al., 1992; Blechert et al., 1995; Kauss et al., 1992). Addition of MJ initiates de novo transcription of genes, such as phenylalanine ammonia lyase and peroxidases, which are involved in some chemical defense mechanisms of plants (Gundlach et al., 1992) and the synthesis of early intermediates of secondary products, including the N-benzoyl-3-phenylisoserine sidechain of taxol. MJ induced rosmarinic acid

biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures (Dörnenburg and Knorr, 1995) and shikonin, the red naphthoquinone pigments of the root, and dihydroeihenofuran, an abnormal benzofuran metabolite (Yazaki et al., 1997). Wounding-induced anthocyanin and flavonoid synthesis in petunia was enhanced by MJ (Tamari et al., 1995). Cooperative stimulation by ethylene and MJ of taxol formation in *T. cuspidata* (Mirjalili and Linden, 1996) and *T. canadensis* (Phisalaphong and Linden, 1998) has been reported.

Co-mediation of oligosaccharides and MJ has been demonstrated in the rice system in the induction of phytoalexin (Nojiri et al., 1995). Exogenously applied MJ to elicited cells increased production of momilactone A to levels higher than those elicited with N-acetylchitoheptaose alone. In suspension-cultured cells of parsley the influence of MJ on elicitation using cell walls of *Phytophtora megasperma* (Pmg elicitor) and chitosan was demonstrated (Kauss et al., 1994). These results suggested MJ conditioned the parsley suspension cells in a time-dependent manner to become more responsive to elicitation. Also using parsley suspension cultures, Ellard-Ivey and Douglas showed the elicitor response could be partially mimicked by MJ treatment in expression of phenylpropanoid genes (Ellard- Ivey and Douglas, 1996).

Taxol is a plant derived drug used in the treatment of breast, ovarian and lung cancers. Several papers now present results showing that MJ could enhance taxol production (Mirjalili and Linden, 1996; Yukimune et al., 1996; Ketchum et al., 1998) from several *Taxus* species. The contribution of this document is the study of the interaction of MJ with chitin and chitosan-dervied oligosaccharides to stimulate taxol production.

4.1 Experimental Studies

Experimental Studies

For studies of the interdependence of MJ with chitin and chitosan derived elicitors on formation of taxol and other taxanes, experiments were conducted using 3.0 mL of 8 day old suspensions in individual wells of Falcon (Lincoln Park, NJ) twelve-well plates. Aqueous carbohydrate preparations and ethanolic methyl jasmonate solutions (1% of suspension) were added to each well according to the experimental design. The multi-well plates were individually sealed in their original paper wrappers. If the headspace gas mixture was controlled (optimum combinations of N_2, O_2, CO_2, and C_2H_4), filtered inlet and outlet of gas mixture lines were then inserted into the container for each plate. If the headspace gas mixture composition was not controlled, the plates simply were incubated without the wrappers in an incubator in dark at 25°C with 130 rpm shaking. Samples were taken for analyses of taxanes and sugars 13 days after elicitation. The experiments were done in duplicate and the samples for analysis were taken from each well of the multi-well plates.

Elicitor Preparations

Solutions of methyl jasmonate (Bedoukian Research Inc., Danbury, CT) were prepared in ethanol in such a way that constant volume aliquots were added to each well of the multi-well plate to make all suspensions 1 (v/v) percent in ethanol. At the same time as MJ addition, equal volumes of aqueous solutions of N-acetylchitohexaose (Seikagaku Corporation, Tokyo 103, JAPAN), chitosan-derived elicitors (Aeroponics International, Inc., Berthoud, CO) or suspensions of colloidal chitin were added to each well according to the

experimental design. Preparation of colloidal chitin was based on the methanesulfonic acid method of Hirano and Nagao (Hirano and Nagao, 1988) using practical grade chitin (Sigma Chemical, St. Louis, MO) that was milled to a 60 mesh flour.

4.2 Results

4.2.1 Characterization of Oligosaccharide Elicitors

The Ultrahydrogel column chemistry separates as a molecular sieve; materials of greater molecular weight elute prior to those of lesser molecular size. The supernatants of these suspensions were analyzed using a Waters Associates Ultrahydrogel Linear Column; eluant for this chemistry was 0.5 mM sodium sulfate in distilled water at 25°C flowing at 0.6 mL/min. Overlaying the chromatogram of N-acetylchitohexaose with that of the chitosan hydrolysate helps identify the retention times based on the average degree of polymerization of the oligosaccharides in the chitosan hydrolysate preparation. A considerable amount of material is pentasaccharide, and a lesser amount is tetrasaccharide.

The quantity of oligosaccharide with DP 6 in the undiluted chitosan preparation was estimated as 8.0 mg/mL was based on standard curve quantiation using a N-acetylchitohexaose standard curve in the HPLC analaysis. The concentration of the chitin oligosaccharides, that are considered optimal in some elicitation systems (Ito et al., 1997). The degree of acetylation of the chitosan hydrolysate was analyzed by proton NMR spectra obtained at the analytical service center in the Colorado State University Department of Chemistry. The -NH, -CH and $-CH_3$ proton intensities from 400 MHz spectra obtained from a 5 mM N-acetylchitohexaose solution in 10 (v/v) percent D_2O were

compared with those of the unknown chitosan hydrolysate solution. The relative methyl proton intensities from the chitosan hydrolysate were approximately 20 percent as great as those from the fully acetylated N-acetylchitohexaose spectrum. The results indicate that an average of 1 in 5 of the glycosyl residues in the chitosan hydrolysate were acetylated.

4.2.2 Elicitation Experiments

The hypothesis applied to this study was based on knowledge of evolutionarily conserved binding proteins that are important in chitin oligosaccharide reception and subsequent signal transduction (Kaku et al., 1997). Induction of enzymes for the formation of taxol and related taxanes in suspension cultures of *T. canadensis* by chitin and chitosan-oligosaccharide preparations with and without application of MJ and ethylene were studied in the following series of experiments.

4.2.2.1. N-acetylchitohexaose Co-Mediation with MJ in Poor Production Culture and Without Headspace Gas Regulation

N-acetylchitohexaose at concentrations between 0 and 6.3 mg/L were added eight days after cell inoculation into shake flasks to which either 0 or 100 μM MJ were added at the same time (Table 4.1). Results from analysis of taxanes in the culture supernatants after 21 days of incubation with added MJ showed a 13-fold increase in concentration of taxol compared to controls. Elicitation with 0.63 mg/L of N-acetylchitohexaose and 100 μM MJ as co-mediators showed even greater induction of of taxane production. Taxol, baccatin III and 10-deacetylbaccatin III concentrations were about 15-fold greater than the 100 μM MJ treatment-controls and more than 36-fold greater than controls. The N-acetylchitohexaose

oligosaccharide alone at low and at high concentrations was ineffective in induction of taxane production (Table 4.1 and Figure 4.1). Notice differential induction at 100 μM MJ by the oligosaccharide. Taxanes with the N-benzoyl-phenylalanyl sidechain (taxol and 10-deacetyltaxol) were stimulated >10-fold, while the baccatin III taxanes without the sidechain were both <10-fold greater.

Table 4.1. N-acetylchitohexaose and methyl jasmonate as co-medicator in poor production culture.

Treatment		Results			
N-acetylchito Hexaose(mg/L)	MJ (μM)	Taxol (mg/L)	10-DAT (mg/L)	Baccatin III (mg/L)	10-DAB (mg/L)
0	0	0.050	0.073	0.157	0.852
0	100	0.667	1.600	0.174	0.851
0.63	100	8.667	29.089	1.391	2.556
6.30	0	0.083	0.181	0.348	0.681
3.15	0	0.075	0.131	0.478	0.681
0.63	0	0.075	0.109	0.304	0.852
0.32	0	0.067	0.327	0.348	1.022

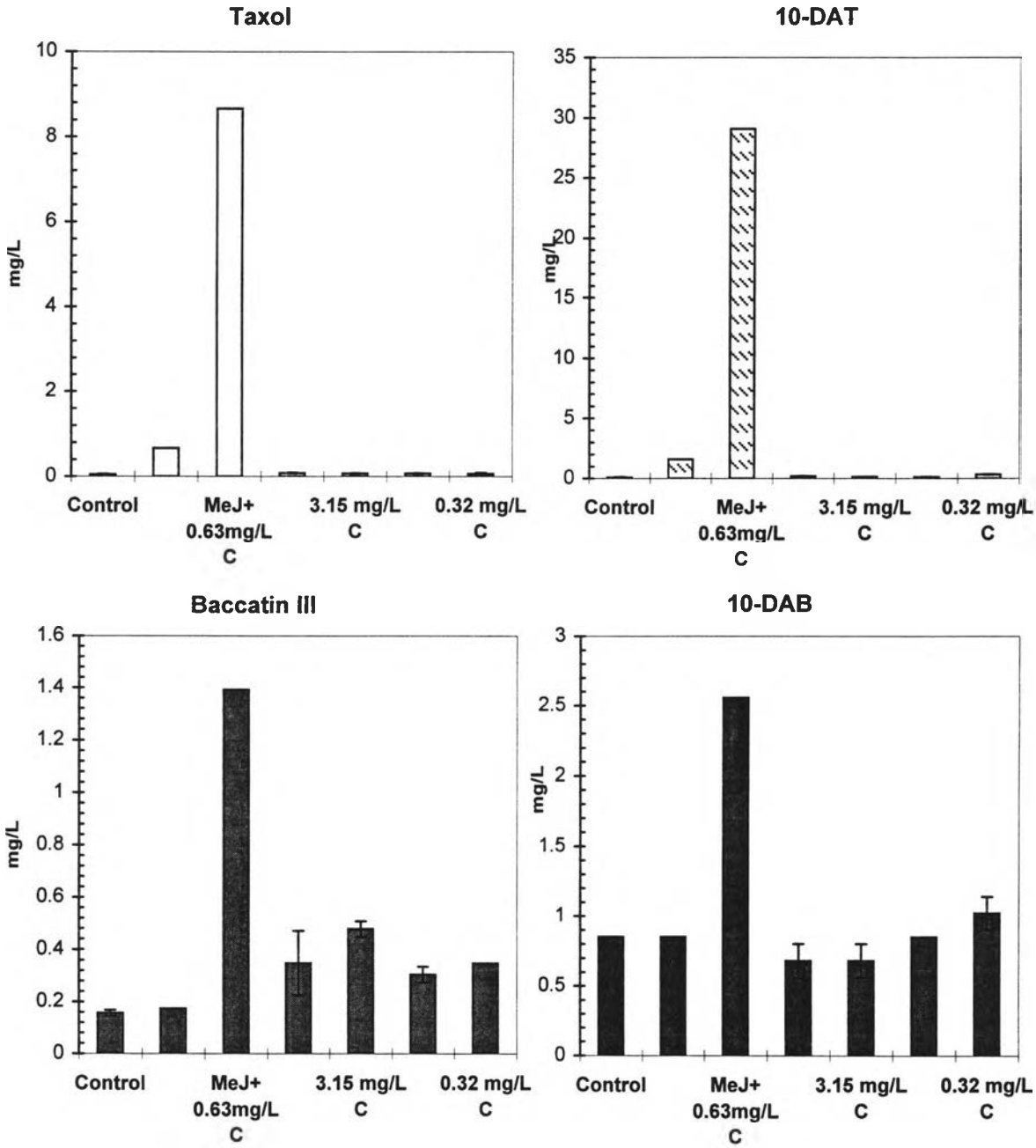


Figure 4.1 The effects of N-acetylchitohexaose (C) co-mediation with methyl jasmonate on production of taxanes after 14 days of elicitation (21 days of cell transfers) in poor production culture (*T. canadensis* (C93AD)) without headspace gas control.

4.2.2.2 N-acetylchitohexaose at Several Concentrations with 100μM MJ and Without Headspace Gas Regulation

A second experiment to define the optimum concentration of oligosaccharide was conducted; the productivity of the culture in terms of taxane levels without the oligosaccharide (Table 4.2) were similar to those obtained from the comparable treatment. Data for this treatment in Table 4.2.1 and Figure 4.2 shows taxanes (10-DAT and taxol) concentrations three times greater and baccatins (10-DAB and baccatin III) two times greater than the MJ elicited controls. Stimulation of each taxane level by addition of 0.63 mg/L (0.5 μM) oligosaccharide was only three-fold given the better condition of the cell culture in this case. No significant effect of the oligosaccharide on taxane ratio was observed (Figure 4.3).

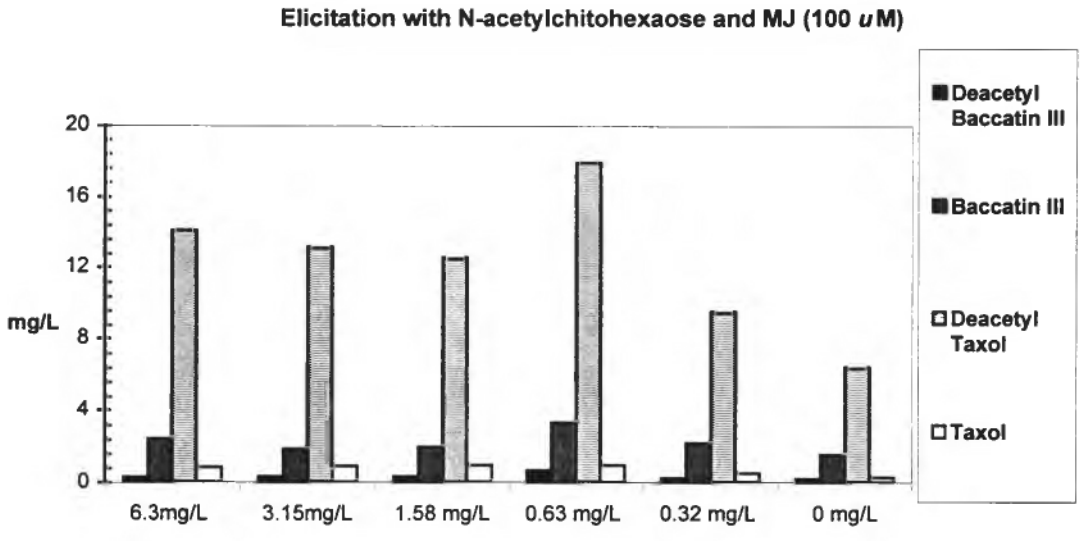


Figure 4.2. Effect of N-acetylchitohexaose and MJ (100 μM) as co-mediators of taxane formation by *T. canadensis* (C93AD) after 21 days of cell transfer in the systems without headspace gas control.

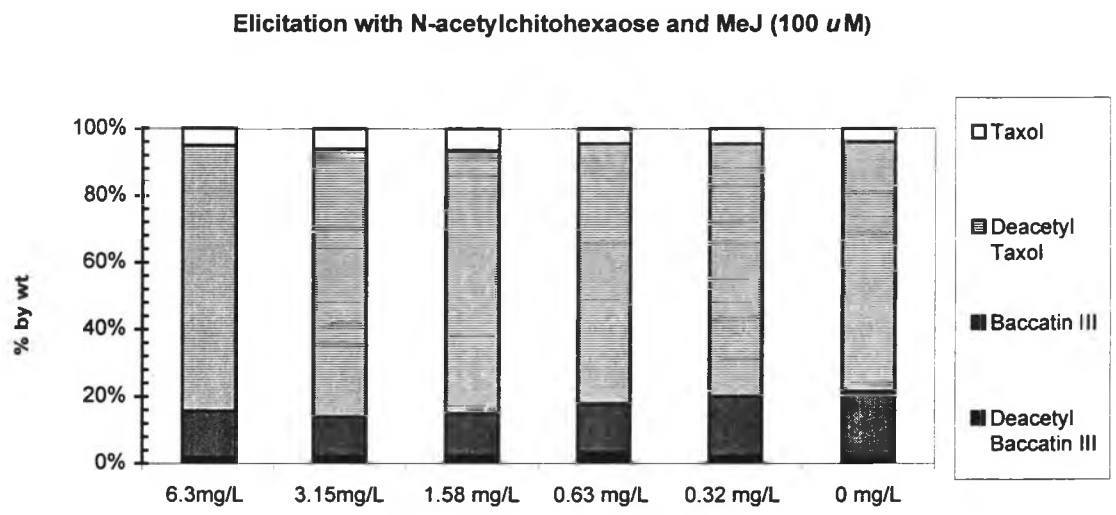


Figure 4.3 Effects of N-acetylchitohexaose and MJ as co-mediators on taxane ratio at 21 days of cell transfer in the systems without headspace gas control.

4.2.2.3 Chitosan Hydrolysate Co-Mediation with 100μM MJ and Without Headspace Gas Regulation.

Similarly, application of various chitosan hydrolysate concentrations with MJ eight days after culture transfer, revealed a dose dependence in taxane concentrations (Table 4.2.2). In this case, the relationship with oligosaccharide concentration appears exponential over this concentration range. The increase in concentrations of the baccatins may also follow this trend. Compared to the treatment with only 100μM MJ added on day 8, the maximum increase in taxane concentration was about six-fold using the lowest dilution of the chitosan hydrolysate with an estimated oligosaccharide concentration of 75 μg/mL. The stimulation under these conditions was equivalent to that observed using 0.63 μg/mL (0.5 μM) N-acetylchitohexaose co-mediation with MJ in the poor condition culture (Figure 4.1).

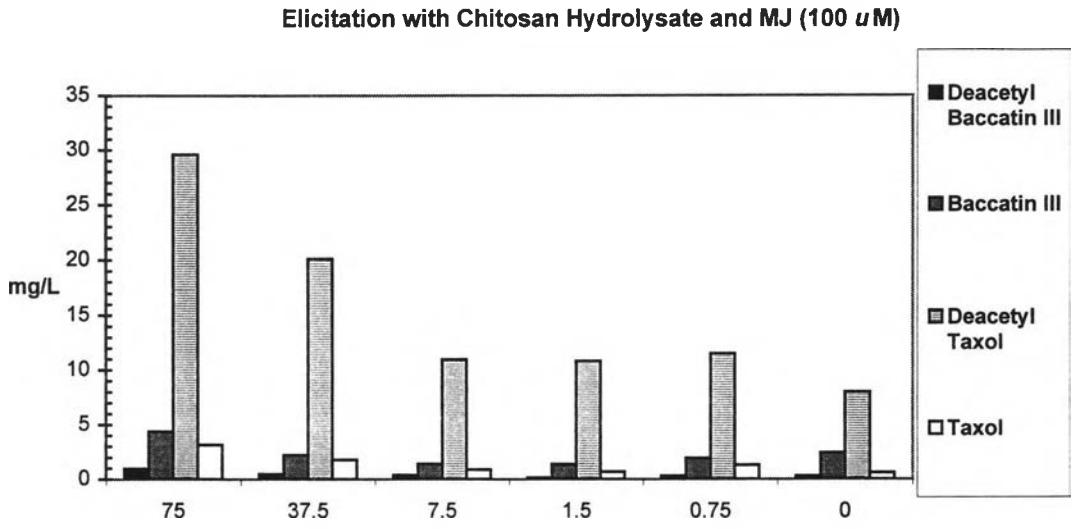


Figure 4.4. Effect of chitosan hydrolysate and MJ (100 μM) as co-mediators of taxane formation by *T. canadensis* (C93AD) after 21 days of cell transfer in the systems without headspace gas control.

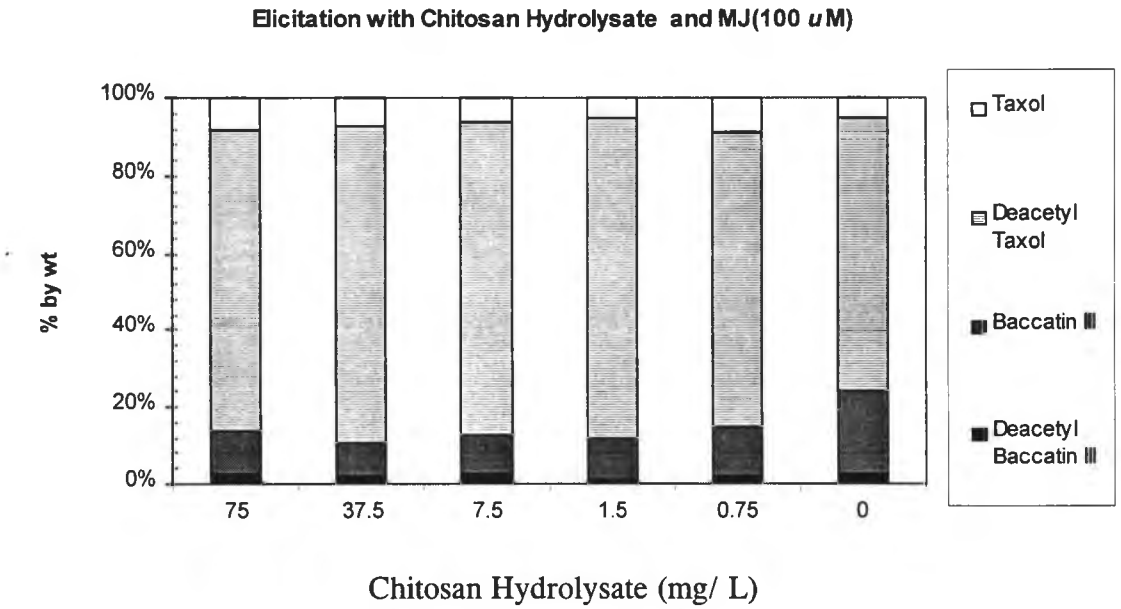


Figure 4.5 Effects of chitosan hydrolysate and MJ as co-mediators on taxane ratio at 21 days of cell transfer in the systems without headspace gas control.

4.2.2.4. Colloidal Chitin Co-Mediation with 100 μM MJ and Without Headspace Gas Regulation

This preparation of chitin was a mixture of solids and very high molecular weight chitin molecules that were presented to the cultures. HPLC analysis of the supernatant did not reveal soluble oligosaccharides. Data for this experiment are given in Table IIB. Essentially, the 21-day increased production was stimulated approximately two-fold by 100μM MJ at all of the colloidal chitin concentrations studied. It is important to note that the ratio of increase in taxane and baccatin concentration in each treatment were nearly the same (Figure 4.7).

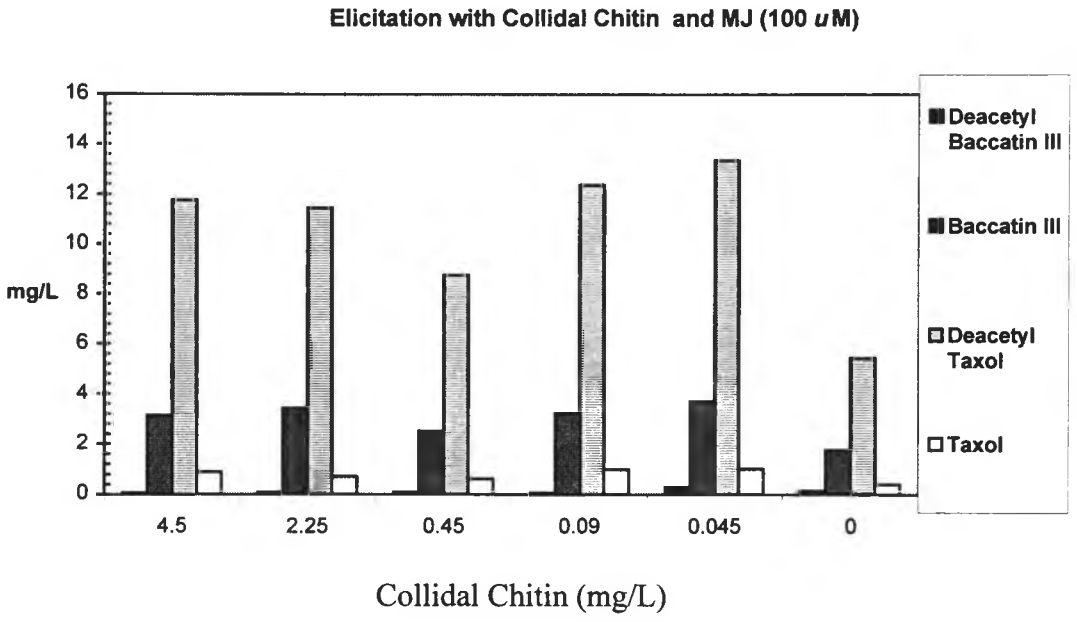


Figure 4.6. Effect of collogal chitin and MJ (100 μM) as co-mediators of taxane formation by *T. canadensis* (C93AD) after 21 days of cell transfers in the systems without headspace gas control.

Table 4.2 N-acetylchitohexose, colloidal chitosan or chitosan hydrolysate with 100μM methyl jasmonate as co- medicator in the system without head space gas control.

1) N-acetylchitohexaose and 100 μM Methyl jasmonate

Treatments Results

N-acetylchitohexaose (μg/ mL) (together with 100μM MJ)	Taxol (mg/L)	Deacetyl Taxol (mg/L)	Baccatin III (mg/L)	Deacetyl Baccatin III (mg/L)
6.3	0.898	14.156	2.480	0.322
3.15	0.975	13.157	1.923	0.369
1.57	1.026	12.548	2.024	0.369
0.63	1.026	17.932	3.401	0.738
0.31	0.575	9.550	2.267	0.295
0.00	0.342	6.432	1.620	0.243

2) Chitosan hydrolysate and 100 μM Methyl jasmonate

Treatments Results

Chitosan hydrolysate (μg/ mL) (together with 100μM MJ)	Taxol (mg/L)	Deacetyl Taxol (mg/L)	Baccatin III (mg/L)	Deacetyl Baccatin III (mg/L)
75.00	3.172	29.591	4.393	0.968
37.50	1.796	20.101	2.227	0.461
7.50	0.872	10.964	1.417	0.369
1.50	0.684	10.818	1.377	0.148
0.75	1.300	11.500	1.943	0.295
0	0.575	7.991	2.429	0.295

3) Collidal Chitosan and 100 μM Methyl jasmonate

Treatments Results

Collidal Chitosan (μg/ mL) (together with 100μM MJ)	Taxol (mg/L)	Deacetyl Taxol (mg/L)	Baccatin III (mg/L)	Deacetyl Baccatin III (mg/L)
4.500	0.898	11.768	3.118	0.065
2.250	0.718	11.451	3.441	0.092
0.450	0.633	8.771	2.531	0.092
0.090	0.999	12.377	3.239	0.074
0.045	1.026	13.352	3.725	0.295
0	0.410	5.458	1.781	0.148

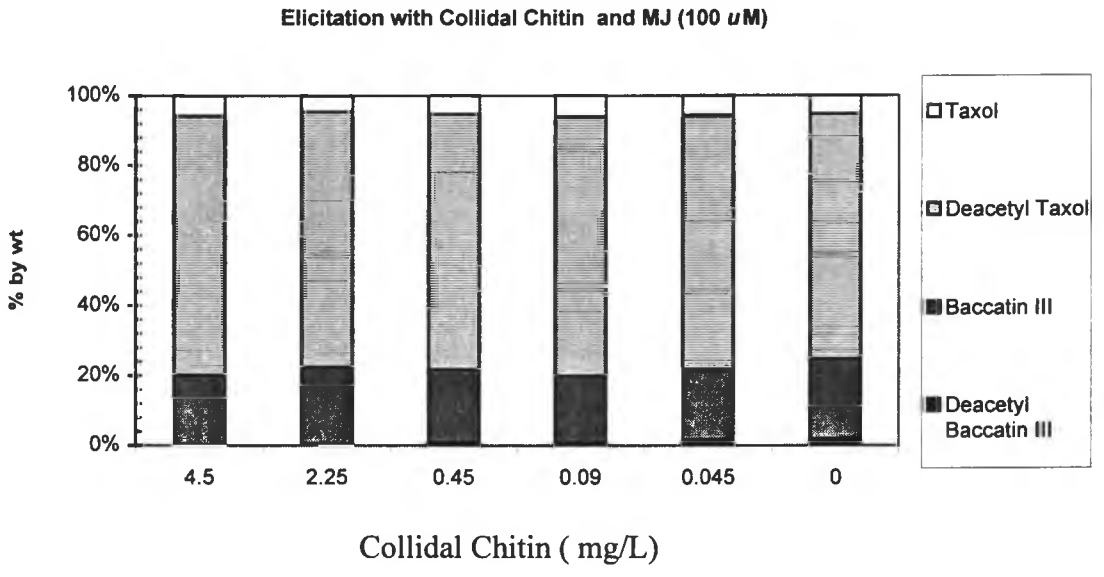


Figure 4.7 Effects of collidal chitin and MJ as co-mediators on taxane ratio at 21 days of cell transfers in the systems without headspace gas control.

4.2.2.5 N-acetylchitohexaose, Colloidal Chitin and Chitosan Hydrolysate Comediation with Various MJ Concentrations and with Defined Headspace Gas Mixture Composition.

The environmental presence of a defined headspace gas mixture composition in this experiment appears to affect the oligosaccharide elicitation process. The head space gas in this study was controlled at the optimal concentrations (6 ppm C_2H_4, 10% O_2 and 0.5% CO_2) determined from previous studies (Mirjalili and Linden, 1995). Using the concentrations of N-acetylchitohexaose, colloidal chitin and chitosan hydrolysate that gave the best results from the experiments above: 0.63 μg/mL (0.5 μM) N-acetylchitohexasoe, 0.45 μg/mL of our colloidal chitosan preparation and 75 μg/mL of the aeroponics chitosan hydrolysate were used to elicite the suspension cultures of *T. canadensis* together with various MJ concentrations after eight days of growth.

These data, obtained to find the optimal range of MJ as a co-mediator, are shown in Table 4.3. With all three elicitors, the optimal concentration of MJ to stimulate taxol formation with the chitosan hydrolysate was 200 μM; this is the same optimum concentration from elicitation with MJ alone (Yazaki et al., 1997; Ketchum et al., 1998). In fact, data on the bottom lines of each of Table 3.1, 3.2 and 3.3 indicate 50 to 90 percent of the level of taxane production using 200 μM MJ without elicitors as compared to values obtained with elicitors. The relative stimulation in this experiment (Figure 4.8 and Figure 4.9) was not as striking as in previous examples. Previous work (Mirjalili and Linden, 1996) has shown taxane production to be dependent on ethylene and MJ concentrations. In the controlled gas environment, ethylene may be substituting for the oligosaccharde in co-mediation with MJ.

The experimental data showed strong inhibition when MJ concentration was increased to 400 μM. Elicitation at 200 μM MJ with collidal chitin or or the chitosan solution induced taxane production up to three and fifteen-fold compared to the treatments with elicitors, but without MJ. Also notice the ratio of taxanes relative to total taxanes + baccatins increase with MJ concentrations where taxanes represents taxol + 10-DAT and baccatins are baccatin III +10-DAB.

Table 4.3 N-acetylchitohexose, colloidal chitosan or chitosan hydrolysate with various methyl jasmonate concentrations in the system with 6 ppm ethylene, 15% oxygen and 0.5% carbon dioxide.

1) N-acetylchitohexose with various concentration of MJ

Treatments Results

N-acetylchitohexose (μg/ mL)	MJ (μM)	Taxol (mg/L)	DAT (mg/L)	Baccatin III (mg/L)	DAB (mg/L)
0	0	0.150	0	0	0.765
0.635	0	0	0	0.664	1.093
0.635	25	0.407	0.094	1.714	1.093
0.635	50	0.732	0.338	2.102	0.765
0.635	100	1.163	0.350	1.493	0.410
0.635	200	2.189	1.814	2.102	0.178
0.635	400	0.100	0.100	0.221	0.014
0	200	2.001	1.751	3.318	0.355

2) Chitosan Hydrolysate with various concentration of MJ

Treatments Results

Chitosan ydrolysate (μg/mL)	MJ (μM)	Taxol (mg/L)	DAT (mg/L)	Baccatin III (mg/L)	DAB (mg/L)
0	0	0	3.332	1.549	1.284
75	0	0.500	7.806	3.208	0
75	25	0.919	9.424	3.208	0
75	50	0.594	5.378	2.102	0
75	100	0.626	6.187	2.323	0.008
75	200	1.595	7.568	2.212	0.008
75	400	0.813	4.855	1.1061	0
0	200	1.126	8.472	1.770	0.437

3) Colloidal Chitosan with various concentration of MJ

Treatments Results

Colloidal Chitosan (μg/mL)	MJ (μM)	Taxol (mg/L)	DAT (mg/L)	Baccatin III (mg/L)	DAB (mg/L)
0	0	0.044	0.286	1.549	1.366
0.45	0	0.125	8.377	3.540	0
0.45	25	0.938	8.900	3.540	0
0.45	50	0.751	5.473	2.212	0
0.45	100	1.126	7.710	1.991	0.006
0.45	200	1.877	7.853	1.449	0
0.45	400	0.025	0.247	0.111	0
0	200	0.876	7.044	1.327	0.273

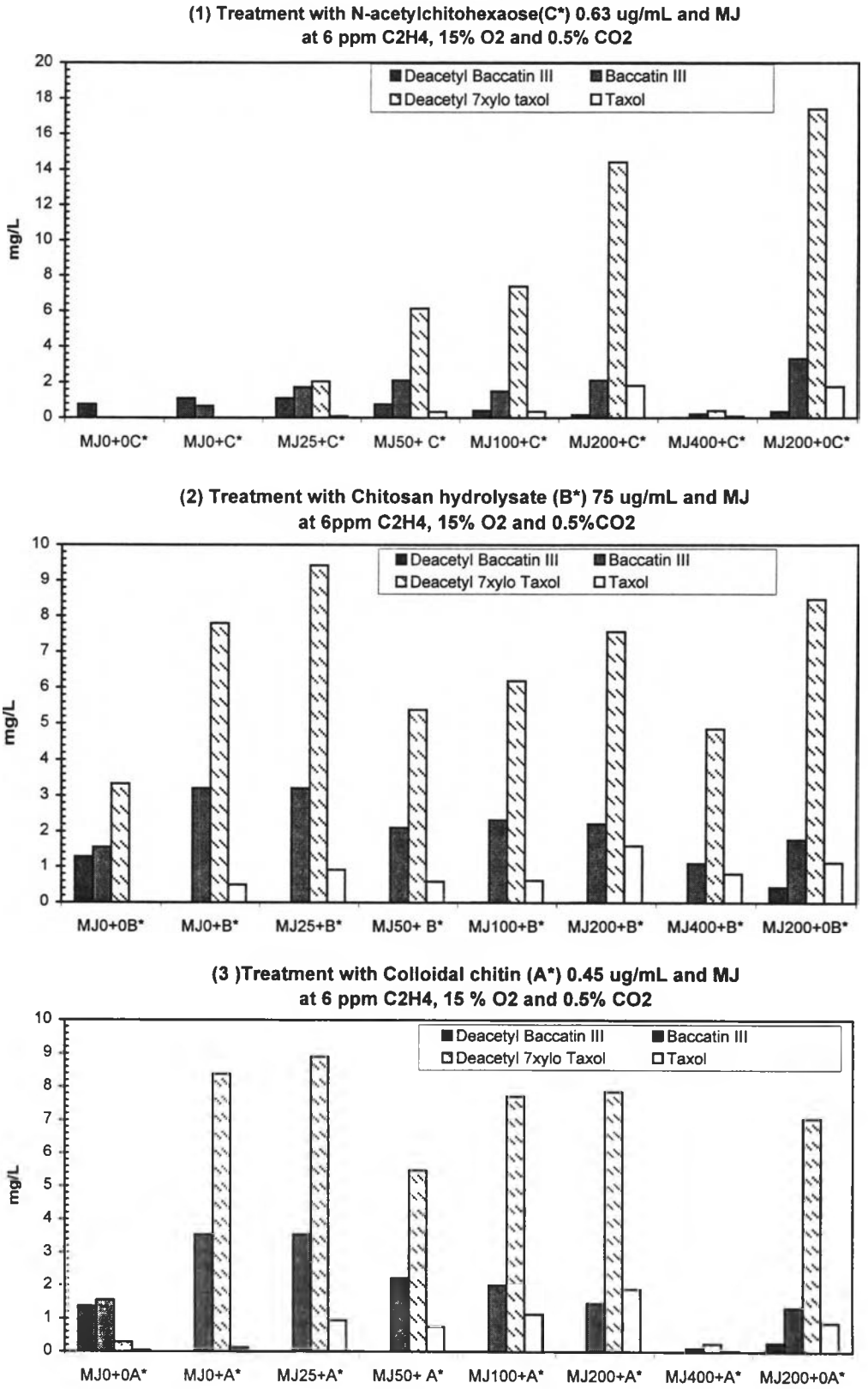


Figure 4.8 Effects of N-acetylchitohexaose,colloidal chitin and chitosan hydrolysate comediation with various MJ concentration (0- 400 *u*M) and with defined headspace gas mixture composition on taxane formation by *T. canadensis* after 21 days of cell transfer.

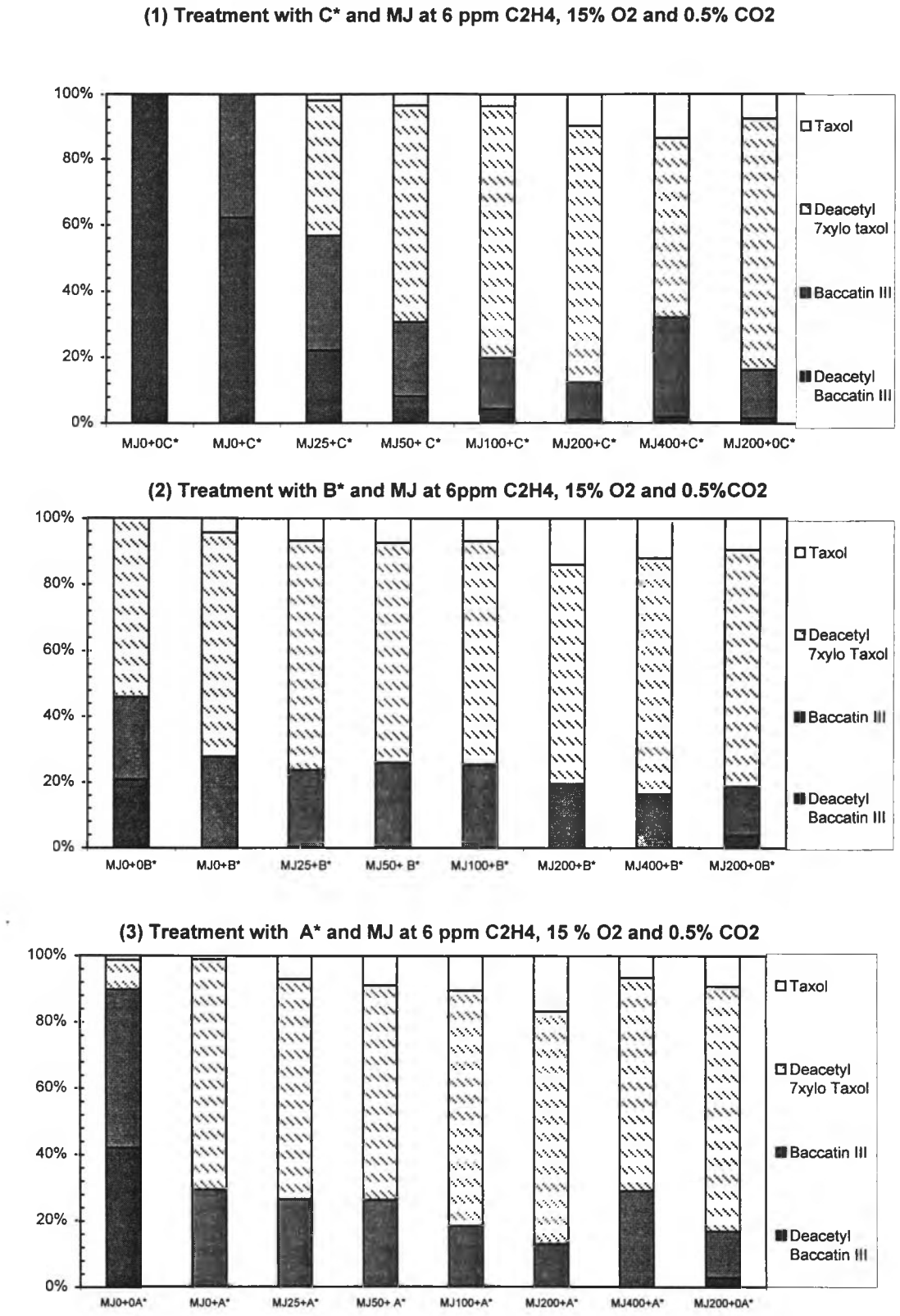


Figure 4.9 Effects of N-acetylchitohexaose,colloidal chitin and chitosan hydrolysate comediation with various MJ concentration (0-400 *u*M) and with defined headspace gas mixture composition on taxane ratios.

4.2.2 6 Full Factorial Design Experiments with N-acetylchitohexaose and MJ.

F1) Without headspace gas regulation.

Bar graphs in Figure 4.10 give representations of taxane concentrations at 0, 50 and 100 μM MJ when using 0, 0.16, 0.63 and 1.6 mg/L N-acetylchitohexaose. In microtiter wells containing 0.16 mg/L oligosaccharide, the greatest accumulations appeared when co-elicited with 50 μM MJ and also with 1.6 mg/L oligosaccharide and 100 μM MJ. Such co-dependence has been reported earlier (Chen and Bleecker, 1995; Rajamohanan et al., 1996). The previously determined optimum oligosaccharde concentration (0.63 mg/L) yielded only slight concentrations of taxanes. All other combinations of elitication conditions yielded approximately equal quantities of baccatins. The ratios of taxanes (taxol + 10-DAT) relative to total (taxanes + baccatins) increase with MJ concentrations (Figure 4.11). Also ratios of taxanes relative to total taxanes + baccatins in the optimum of the system with MJ and chitin co-mediation was greater than in the system elicited with MJ alone (Figure 4.11).

F2) With defined headspace gas mixture composition.

Another experiment was conducted with the use of the gas mixing station to provide optimum concentrations of the headspace gas (7 ppm C_2H_4 15%, O_2 and 0.5% CO_2) for stimulation of taxane production by *T. canadensis* cultures. Four MJ concentrations (0, 50, 100 and 200 μM) and six N-acetylchitohexaose concentrations (0, 0.32, 0.63, 1.26, 3.2 and 6.3 mg/L) were examined in a duplicated full factorial design experiment. The graph from this work in Figure 4.12 distinctly showed significant stimulation of taxane production in replicates containing no oligosaccharide and the 100 μM optimum MJ elicitation concentration under the given culture conditions (3 mg/L compared to < 1 mg/L in all other

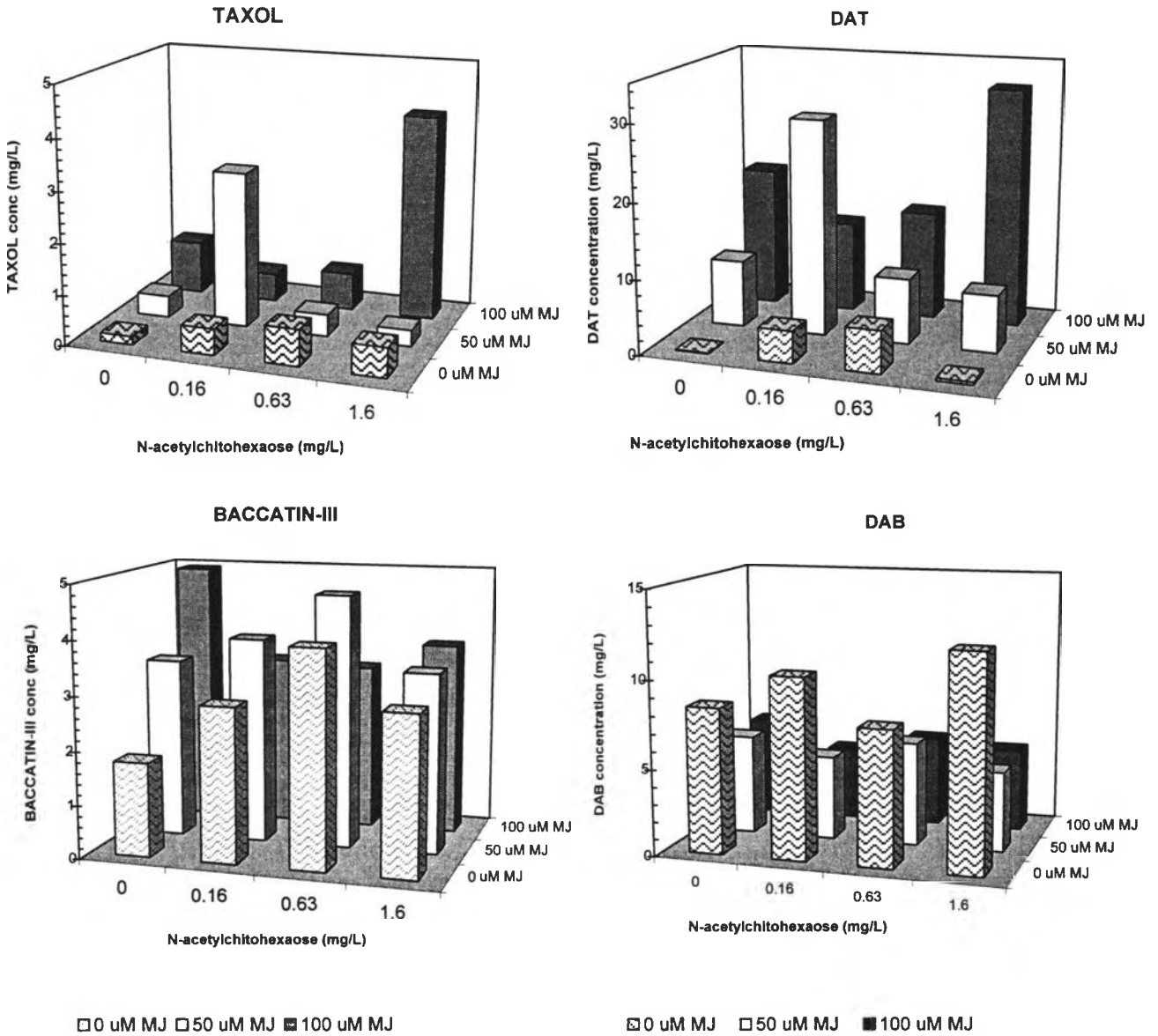


Figure 4.10 Taxanes concentrations after 21 days of cell transfer in the full factorial design experiments with various concentrations of N-acetylchitohexaose and MJ without headspace gas regulation.

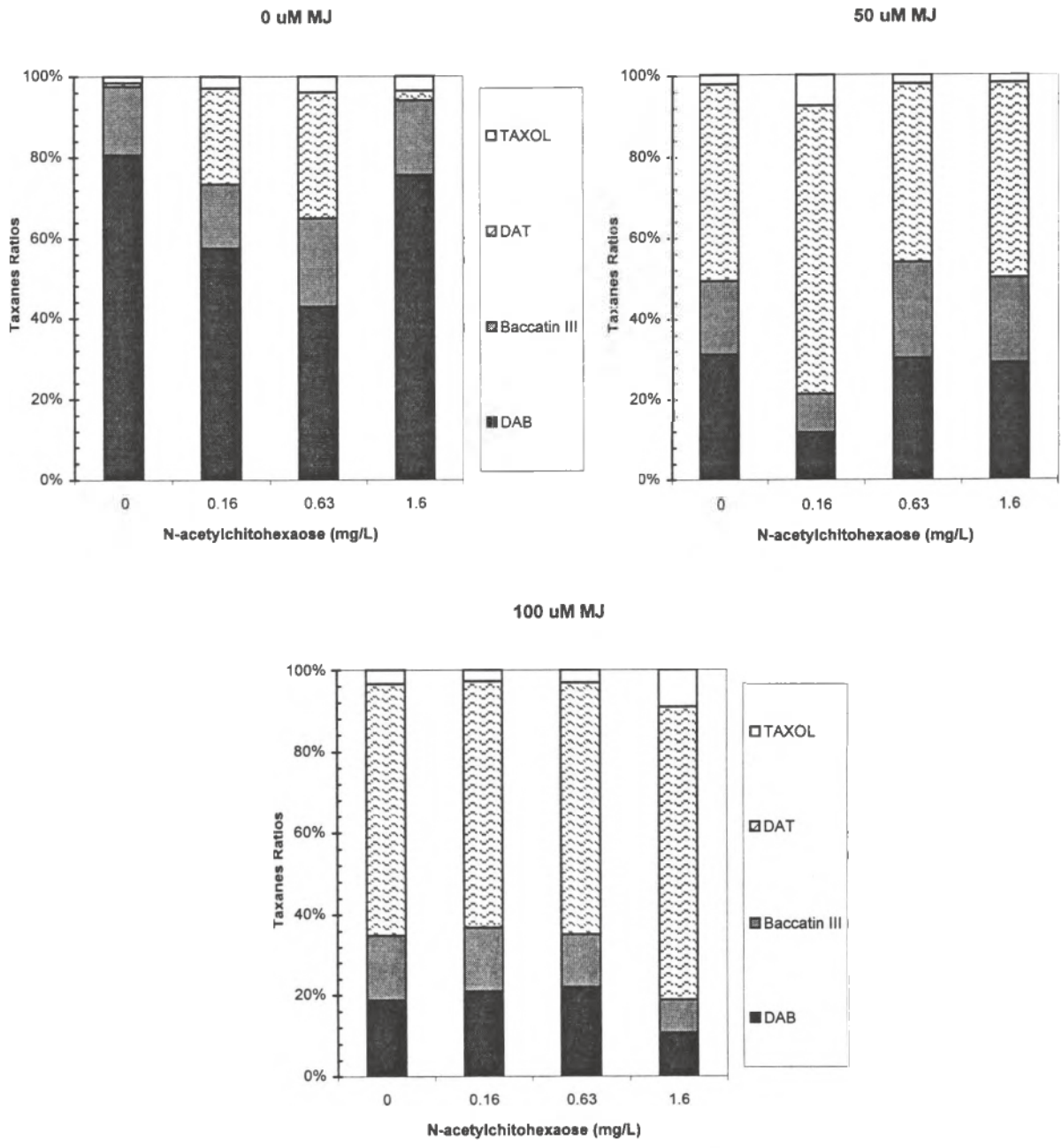


Figure 4.11 Ratios of taxanes after 21 days of cell transfer in the full factorial design experiments with various concentrations of N-acetylchitohexaose and MJ without headspace gas regulation.

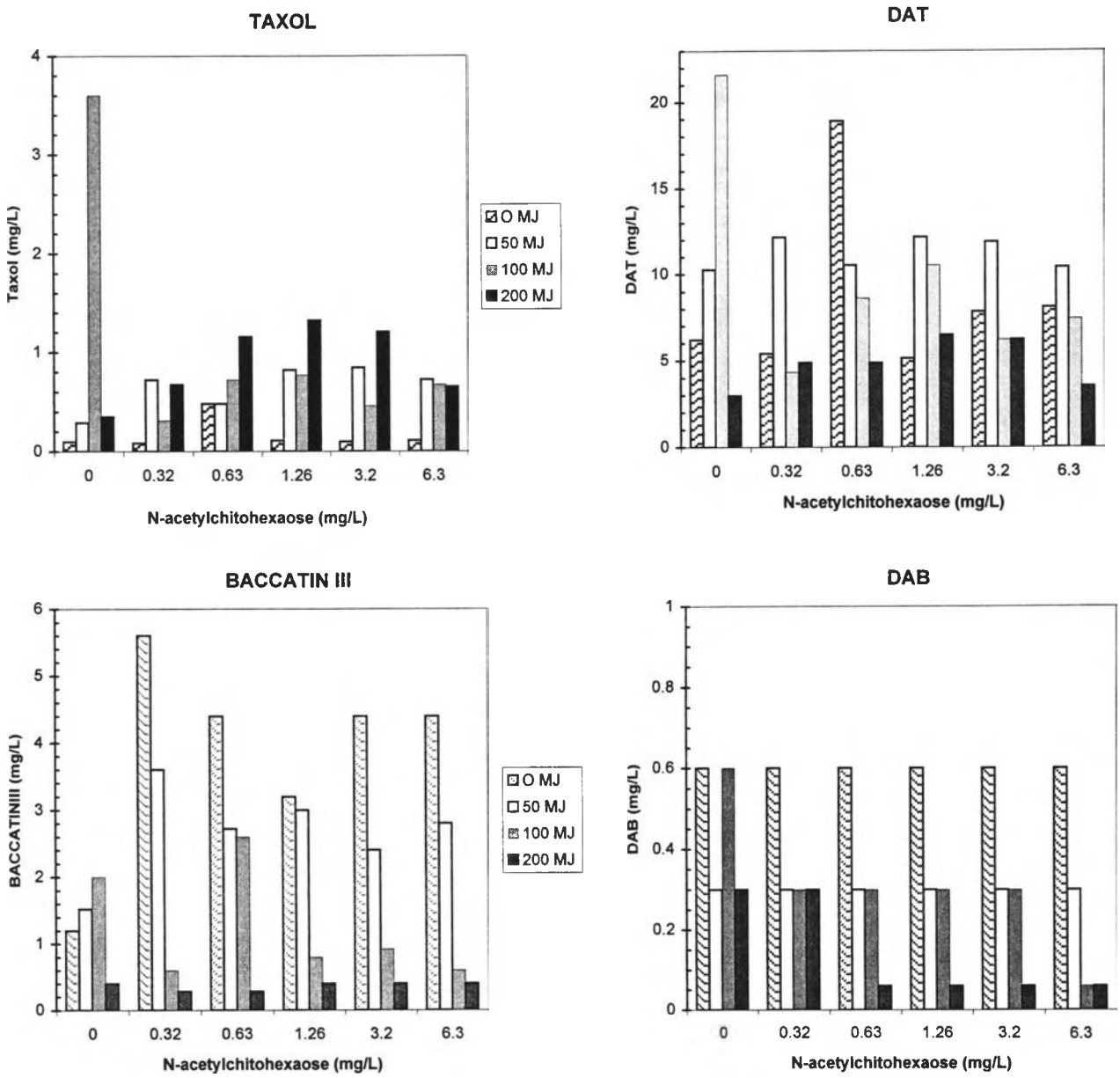


Figure 4.12 Taxanes concentration after 21 days of cell transfer in the full factorial design experiment with various N-acetylchitohexaose and MJ concentrations and defined headspace gas mixture composition.

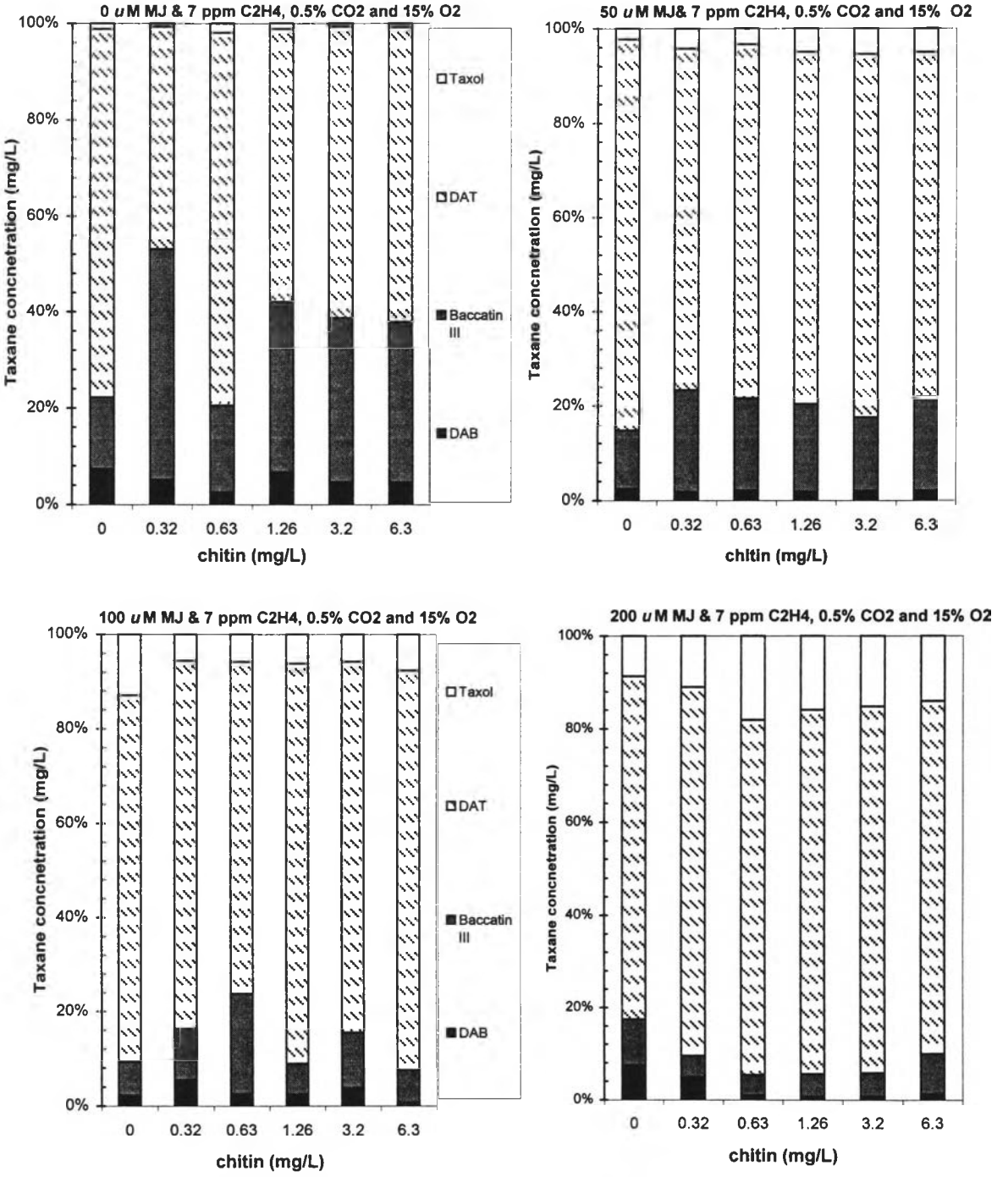


Figure 4.13 Ratios of taxanes after 21 days of cell transfer in the full factorial design experiment with various N-acetylchitohexaose & MJ concentrations and defined headspace gas mixture composition.

treatments). Here again the headspace gas composition, and more specifically the ethylene component, appeared to result in efficient co-mediation of elicitation.

4.3 Discussion

Induction of taxane formation in suspension cultures of *T. canadensis* by three chitin and chitosan derivatives together with or without MJ in multi-well plate systems were examined with or without headspace gas composition regulation. In several of the experiments, only when MJ was applied at the same time as the chitin hexasaccharide, colloidal chitin and chitosan hydrolysate was the stimulation of taxol production and other related taxanes observed. Variability appears to be related to the condition of the respective cultures; cultures that were not producing even moderate levels of taxol responded better to the combination of elicitors than did cultures that were in generally good condition. Stressed cultures may have been producing greater amounts of ethylene than the cultures in good condition; co-mediation appeared more dramatic.

The best reported case production from plant cell culture is 117 mg/L of taxol within five days of elicitation (23.4 mg L^{-1} day^{-1}). Control cultures, with no MJ, produced about 1 mg/L (Ketchum et al., 1998). Various cellular responses, which relate expression of genes to pathogen-related reactions, including early membrane responses such as the changes in membrane potential, ion flux, oxidative burst, protein phosphorylation, induction of jasmonic acid, are induced by oligosaccharide elicitors (Cote and Hahn, 1994). Kuchitsu et al. (1997) have studied N-acetylchitooligosaccharide elicitor effects on transient ion fluxes through the plasma membrane in suspension rice cell cultures in conjunction with phytoalexin

production. Using purified oligosaccharides, elicitation occured only with oligomers with degree of polymerization greater than five. Similar phenomena are reported by Felix et al. (1998) using suspension cultured tomato cells, except that effects from chitin oligomers of DP5 = DP4 >>DP3 >> DP2 >DP1. While deacetylated oligomers were not active in rice, both chitin and chitosan derivatives function in the presence of MJ act as modifiers of secondary metabolite production in the *Taxus* system studied here.

The effect of N-acetylchitohexaose in co-mediation with MJ in certain combinations and under certain cell culture conditions is difficult to understand. Ethylene in the headspace gas composition is the one variable that consistently is different in cases that co-mediation of MJ and N-acetylchitohexaose does not act better than MJ alone. Experiments, in which ethylene is not supplied to the headspace and in which the closure on the growth vessels are not tight so as to keep ethylene produced by the plant cell from diffusing from the immediate headspace of the culture, demonstrate remarkably better co-mediated elicitation of taxane production, relative to controls. There is a distinct possibility that the chitosan and chitin-oligosaccharides used in this study promote ethylene biosynthesis by the plant cell culture. In this case ethylene concentration may be effectively great enough at the site of action in the cells to promote the MJ/ethylene co-mediation. Possible reasons for this and for observed inhibition of elicitation at high MJ concentrations has been presented earlier (Chapter 3).

Finally, questions about changes in sensitivity to elicitation arise with such observations. Halevy et al. (1996) find changes in sensitivity to ethylene by the involvement of short chain fatty acid in petal senescence following pollination. Similarly, Saniewski et al. (1994) have

described relationships of MJ action with fatty acid and sterols in elicitation processes. Kauss and coworkers discussed conditioning plant cell cultures and hypocotyls of etiolated cucumber seedlings to pathogen-related defense responses using partially acetylated chitosan (Kauss et al., 1997), salicylic acid (Kauss and Jeblick, 1996), fungal cell wall derived oligosaccharides (Kastner et al., 1998), low molecular weight lignin fragments (Franke et al., 1998), benzothiazole (Kaestner et al., 1998; Katz et al., 1998) and most recently cutin monomers and surface wax constituents (Fauth et al., 1998). Titaranko et al. (1997) and O'Donnell et al. (1996) relate wound dependent pathways with MJ action; presumably, wound induced ethylene biosynthesis is involved. Boller and coworkers document desensitization of primary defense responses by repeated treatments with chitin oligosaccharides as related to surface binding phenomena (Felix et al., 1998). There is also evidence for the possibility of sensitivity effects by association of downstream ethylene signal transduction components (Clark et al., 1998).

4.4 Conclusions

The interdependence of methyl jasmonate with chitin and chitosan derived elicitors was studied using plant cell suspension cultures of *Taxus canadensis*. Induction of the biosynthesis of taxol and other taxanes was enhanced when methyl jasmonate and elicitors were added eight days after culture transfer compared to treatments in which only methyl jasmonate or only elicitor were added. The optimal elicitor concentration using N-acetylchitohexaose was 500 nM, but only in the presence of methyl jasmonate. Little,

if any, induction of taxane formation occurred with the oligosaccharide alone. The optimal methyl jasmonate concentration was 200 μM using colloidal chitin or oligosaccharides of chitin and chitosan as elicitors. Differential induction of taxanes with the N-benzoyl-L-phenylisoserine sidechain compared to that of taxanes without the sidechain was dependent on the presence of oligosaccharides. Systems to which ethylene was provided continuously to the headspace of the culture did not require oligosaccharides for methyl jasmonate co-mediation of elicitation.

CHAPTER 5

PRECURSOR STUDY WITH METHYL JASMONATE ELICITATION AND APPROACH TO UNDERSTANDING BIOSYNTHETIC PATHWAY FOR TAXOL

Recent research on plant cell culture has focused on determining the control of secondary metabolism by identifying the rate-limiting steps of biosynthetic pathways. Taxol and related taxanes are functionalized diterpenes derived from the universal diterpene precursor, geranylgeranyl pyrophosphate (GGPP). The biosynthetic pathway to taxol is unknown, but significant progress has been made. To obtain detailed information, the taxol biosynthetic pathway has been characterized using many techniques including elicitation, inhibition of metabolic steps and precursor feeding. The first three steps of taxol biosynthesis have been defined and the responsible enzymes are described in the literature review (Chapter 1). The tricyclic taxoid arises from GGPP by sequential intramolecular cyclizations of double bonds to form a simple diterpene skeleton (Hezari et al., 1997). Recent advancements in understanding of the final steps of biosynthesis of unfunctionalized taxanes (Koeep et. al., 1995) containing the 4, 5, 20 oxetane ring has been described by Hefner et al. (1996), based on intermediates synthesized by Rubenstein and Williams (1995). The baccatin III diterpenoid with oxetane ring becomes a taxane

when the benzoyl-3-phenylisoserine sidechain is attached to C-13 hydroxyl of 10-deacetylbaccatin-III. The presence of an enzyme in T. *brevifolia* tissue, which is capable of linking a phenylpropanoid side chain to C-13 of baccatin III, was reported (Fett-Neto and DiCosmo, 1996).

One intermediate reaction was thought to be the acetylation of 10-deacetylbaccatin III (10-DAB) to baccatin III, an assumed precursor of taxol. The activity of acetyl-transferases converting 10-deacetylbaccatin-III to baccatin III was reported (Fett-Neto and DiCosmo, 1996 and Zocher et. al., 1996). However, some recent reports suggested that baccatin III may be synthesized from an intermediate common to taxol, but along a separate branch of the pathway. This hypothesis was supported by a paper from Shuler's group (Srinivasan et al., 1996) that baccatin III appears to have cytoplasmic and plastidic biosynthetic components, while taxol production is essentially plastidic. Treatment of *Taxus sp.* cells with arachidonic acid specifically stimulated taxol production but did not have a significant effect on baccatin III yield. The report from Ketchum et al. (1997) that baccatin-III continues to accumulate in the culture even as the cells are dying might suggest that baccatin-III may also be formed as a degradation product of taxol.

The examination of taxane profiles in some cell lines indicated a larger amount of baccatin-III and 10-deacetyl baccatin-III (10-DAB) compared to taxol and cephalomannine indicated the occurrence of a metabolic block at the point of side chain addition. Improved taxol yields in callus and cell suspensions of *T. cuspidata* were obtained by feeding phenylalanine and other potential taxol side-chain precursors such as benzoic acid, N-benzoylglycine, L-serine, and glycine (Fett-Neto and DiCosmo, 1996).

5.1 Experimental Studies

In the studies, the suspension cultures of *Taxus canadensis* were treated with different compounds, which were expected to be precursors in the taxol biosynthesis pathway either individually or in combination with methyl jasmonate (MJ) elicitation. Prepared precursor and MJ solutions were fed into 3 mL of 8 day old suspension in individual wells of Falcon (Lincoln Park, NJ) twelve well plates according to the experimental design given in Table 5.1. The precursors were all obtained from Sigma Chemical (St. Louis, MO) and dissolved as stock solutions that were diltuted to the given concentrations in the 3 mL of culture medium. The plates were then incubated in an incubator in the dark at 25 ° C with 130 rpm rotating under 15% (V/V) oxygen, 0.5% (V/V) carbon dioxide and 7 ppm ethylene. The headspace gas concentrations were measured by GC and were those that resulted in the highest taxol production as discussed in Chapter 3. The apparatus and procedure used for controlling the mixture of gases flowing to the headspace of the incubator were according to the gas mixing apparatus in Chapter 2. Samples of the cultures were taken for analysis of taxanes after 13 days elicitation or 21 days after culture transfer. The experiments were all duplicated. The concentration ranges for each precursor was chosen from the optimal ranges reported from other studies (Fett-Neto and DiCosmo, 1996). The concentration of elicitors used in the studies (200 μM MJ and 500 μM N-acetylchitohexaose) were the optimal concentrations for taxol production obtained from the previous studies (Chapter 3 and Chapter 4).

Table 5.1 Treatment conditions for precursor studies in Chapter 5.

Experiment	Precursor		Elicitor	
	Compound	Concentration	Methyl jasmonate (μM)	N-acetylchito-hexaose (μM)
A	Geranylgeranyl pyrophosphate (GGPP)	0, 0.025, 0.05, 0.1, 0.15 g/L	0 or 100	0
B	Precursor of taxol side chain • L-phenylalanine • L-serine • L-glycine • benzoic acid •N-benzoylglycine	0, 0.05, 0.1, 0.2, 0.5 mM	0 or 100	0
C	N-benzoyl L-phenyl isoserine (taxol side chain)	0, 0.025, 0.063, 0.125, 0.25, 0.5 mM	0 or 200	0 or 500
D	Acetyl CoA	0, 0.005, 0.0125, 0.025, 0.05, 0.1 mM	0 or 200	0 or 500

5.2 Results and Discussions

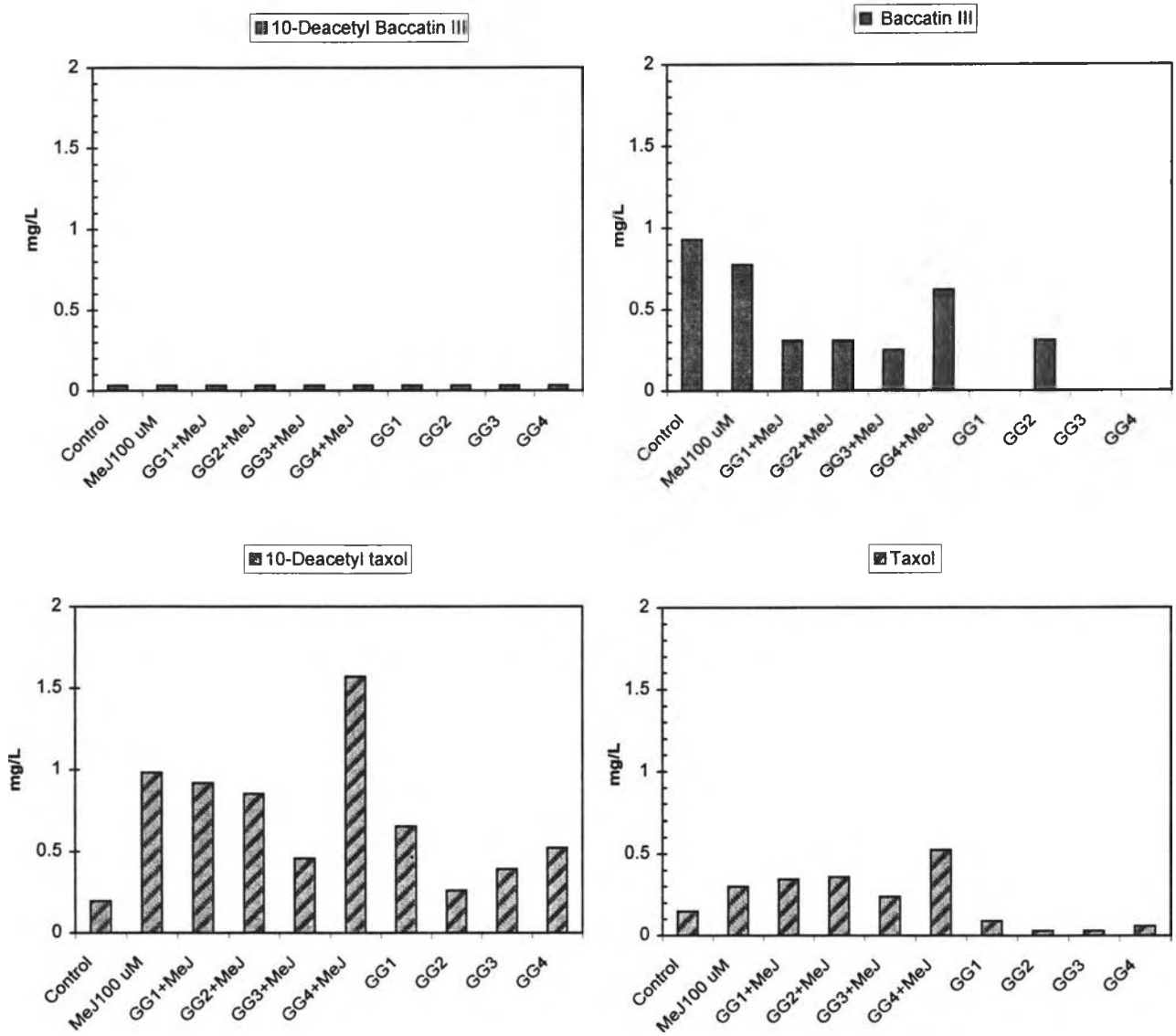
5.2.1. Geranylgeranyl Pyrophosphate

The biosynthesis of taxol (paclitaxel) and related taxoids in Pacific yew *(Taxus brevifolia)* is thought to involve the cyclization of geranylgeranyl diphosphate to a taxadiene followed by extensive oxygenation of this diterpene olefin intermediate. A cell-free preparation from sapling yew stems catalyzed the conversion of (1-3H) geranylgeranyl diphosphate to a cyclic diterpene olefin, that when incubated with stem sections, was converted in good radiochemical yield to several highly functionalized taxanes, including 10-deacetyl baccatin III and taxol itself (Koepp et al., 1995).

In this study, effects of addition of a precursor of taxadiene, geranylgeranyl pyrophosphate (GGPP), on taxane production were examined. Because of the insolubility of GGPP in water, GGPP was added in a form of ethanolic solution (0.5% of total culture volume) at day 8 after cell transfer. All treatments, including controls, were given equal quantities of ethanol, as this reagent has been shown to affect culture physiology (Mirjalili and Linden, 1996). MJ was also added as an ethanol solution in the same manner.

Addition 0.025-0.15 g/L GGPP using ethanol as a carrier did not significantly improve taxol and other taxane yields in the system either with or without MJ (Figure 5.1). According to a report by Hirasuna et al. (1996), addition of 0.01-0.1 g/L of geraniol, presumptive precursor of GGPP or pinene, which is an end product in monoterpene biosynthesis, showed minor enhancements in production of taxol and other taxanes. However, no significant effect on taxane production was observed if ethanol was used as a carrier for geraniol and pinene addition to a high producing cell line.

Supplementation of GGPP did not significantly improve yields of taxol and other taxanes in the system with or without MJ. These results may imply that GGPP is not the limiting substrate of taxol pathway and MJ stimulated the reaction at a step downstream of GGPP formation. However, from the fact that phosphorylated compounds are difficult to load into cells or vacuoles where biosynthetic reactions occur, possibly GGPP was not crossing the cytoplasmic on tonoplastic membranes. Therefore, the effect of feeding this precursor on taxane production might either depend on whether the added precursor can reach the site of the biosynthetic pathway or whether the pathway has the enzymatic capacity to utilize more of this precursor.



Precursor = Geranylgeranyl pyrophosphate (1) =0.15 g/L ; (2) =0.10 g/L ; (3) = 0.05 g/L ; (4) = 0.025 g/L

Figure 5.1 Taxane yield of suspension cultures of *Taxus canadensis* (C93AD) grown in the presence of various concentrations of geranylgeranyl pyrophosphate with and without methyl jasmonate (100 *u*M elicitation).

5.2.2 Potential Taxol Side-Chain Precursors, (L-phenylalanine, Serine, L-glycine, Benzoic Acid and N-benzoylglycine)

From the structures shown in Figure 5.2, it may be seen that the taxol molecule could arise from the mevalonate pathway (terpenoid moiety) and from the shikimic acid pathway (benzoyl groups both of the side chain and of the terpenoid moiety) (Fett-Neto and DiCosmo, 1996). Semisynthesis of taxol has been successfully developed using plant-derived precursors such as 10-deacetylbaccatin III and baccatin III by attachment of N-benzoylphenylisoserine, the side chain of taxol, to the diterpene ring system.

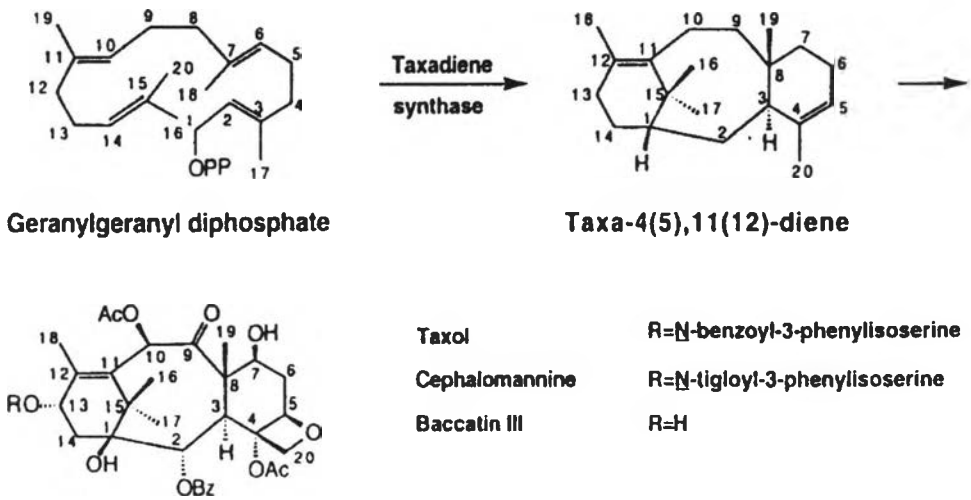
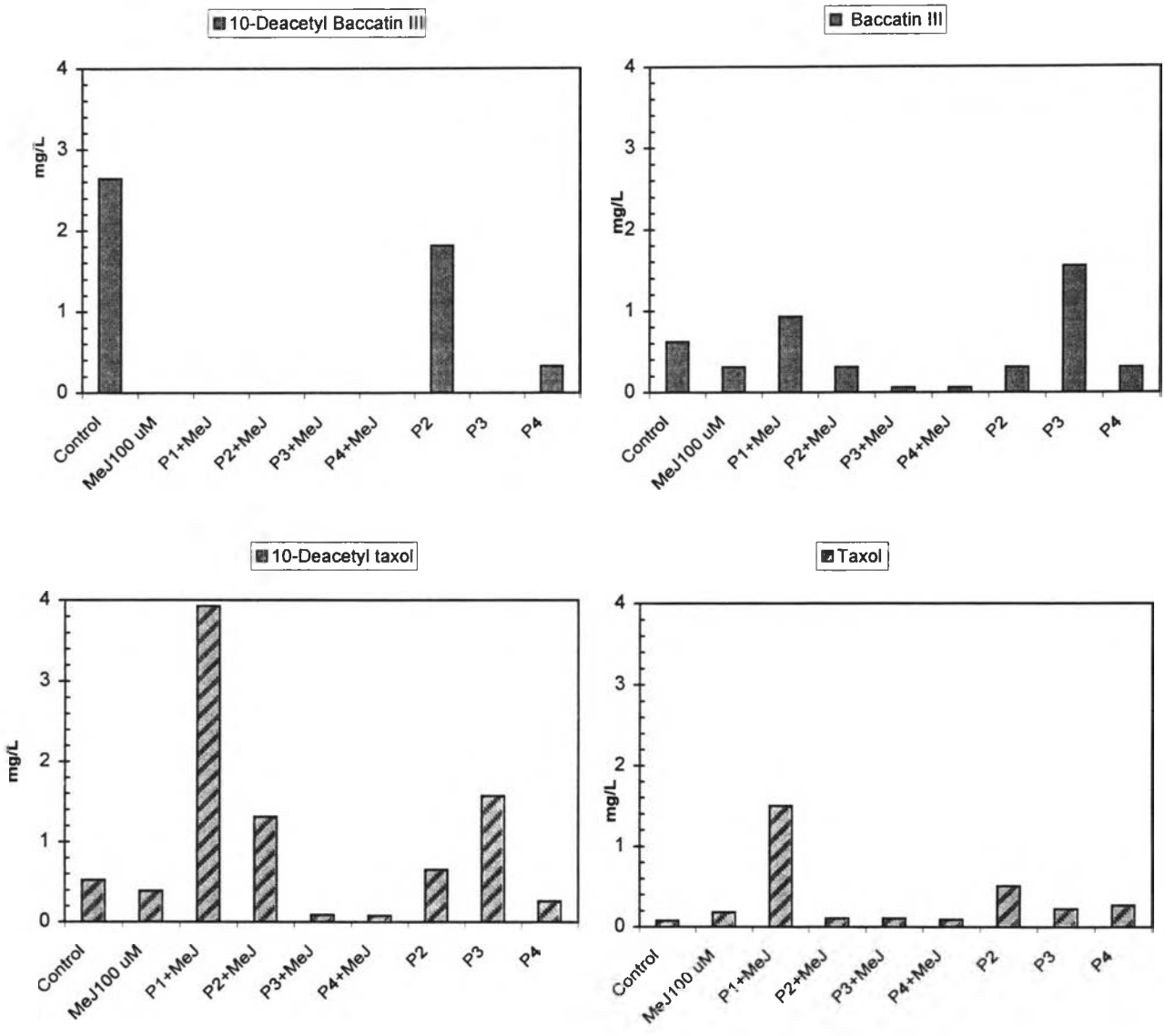


Figure 5.2 Cyclization of geranylgeranyl diphosphate to taxa-4(5), 11 (12)-diene and elaboration of olefin to taxol, cephalomannine or baccatin III. OPP denotes the diphosphate moiety, Bz the benzoyl group, and Ac the acetyl group (Hezari and Croteau, 1997).

The presence of an enzyme in *T. brevifolia* tissue, which is capable of linking a phenylpropanoid side chain to C-13 of baccatin III was reported (Fett-Neto and DiCosmo, 1996). Incorporation of phenylalanine in the side chain of taxol was also reported by Strobel et al. (1992) in *T. brevifolia.* The N-benzoylphenylisoserine side chain of taxol was suggested to arise from phenylalanine via β-phenylalanine (Fleming et al., 1993). The last step in taxol biosynthesis was believed to be benzoylation of N-debenzoyltaxol at the ester in position 2 of the diterpenoid ring.

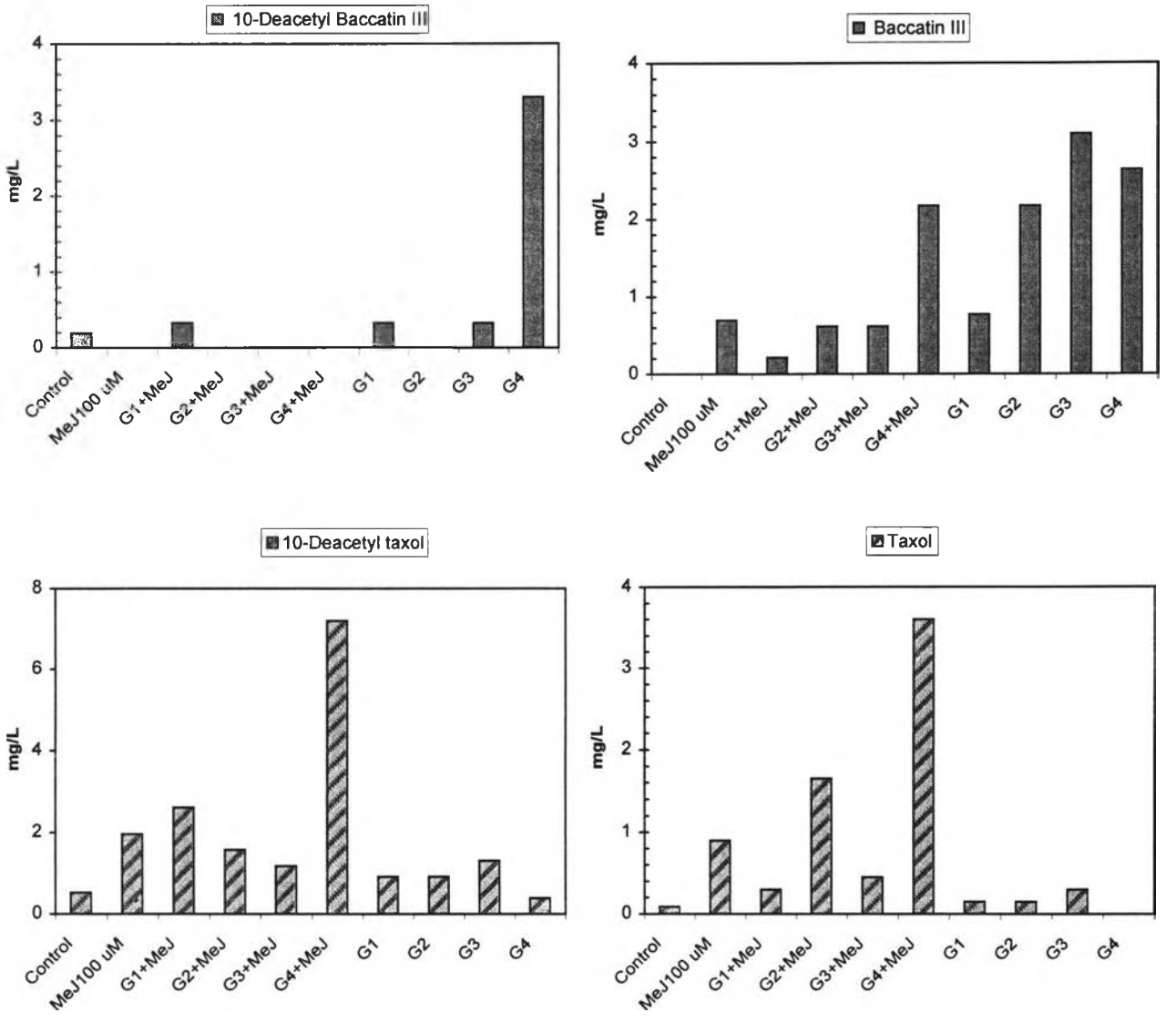
The examination of taxane profiles in some of cell culture lines indicated a larger amount of baccatin III and 10-deacetylbaccatin III (10-DAB) as compared with taxol and cephalomanine (Fett-Neto et al. (1994)). This was also characteristic of our *T. canadensis* (C93AD) cultures without MJ elicitation. By using a simplified form of the conceptual model (Srinivasan, 1995), the yield of taxol was solely dependent on the levels and ratio of side-chain precursor to taxane precursor. Therefore, improved taxol yields could be obtained following feeding of phenylalanine or other potential taxol side-chain precursors together with MJ elicitation.

Significant increases in yields of taxol and other taxanes in cell suspension cultures of *T. canadensis* were observed following feeding of L-phenylalanine, glycine and N-benzoylglycine at different concentrations in both systems with and without 100 μM MJ (Figure 5.3, 5.4 and 5.5). However, no increase in production of taxol or other taxanes when feeding benzoate (0.05-0.1 mM) (Figure 5.6) or L-serine (0.05-0.5 mM) (Figure 5.7). The overall comparison of increasing yield by treatment of each precursor is presented in Figures 5.8 and 5.9.



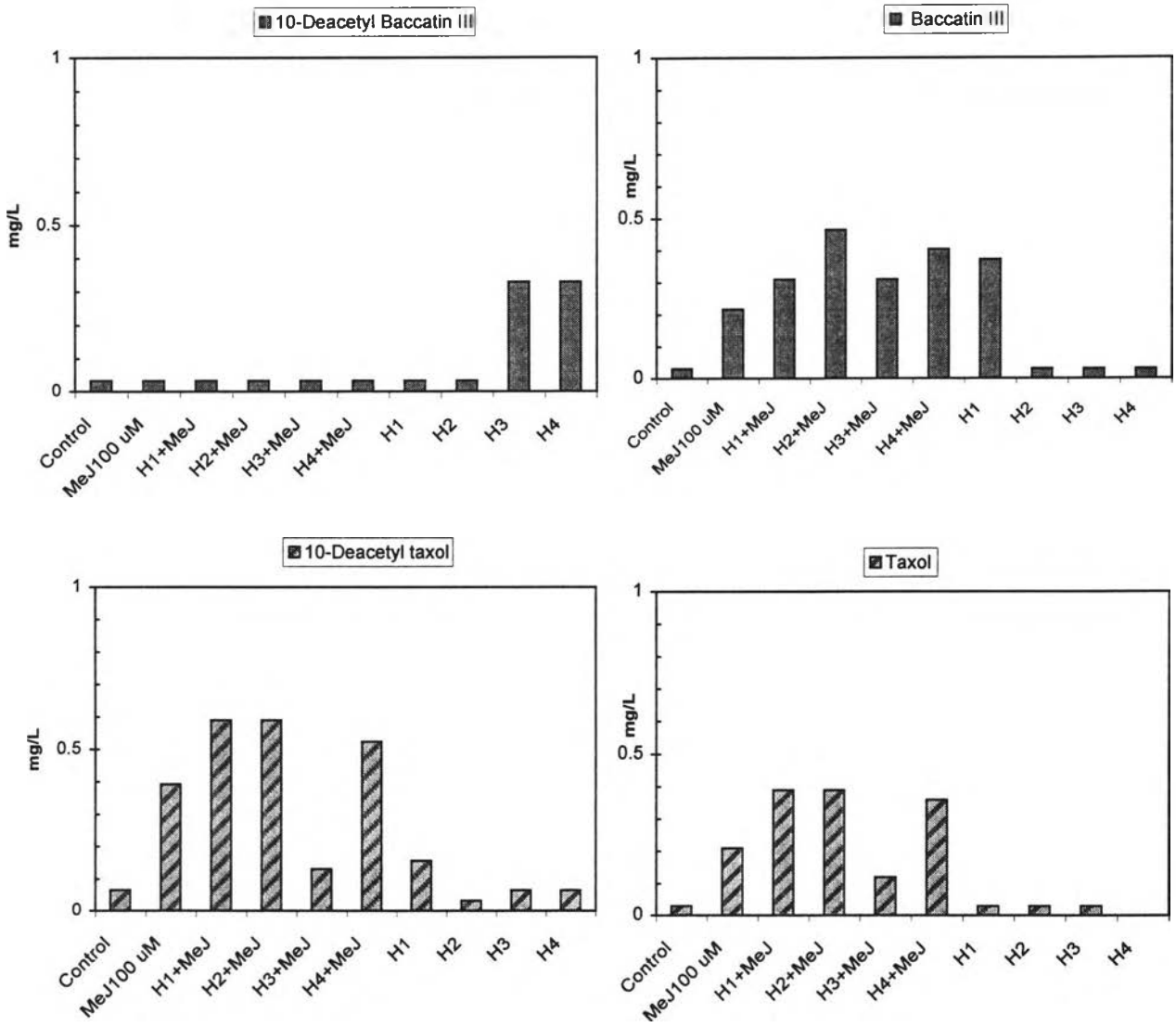
Precursor = L-Phenylalanine (1) = 0.5 mM ; (2) = 0.2 mM ; (3) = 0.1 mM ; (4) = 0.05 mM

Figure 5.3 Taxane yield of suspension cultures of *Taxus canadensis* (C93AD) grown in the presence of various concentrations of L-phenylalanine with and without methyl jasmonate (100 *u*M elicitation).



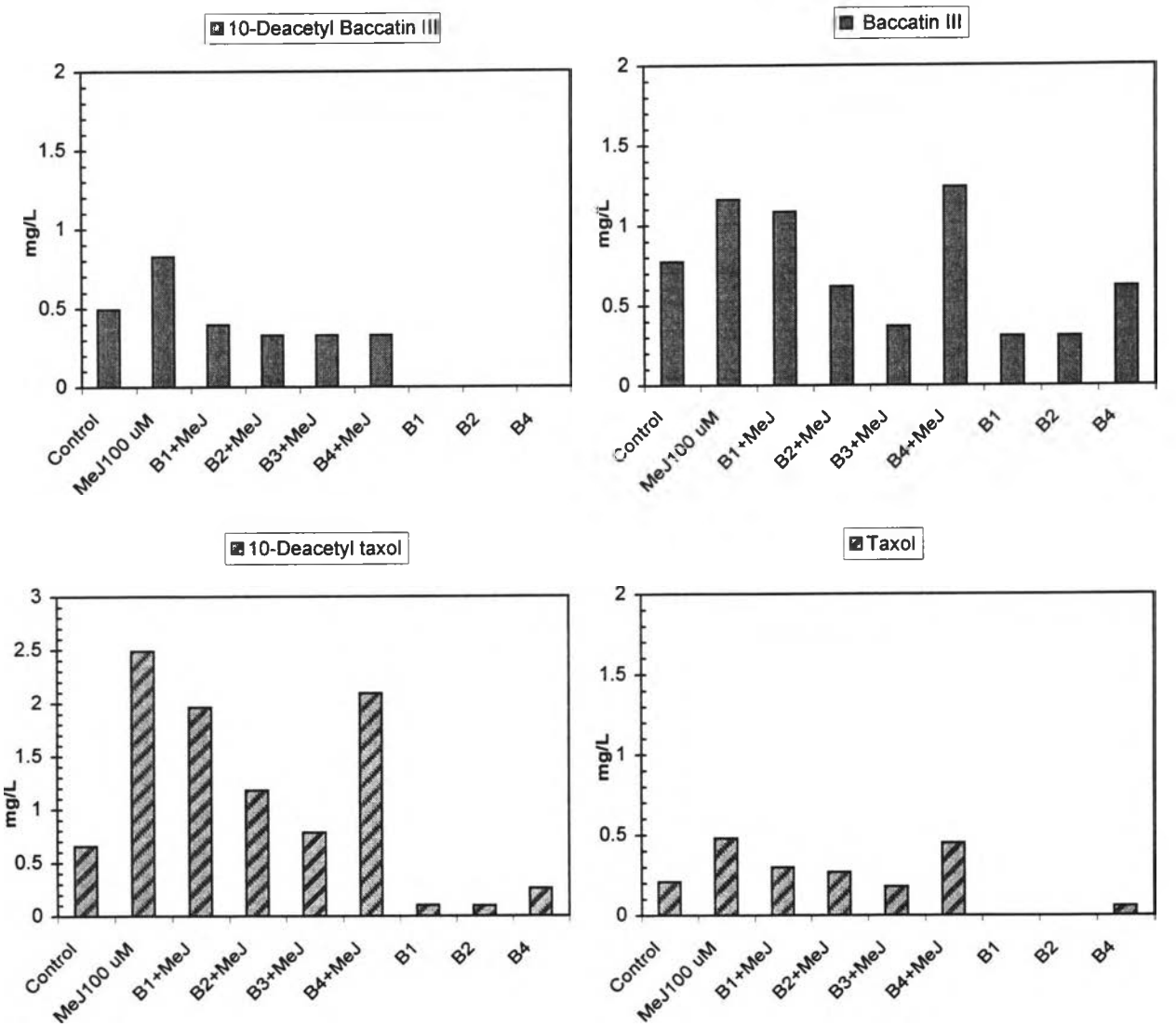
Precursor = Glycine (1) = 0.5 mM ; (2) = 0.2 mM ; (3) = 0.1 mM ; (4) = 0.05 mM

Figure 5.4 Taxane yield of suspension cultures of *Taxus canadensis* (C93AD) grown in the presence of various concentrations of glycine with and without methyl jasmonate (100 *u*M elicitation).



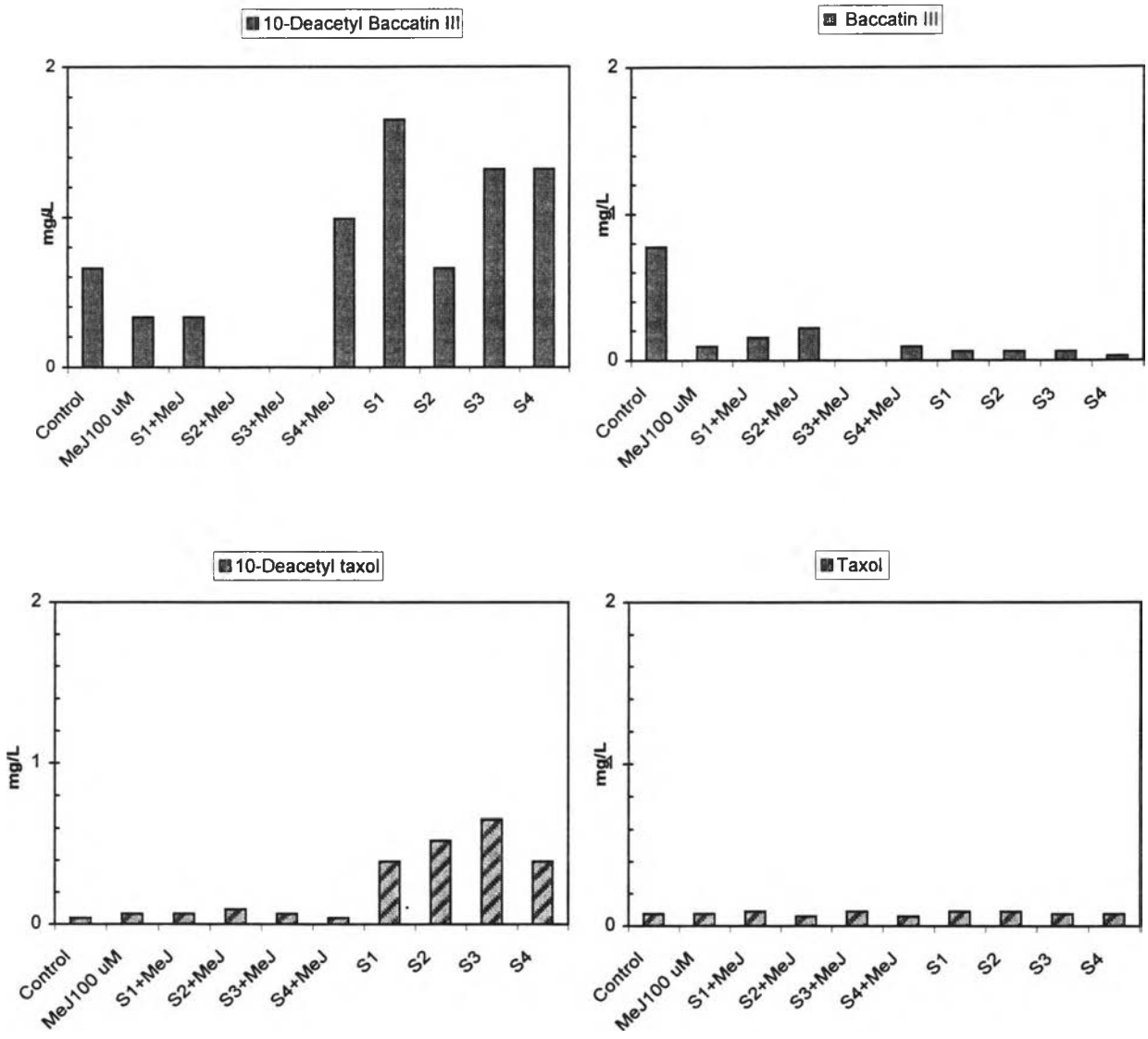
Precursor = H= Hippuric acid (N-Benzoylglycine) (1) = 0.5 mM; (2) = 0.2 mM; (3) = 0.1 mM; (4) = 0.05 mM

Figure 5.5 Taxane yield of suspension cultures of *Taxus canadensis* (C93AD) grown in the presence of various concentrations of N-benzoylglycine with and without methyl jasmonate (100 *u*M elicitation).



Precursor = Benzoate (1) = 0.5 mM ; (2) = 0.2 mM ; (3) = 0.1 mM ; (4) = 0.05 mM

Figure 5.6Taxane yield of suspension cultures of *Taxus canadensis* (C93AD) grown in the presence of various concentrations of benzoate with and without methyl jasmonate (100 *u*M elicitation).



Precursor = S = Serine (1) = 0.5 mM ; (2) = 0.2 mM ; (3) = 0.1 mM ; (4) = 0.05 mM

Figure 5.7 Taxane yield of suspension cultures of *Taxus canadensis* (C93AD) grown in the presence of various concentrations of serine with and without methyl jasmonate (100 *u*M elicitation).

The enhancement of taxol accumulation using precursor supplements was also reported by Fett-Neto et al. (1994) in *T. cuspidata* cultures. Taxol yields increased up to 5 fold by feeding aromatic compounds and nonaromatic amino acids. The optimal ranges for the aromatic compounds using phenylalanine were 1mM for callus and 0.05-0.20 mM for suspensions, using benzoic acid: 0.2-1.0 mM for callus and 0.05-0.20 mM for suspensions, and using N-benzoylglycine: 0.2-10.0 mM for callus and 0.05 mM for suspensions. The optimal ranges for the nonaromatic amino acids using glycine was 0.20 mM for callus and suspensions and using serine: 0.2 mM for callus and 0.05-0.2 mM for suspensions.

Supplementation of L-phenylalanine at 0.1-0.5 mM (Figure 5.3) resulted in improved taxol, 10-deacetyltaxol (10-DAT) and baccatin III yields by 3.4, 1.7, and 1.2 fold in the system without MJ and 3.2, 3.4 and 1.1 fold in the system with MJ. The enhanced production of taxol and 10-DAT by L-phenylalanine and other potential taxol side-chain precursors is probably related to its involvement as a precursor for N-benzoyl-L-phenyl-isoserine, the side chain of taxol at C13.

In this study, benzoic acid supplementation did not promote taxol or other taxanes production but rather inhibited cell growth. This failure is possible due to the improper test of the benzoic acid concentration range. The negative effect of benzoic acid on the pH of the system might be responsible for growth inhibition.

By feeding N-benzoylglycine (0.05-0.5 mM) in 100 μM MJ elicited system, taxol, 10-DAT and baccatin III increased by 1.7, 1.5 and 1.6 fold, but not in the unelicited system.

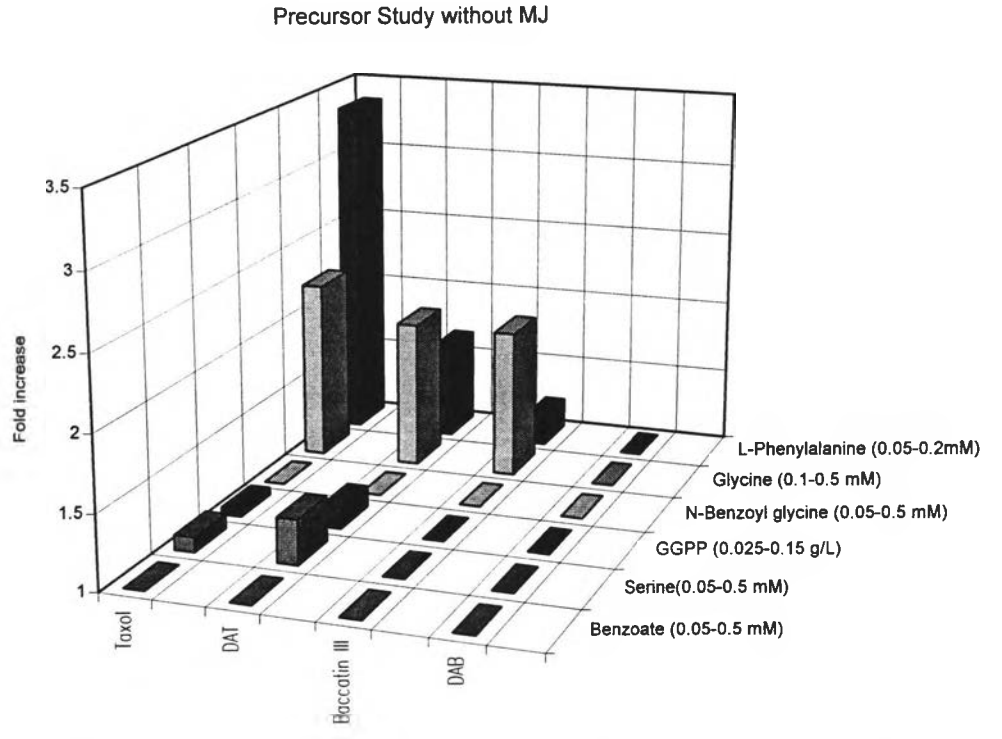


Figure 5.8 Average increase of best in series compared to control of taxanes at 21 days of cell transfer with different precursor supplementation in the system without MJ.

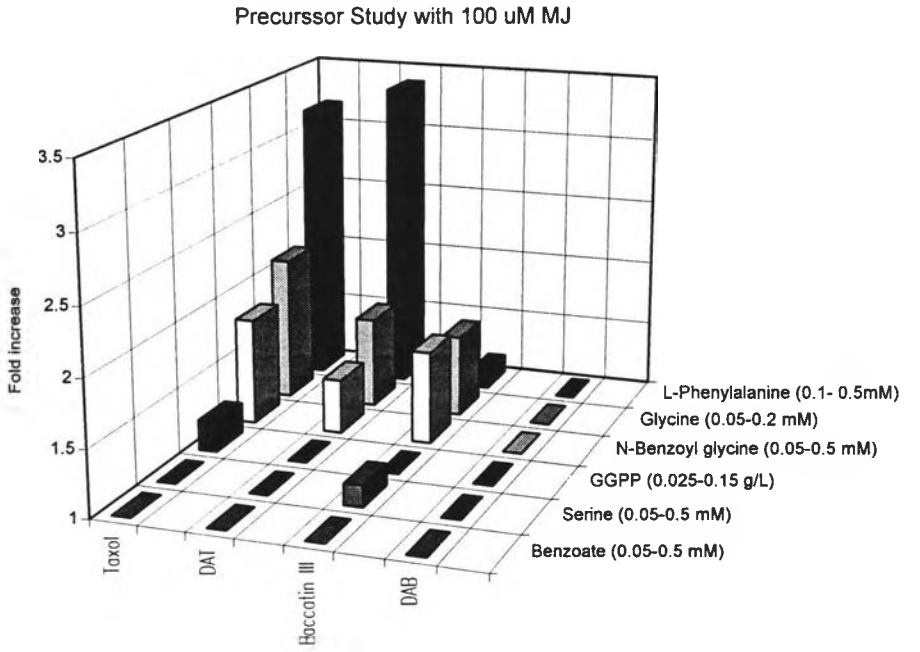


Figure 5.9 Average increase of best in series compared to control of taxanes at 21 days of cell transfer with different precursor supplementation in the system with MJ.

Supplementation of glycine increased production yields of taxol, 10-DAT and baccatin III by 2.2, 2.0 and 2.0 fold, respectively in system without MJ and 2.1, 1.7 and 1.6 fold in the system with MJ. Increased taxol yield in the presence of glycine might be probably related its catabolism, since this amino acids could enter the shikimic acid pathway, leading to phenylalanine and/or benzoic acid synthesis.

The concentrations of taxol, 10-deacetyl taxol and baccatin-III obtained in the system elicited with MJ were much more than the system without MJ under the same treatment. The overall average ratio of taxol concentration in the systems with MJ elicitation to the systems without MJ was about 5. These results may imply that the precursors of the side chain might not be the limiting compounds in the taxol biosynthesis pathway, but excess supplementation of these compounds might be able to move the equilibrium of the reaction toward taxol production. The effect of feeding this precursor on taxane production also depends on whether the pathway has the enzymatic capacity to utilize more of this precursor; MJ might stimulate the production of enzymes of the pathway.

10-DAB is the only observed taxane that did not increase with the supplementation of the side chain precursors. Since both 10-DAB and baccatin-III structures are without the side chain, the results may imply that the synthesis of taxol occurs after the synthesis of 10-DAB and that baccatin-III could form as a degradation product of taxol. The latter conclusion is in agreement with interpretation of Ketchum et al. (1998).

5.2.3. Taxol Side-Chain Precursor (N-benzoyl-L-phenyl-isoserine)

N-benzoyl-L-phenyl-isoserine is the side chain of taxol and that of some related taxoids, e.g., 10-deacetyl taxol. The sequence of attachment of the side chain is still unclear. However, if the side-chain precursor is a limiting substrate, but not the taxadiene,

then we would expect to increase the level of 10-deacetyl taxol (10-DAT) and taxol (taxanes with side chains) yields and decrease of 10-deacetyl baccatin III (10-DAB) (non-side-chain taxane).

The examination of taxane profiles in our study in the system without elicitation sometimes indicated a large amount of 10-DAB compared to taxol and 10-DAT. Side chain biosynthesis could be limited by availability of side chain precursors. L-Phenylalanine feeding in both systems with and without elicitation of MJ (section 5.2.2) results in a significant increase in taxol yields (more than 300%). The enhanced yields may be related to the involvement of phenylalanine in the biosynthesis of N-benzoyl-L-phenyl-isoserine.

In our studies discussed in Chapter 4, co-mediated elicitation by N-acetylchitohexaose and MJ promoted taxol production. The study by Dörnenburg and Knorr (1995) suggested that chitosan might improve transfer of feeding compounds through the cell membrane by causing permeabilization. Therefore, in this chapter, examinations of feeding the taxol side chain were conducted in the system with and without MJ and with and without N-acetylchitohexaose and with the controlled headspace gas following the experimental design in Table 5.1.

Unfortunately, absolute taxol values were low for this set of experiments; no taxol accumulation was observed in the control. No significant increases of production of taxol and other taxane were observed in the presence of N-benzoyl-L-phenyl-isoserine as shown in Figure 5.10 and 5.11. The organization of each Figure is as follows: a) presents results without elicitation; b) with 0.63 mg/L N-acetylchitohexaose; c) with 200 μM MJ

and d) with 0.63 mg/L N-acetylchitohexaose and 200 μM MJ.

Elicitation with 200 μM MJ, which was the optimal concentration in previous studies discussed in Chapter 3 resulted in dramatically decreased taxol and other taxane production in this experiment. The inhibition effect of MJ on taxol production was also observed in the subsequent kinetic study (Chapter 6) at concentrations of 200 μM or more. Addition of N-benzoyl-L-phenyl-isoserine did not significantly improve taxol or 10-DAT yields. There was a 10 % increase of taxol and 10-DAT at 0.025-0.063 mM of N-benzoyl L-phenyl isoserine in cultures without MJ addition. No increase of taxol or other taxanes occurred when adding N-acetylchitohexaose, but inhibition did result when adding N-acetylchitohexaose together with MJ (Figure 5.10 d). Overall, addition of N-benzoyl L-phenyl isoserine showed negative effects on the accumulations 10-DAB and baccatin III (Figure 5.11).

The effect of MJ at 200 μM on taxane accumulation compared to the previous study (Chapter 3) showed obvious changes in cell sensitivity to MJ and the ability to produce taxanes. Both observations may be due to problems of inherent genetic or epigenetic instability of this cell line. The instability of *Taxus* spp. was also reported by the Shuler group (Hirasuna et al., 1996; Ketchum and Gibson, 1996; Ketchum et al., 1998).

The supplementation of the taxol side chain, N-benzoyl-L-phenyl-isoserine did not significantly increase taxol or 10-DAT formation, although the enhancement of yield of taxol and 10-DAT were observed with supplementation of taxol side chain precurrsors, L-phenylalanine. The ineffective effect of feeding N-benzoyl-L-phenyl-isoserine on taxane production in this study was possibly due to poor cell conditions or N-benzoyl-L-

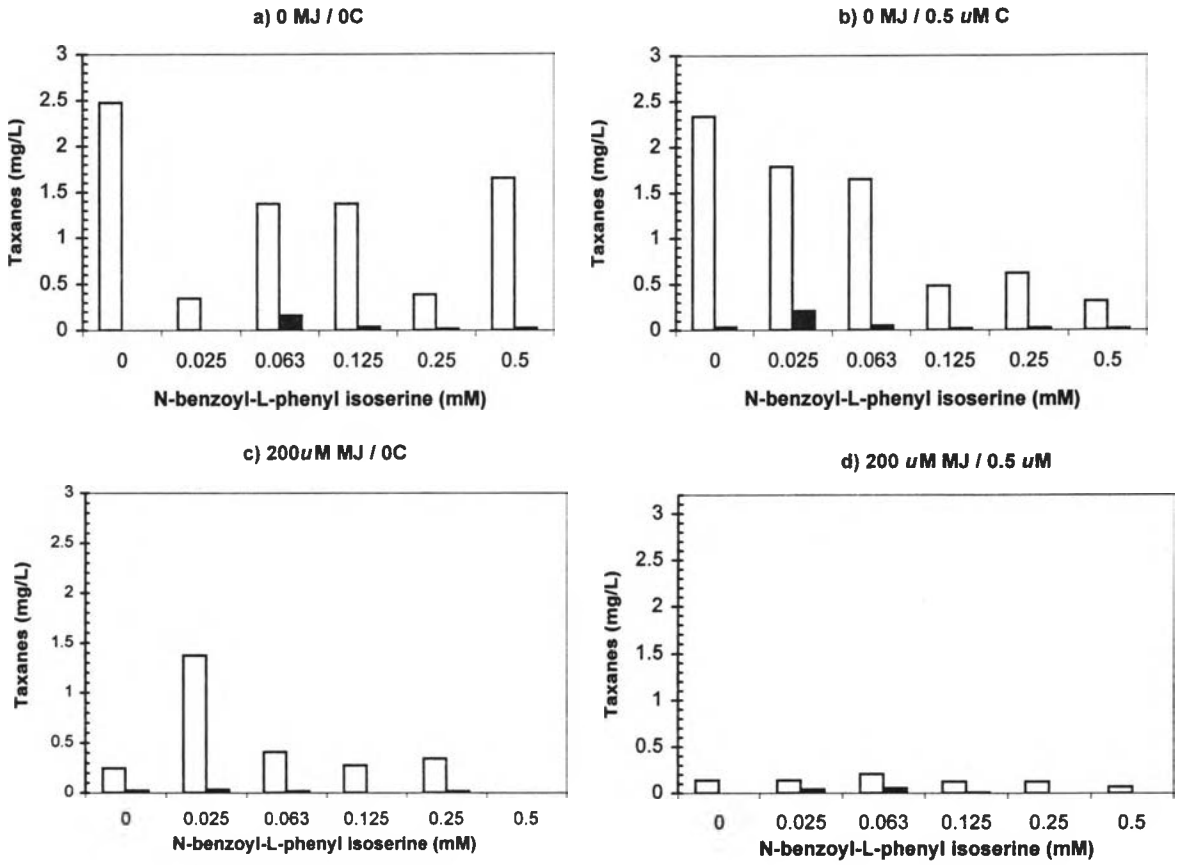


Figure 5.10 Taxol and 10-deacetyl taxol concentrations in suspension cultures of *T. canadensis* at 21 days with various concentrations of N-benzoyl L-phenylisoserine supplementation at day 8 in systems with or without (200 *u*M) MJ and with or without (0.5 *u*M) N-acetylchitohexaose (C).

□ = 10-DAT ■ = TAXOL

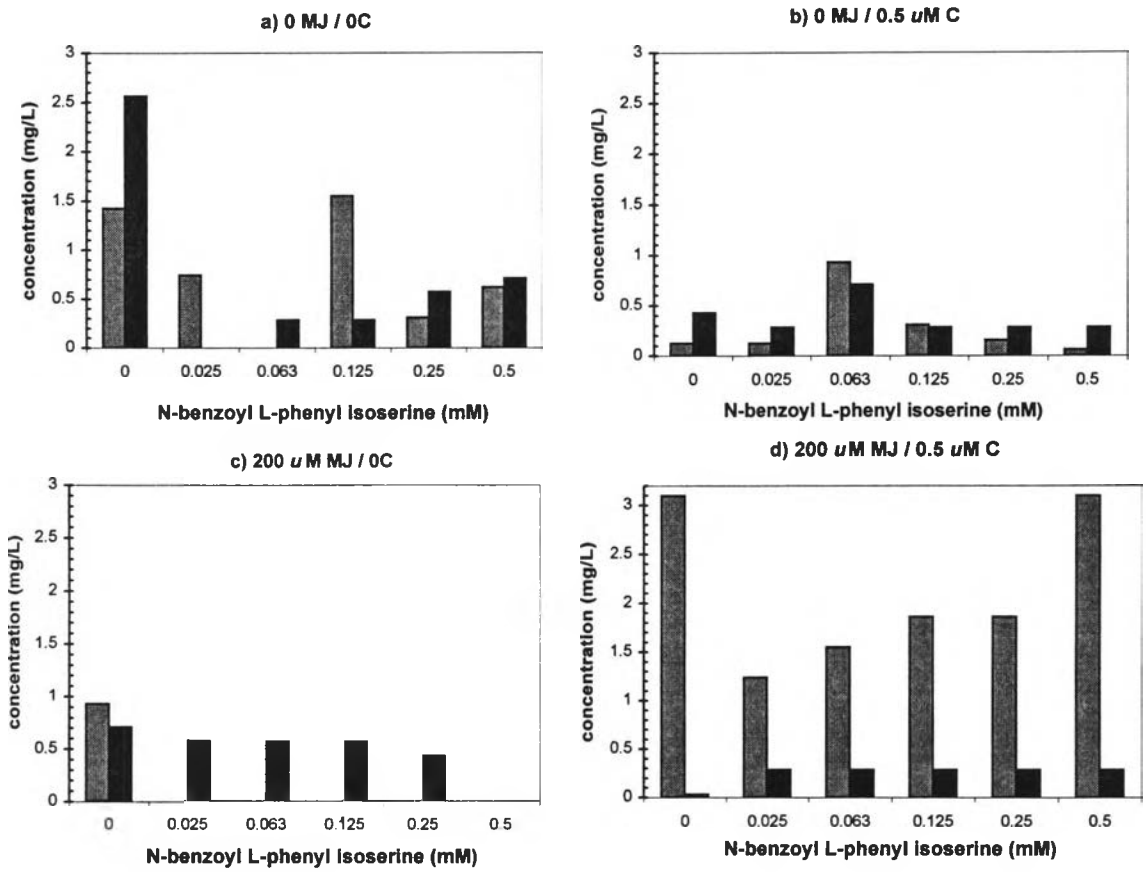


Figure 5.11 Baccatin-III and 10-deacetyl baccatin-III concentrations in suspensions of *T. canadensis* at 21 days with various concentrations of N-benzoyl L-phenyl isoserine supplementation at day 8 in systems with or without (200 uM) MJ and with or without (0.5 *u*M) N-acetylchitohexaose (C).

■ = 10-DAB ■ = BACCATIN III

phenyl- isoserine was not crossing the cytoplasmic or tonoplastic membrane.

5.2.4. Acetyl Co-enzyme A

The biosynthesis of taxol is a multi-step process. One intermediate reaction was suggested by the acetylation of 10-deacetylbaccatin-III (10-DAB) to baccatin-III, an assumed precursor of taxol (Zocher et al., 1996). The study showed acetylation of 10-DAB to baccatin-III using crude extracts from roots of *Taxus baccata* saplings and ^{14}C or ^{3}H-labeled acetyl Co-enzyme A as the acetyl donor.

Examinations of taxane profiles from our study found a larger amount of 10-deacetyl taxol compared to taxol or baccatin-III. However, taxol promotion always occurred with high 10-deacetyl taxol concentration, and the profiles of these two compounds always followed the same trends. From this information, we studied the acetylation of 10-deacetyl taxol to taxol. With this hypothesis and since 10-deacetyl taxol accumulates at high concentrations in the culture medium, its conversion to taxol might be one of the rate limiting steps. Taxol production may increase by supplying excess of acetyl Co-enzyme A to increase the acetylation of 10-DAT.

In this study, acetyl Co-enzyme A was added at concentrations of 0.05 to 0.1 mM on day 8 after cell transfer. The effects of acetyl Co-enzyme A on taxol and other taxane formation are shown in Figures 5.12 and 5.13. As before, in each Figure: a) presents results without elicitation; b) with 0.63 mg/L N-acetylchitohexaose; c) with 200 μM MJ and d) with 0.63 mg/L N-acetylchitohexaose and 200 μM MJ. Overall, taxol values were low for this set of experiments; no taxol accumulation was observed in the control.

Although the inhibition effect of 200 μM MJ on taxane production was observed, taxol accumulation increased by addition of acetyl Co-enzyme A together with elicitation. Addition of N-acetylchitohexaose in the system without MJ showed minor enhancements in the production of taxol and 10-DAT (Figure 5.12 a and 5.12 b) but not in the system with 200 μM MJ. Taxol concentration increased by 2 fold with addition of 0.0125-0.025 mM acetyl CoA in the system elicited with MJ or N-acetylchitohexaose or both. No significant effect of acetyl Co-enzyme A on accumulation of 10-DAB or baccatin III was observed (Figure 5.13). 10-DAB decreased in systems elicited with MJ or/and N-acetylchitohexaose.

Ratios of taxol to 10-deacetyl taxol increased by addition of MJ, N-acetylchitohexaose and acetyl-Co enzyme A as shown in Figure 5.14. The ratios of taxol to 10-DAT increased by 2 fold by elicitation with 0.5 μM N-acetylchitohexaose and 6 fold by elictation with 200 μM MJ.

These results are consistent with other reports. Strobel (1992) reported that radiolabeled acetate incorporated uniformly in labeled taxol, which implied that acetate might work as a precursor in taxol production. [1,2-^{13}C] sodium acetate, added at the same time as methyl jasmonate, is incorporated only in the acetate groups of taxol and other taxanes but not in the diterpenoid ring (Ketchum et al., 1997). The activity of acetyl-transferases converting 10-deacetylbaccatin III to baccatin III and 10-deacetyl taxol to taxol using acetyl-CoA as acetyl donor, in cell-free protein extracts of needles and cell suspensions was detected, however the observed activities were relatively low (Fett-Neto and DiCosmo, 1996). From the study by Hirasuna et al. (1996) in suspension cultures of

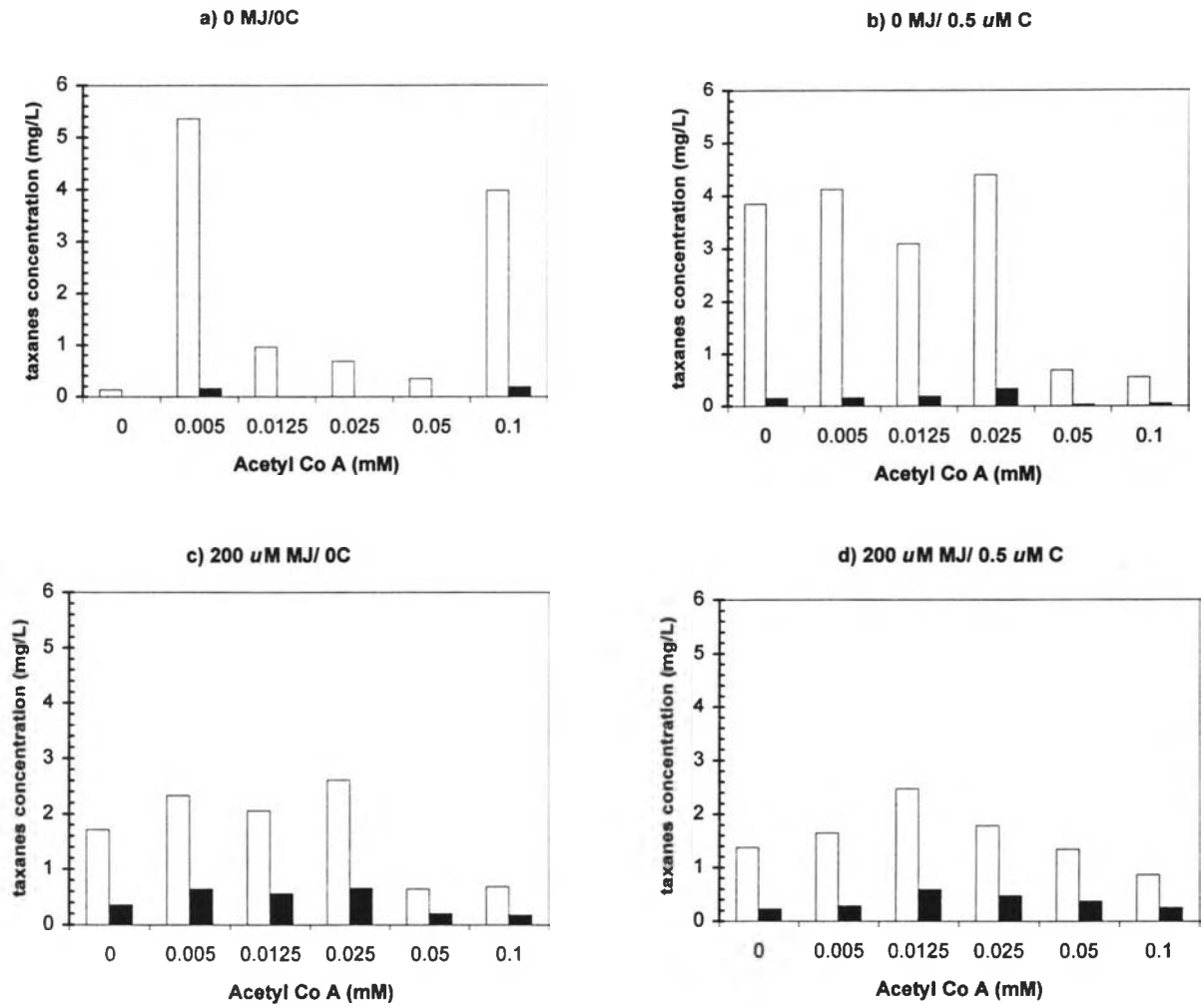


Figure 5.12 Taxol and 10-deacetyl taxol concentrations in suspension cultures of *T. canadensis* at 21 days with various concentrations of acetyl Co A supplementation at day 8 in systems with or without (200 *u*M) MJ and with or without (0.5 *u*M) N-acetylchitohexaose (C).

□ =10- DAT ■ = TAXOL

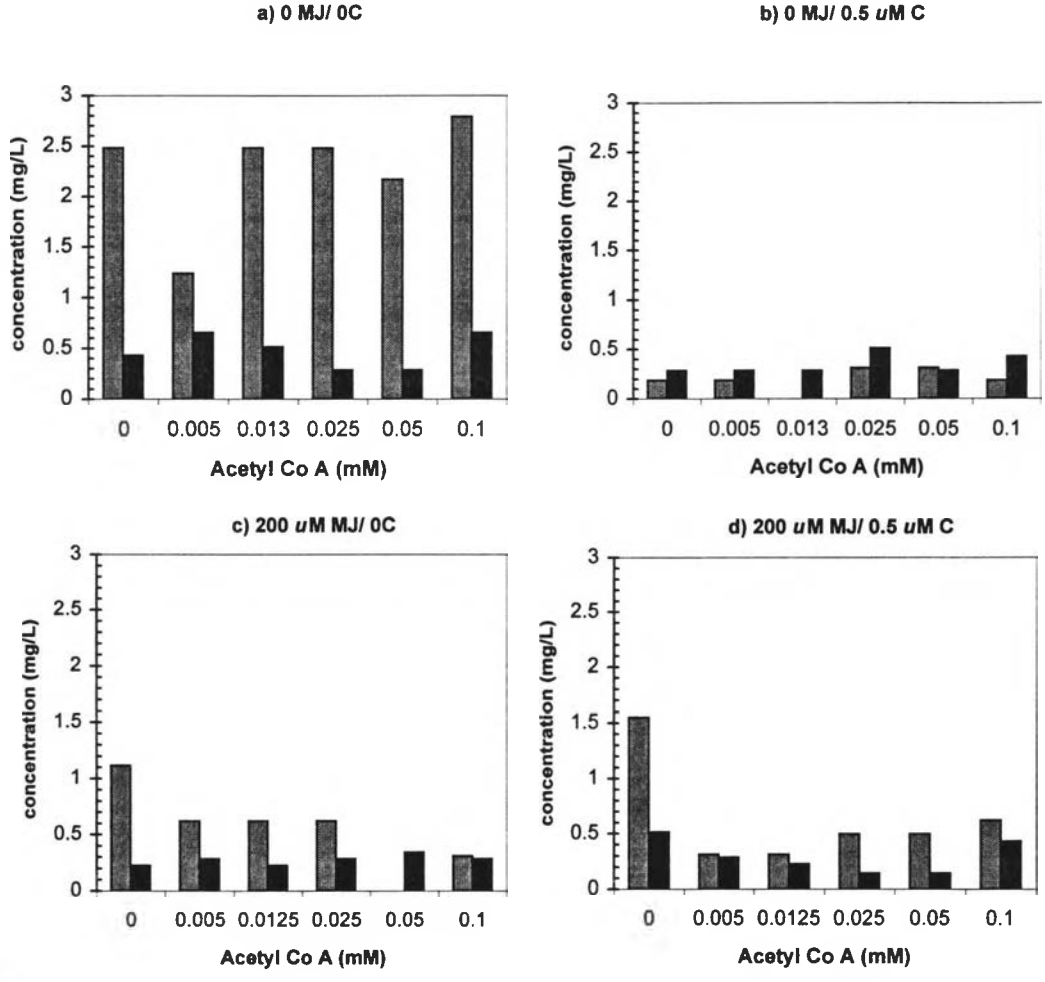
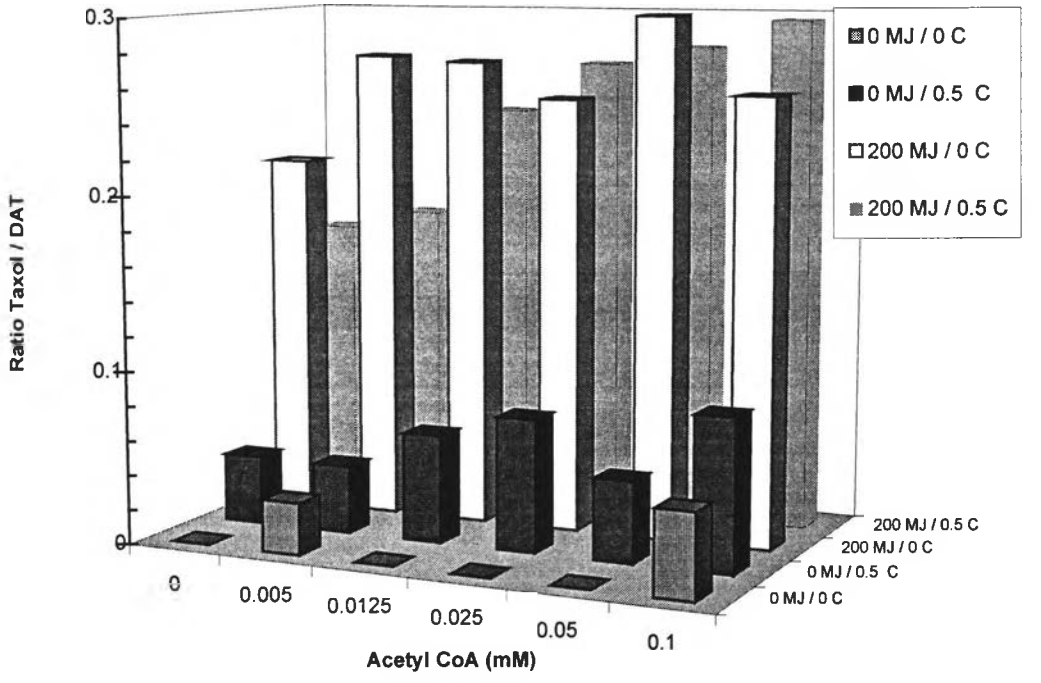


Figure 5.13 Baccatin-III and 10-deacetyl baccatin-III concentrations in suspension cultures of *T. canadensis* at 21 days with various concentrations of acetyl Co A supplementation at day 8 in systems with or without (200 *u*M) MJ and with or without (0.5 *u*M) N-acetylchitohexaose (C).



C = N-acetylchitohexaose (μM); MJ = Methyl Jasmonate (μM)

Figure 5.14 Ratios of taxol to 10-deacetyl taxol at various acetyl Co A concentrations in the system with or without MJ and with or without N-acetylchitohexaose (C).

Taxus baccata (PC2), addition of 1.0 and 10.0 mM acetate showed an increase in the level of taxol from 0.082 mg/L to 0.17 and 0.12 mg/L, respectively.

To confirm the proposed pathway from 10-DAT to taxol, in vitro studies were performed using crude extracts from suspension cells of *T. canadensis* (C93AD). The details of the experimental studies and results are in Appendix B. From the in vitro studies, we detected the activities of acetyl-transferases converting 10-deacetyl baccatin III to baccatin III and 10-deacetyl taxol or 7- xylosyl-10-deacetyl taxol to taxol. No significant difference was detected between using cells with or without MJ elicitation. However, the observed activities of acetyl-transferases were relatively low. Additional experiments using radiolabeled acetyl CoA as acetyl donor are recommended to confirm the existence of these activities.

5.3 Conclusions

- The results from precursor studies suggested that taxol yield can be improved by coupling potential precursor feeding with elicitation (to increase enzyme levels).
- The supplementation of GGPP did not significantly improve taxol or other taxane yields in the system with or without MJ. These results may imply that GGPP is not the limiting substrate of taxol biosynthesis and MJ stimulated the process at a reaction beyond GGPP formation. Another potential reason may be due to the ineffective transport of GGPP crossing the cytoplasmic or tonoplastic membranes.
- Increases of taxol, 10-deacetyl taxol, baccatin III in *Taxus canadensis* were obtained followed feeding phenylalanine and other potential taxol side chain precursors such as N-

benzoylglycine and L-glycine at day eight of growth with and without MJ elicitation.

• Addition of the taxol side-chain (N-benzoyl-L-phenyl-isoserine) did not improve taxol yield as expected.

• Slightly improved taxol yield and significantly increased ratios of taxol to 10-DAT following addition of acetyl Co-enzyme A showed that acetate might work as a precursor in taxol production.

• In this study, elicitation with N-acetylchitohexaose did not significantly improve taxol and other taxane production. The different effect from the studies described in Chapter 4 might relate to whether ethylene was given in the headspace or not. Earlier, the best response was to a poor producing culture without headspace gas control.

• The biosynthesis pathway of taxol from geranylgeranyl pyrophosphate is proposed as shown in Figure 5.15. This proposed pathway is consistent with examinations of taxol and other taxane profiles in our studies (Chapters 3, 4 and 5), together with information from other studies (Strobel, et al., 1992; Fett-Neto et al., 1994; Fett-Neto and DiCosmo, 1996; Srinivasan et al., 1996; Ketchum et al., 1998) as described below:

1) MJ stimulates taxol, 10-DAT, and baccatin-III production but not 10-DAB. The examination of taxane profiles without MJ elicitation indicated a larger amount of 10-DAB and baccatin III compared to taxol and cephalomanine (Fett-Neto et al., 1994). This was also characteristic of our *T. canadensis* (C93AD) cultures without MJ elicitation (Chapters 3, 4 and 5). These observations may imply a separate pathway of taxanes with side chain (10-DAT, taxol and others) from taxanes without side chain (10-DAB and baccatin III), as indicated on the bottom line of Figure 5.15.

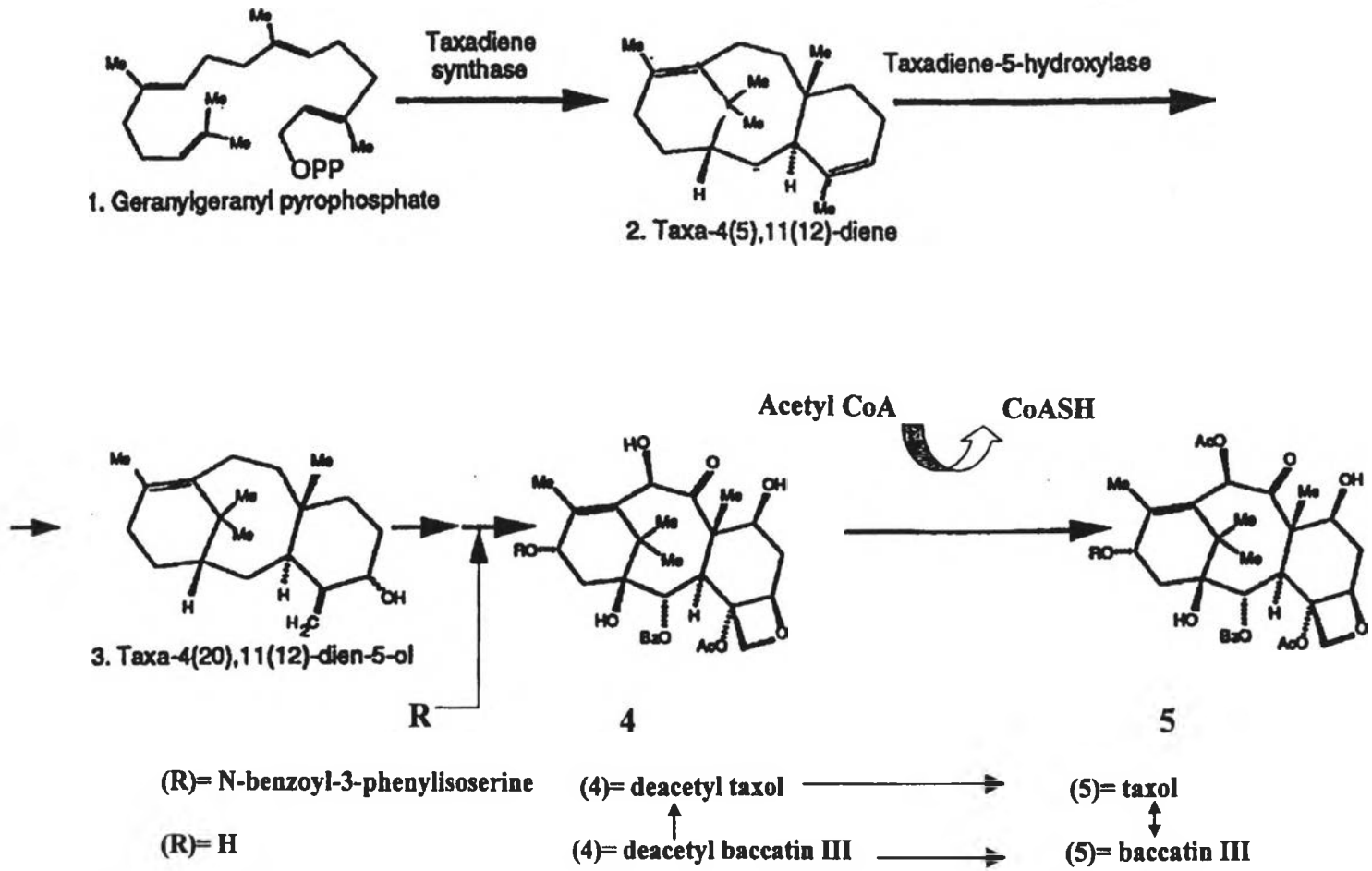


Figure 5.15 The proposed biosynthetic pathway of taxol from geranylgeranyl pyrophosphate.

2) Accumulation of baccatin III increases with increasing 10-DAB and taxol concentrations (Chapter 3). These observations may suggest that both 10-DAB and taxol could be precursors of baccatin III.

3) Taxol accumulation always occurred with high 10-DAT concentration and the profiles of these two compounds followed the same trends (Chapters 3, 4 and 5), which may suggest that 10-DAT could be a direct precursor of taxol.

4) Improved taxol yields and increased ratios of taxol to 10-DAT followed addition of acetyl Coenzyme A (Chapter 5). Therefore, acetate or acetyl-CoA might work as a precursor in taxol production. These results were consistent with the report by Fett-Neto and DiCosmo (1996) on the activity of acetyl-transferases on conversion of 10-DAB to baccatin III and 10-DAT to taxol using acetyl-CoA as acetyl donor, in cell-free protein extracts of needles and cell suspensions. Strobel et al. (1992) also reported radiolabeled acetate incorporated uniformly in taxol. These reports support the proposed pathway from 10-DAT to taxol.

5) The report by Srinivasan et al. (1996) that addition of 10 μM 1-aminobenzotriazole suppressed baccatin III production while doubling the taxol yield, may suggest that baccatin III need not to be a direct precursor of taxol.

6) The report by Ketchum et al. (1998) that baccatin III concentration increases until the cells are dead, and does not seem to follow the same pattern of accumulation as the other taxanes in the medium, may also suggest baccatin III may be a degradation product of taxol.

CHAPTER 6

KINETICS OF TAXANE PRODUCTION, GROWTH, AND NUTRIENT UPTAKE IN CELL SUSPENSIONS OF *TAXUS CANADENSIS*

The accumulation of taxol and other taxanes is thought to be a biological response to specific external stimuli. The studies described in Chapter 3 showed that the interaction between ethylene (C_2H_4) and methyl jasmonate (MJ) played the important role in stimulation of taxol production in *Taxus canadensis*. The results extended the findings in the report on *Taxus cuspidata* suspension cultures by Mirjalili and Linden (1996). Enhancement of taxol and related taxane production using MJ in other *Taxus* species was reported (Yukimune et. al., 1996; Ketchum et. al., 1998). In the studies described in Chapter 4, chitin or chitosan oligosaccharides with MJ as a co-mediator enhanced taxol yields in some systems. Improved yields of taxol and other taxanes following feeding of potential precursors together with elicitation were discussed in Chapter 5.

The cell line, C93AD, initiated from *Taxus canadensis* was used for the studies. The average growth rate was about 2.5 fold in 14 days of the culture cycle (μ= 0.1 day^{-1}). Approximately 80% of the total taxanes in a culture were accumulated in the extra-cellular medium. Suspension cultures of *Taxus canadensis*, responded well to elicitation

with MJ and ethylene as reported in Chapter 3. The optimum of MJ concentration for elicitation at day-8 following transfer of the culture was 200 μM. In order to understand the interrelationships between cell growth, substrate uptake, taxol and related taxane formation, and the effects of elicitation and precursor supplements on the behavior of the system, the kinetic studies described in this chapter were conducted.

6.1 Experimental Studies

In this chapter, sets of experiments to study kinetics of taxol and other taxane production, growth and nutrient uptake in cell suspension of *Taxus canadensis* (C93AD) were performed in 125-mL Erlenmeyer flasks. The cell line used was kindly provided by the laboratories of Prof. M.L. Shuler (School of Chemical Engineering, Cornell University, Ithaca, NY) and Dr. D. Gibson (ARS-USDA, Ithaca, N.Y.). The growth medium used was according to Gibson et al. (1993). The elicitors or a precursor supplement (1% of suspension volume) were added into 50 mL of 8-day old suspension cultures according to the given experimental designs given in Table 6.1. The culture flasks were then incubated in dark at 25°C with 130 rpm shaking. The mixture of gases flowing to the headspace of the incubator was controlled at 6-8 ppm C_2H_4, 15% O_2 and 0.5% CO_2. The samples were taken every 2-3 days to analyze growth and taxane production, as well as the kinetics of carbohydrate utilization. A preliminary kinetic study elicited with 200 μM MJ was first tested to check the pattern of cell activity kinetics. The kinetic studies of the effect of elicitors with supplementation of precursors were then conducted in seven series of experiments. The experiments were all duplicated.

Table 6.1 Treatment conditions for kinetic studies.

Experiment	TREATMENT CONDITIONS			
	Methyl jasmonate (MJ)	N- acetylchito Hexaose (C)	Acetyl coA (AA)	N-benzoyl L-phenyl isoserene (SC)
6.2.1	200 μM	NO	NO	NO
6.2.2	0, 100, 200, 400 μM	NO	NO	NO
6.2.3.1 (=A)	NO	NO	NO	NO
6.2.3.2 (=B)	NO	0.50 μM	NO	NO
6.2.3.3 (=C)	100μM	NO	NO	NO
6.2.3.4 (=D)	100μM	0.50 μM	NO	NO
6.2.3.5 (=E)	100μM	NO	0.02 mM	NO
6.2.3.6 (=F)	100μM	0.50 μM	0.02 mM	NO
6.2.3.7 (=G)	100μM	0.50 μM	0.02 mM	0.02 mM

Remark: Eilicitors and precursors were added to culture at day 8 of cell transfer.

6.2 Results and Discussions

6.2.1 Preliminary Kinetic Study Elicited with 200μM MJ

The role of MJ in secondary product induction has been well established in many plant cell systems. The observation that suspension cell cultures of *Taxus cuspidata* respond well to MJ elicitation was first reported by Mirjalili and Linden (1996). Our studies in Chapter 3 showed the interaction between ethylene (C_2H_4) and MJ played the important role in stimulation of taxol production in *Taxus canadensis*. Suspension cell cultures of

other *Taxus spp* have also been reported to strongly response to elicitation with MJ (Yukimune et al., 1996; Ketchum et al., 1998). In this study, a preliminary kinetic study was conducted to understand the effect of MJ on the kinetic activities of cells in 125 mL Erlenmeyer flasks. Cell suspensions of *Taxus canadensis* (C93AD) were elicited with 200 μM MJ, which was the optimal MJ concentration from experiments given in Chapter 3 on day 8 of the cell cycle and incubated under controlled mixture of gases (6-7 ppm C_2H_4, 15% O_2, 0.5% CO_2) flowing to the headspace.

Sugar utilization

The cell suspensions of *T. candensis* displayed a relatively slow growth rate (about three fold in 21 days). The pattern of sugar utilization is shown in Figure 6.1. Sucrose was rapidly hydrolyzed during the first 12 days and completely hydrolyzed in 15 days. Glucose and fructose were not completely utilized by the cultures during 21 days of growth. The profiles of glucose and fructose followed similar trends however; the concentration of glucose in the system was always less than fructose.

The uptake rate of fructose could be affected by the oxygen supply rate; cells grown at the lower oxygen supply were reportedly unable to utilize fructose (Taticek et al., 1994). This pattern of sugar utilization has been observed using *Taxus* and other plant culture cells (Hirasuna et al., 1996; Wickremesinhe and Arteca, 1994; Srinivasan et al., 1995; Mirjalili and Linden, 1995).

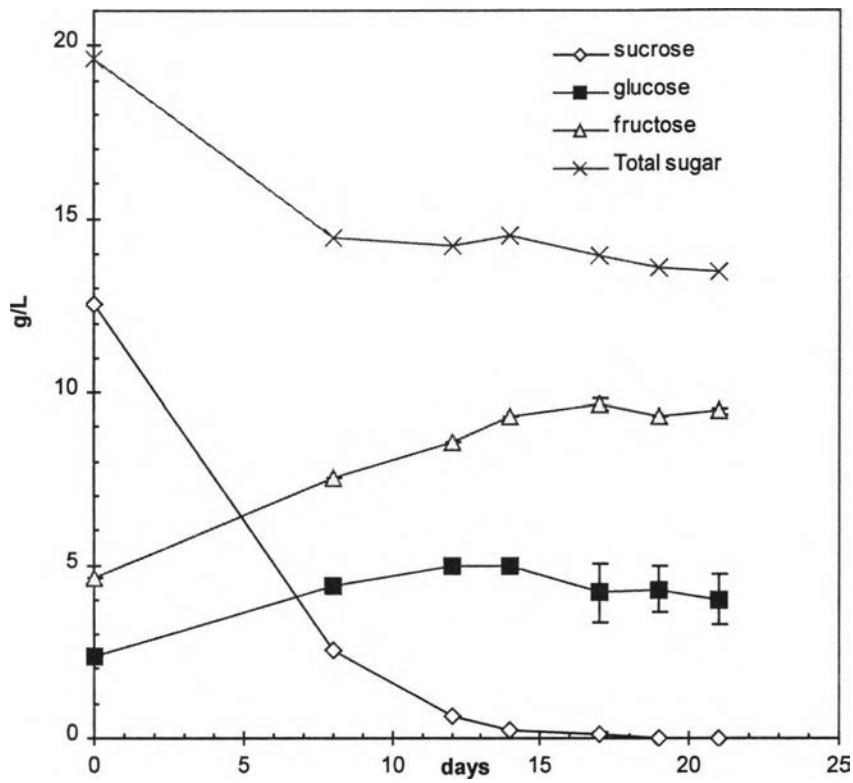


Figure 6.1 :.Sugar profiles of *T. canadensis* (C93AD) in the preliminary kinetic study at 200 uM MJ elicitation under the mixture headspace gas (7 ppm C2H4, 15% O2, 0.5% CO2); MJ was added at day 8 of cell cycle.

Taxanes analysis

The profile of taxanes was shown in Figure 6.2. The greatest concentration of taxanes was found between days 18-21 days (~12 day after elicitation) with 12 mg/L of taxol, 21 mg/L of 10-DAT, 2.5 mg/L of baccatin-III and less than 1 mg/L of 10-DAB. Taxanes started to accumulate at almost a linear rate from day12 (4 days after elicitation) and continued until day 18. Ketchum et al. (1998) observed taxol concentration continues to increase in the *Taxus canadensis* (C93AD) culture up until day 19 or 21 of the culture cycle and began to decline as the cells senesce. The four-day lag phase was also observed by Yukimune et al. (1996). The accumulation of 10-DAB-III, baccatin-III, 10-DAT and taxol trends were analogous to each other (Figure 6.2). However, the relative proportion of 10-DAB and baccatin-III declined from day 12 to 17 and remained constant after that. The 10-DAT proportion increased from day 12 to 14 and slightly decreased after day 17 (Figure 6.3), while the taxol proportion began to increase after day 14 as shown in Figure 6.3. At the end of experiment (17-21 days), the ratio of taxol increased while the ratio of 10-DAT decreased and the ratio of 10-DAB and baccatin-III remained constant; which may imply connections between the metabolites in the pathway from 10-DAT to taxol.

6.2.2 Kinetic Study of the Effect of MJ Concentration

To study the effect of MJ concentration on cell kinetics, the 8 days–old cell suspension culture of *Taxus canadensis* (C93AD) were elicited with different concentrations of MJ and incubated under the same conditions as in section 6.2.1. The cell kinetics of sugar uptake, formation of taxol and other taxanes were compared to non-elicited controls.

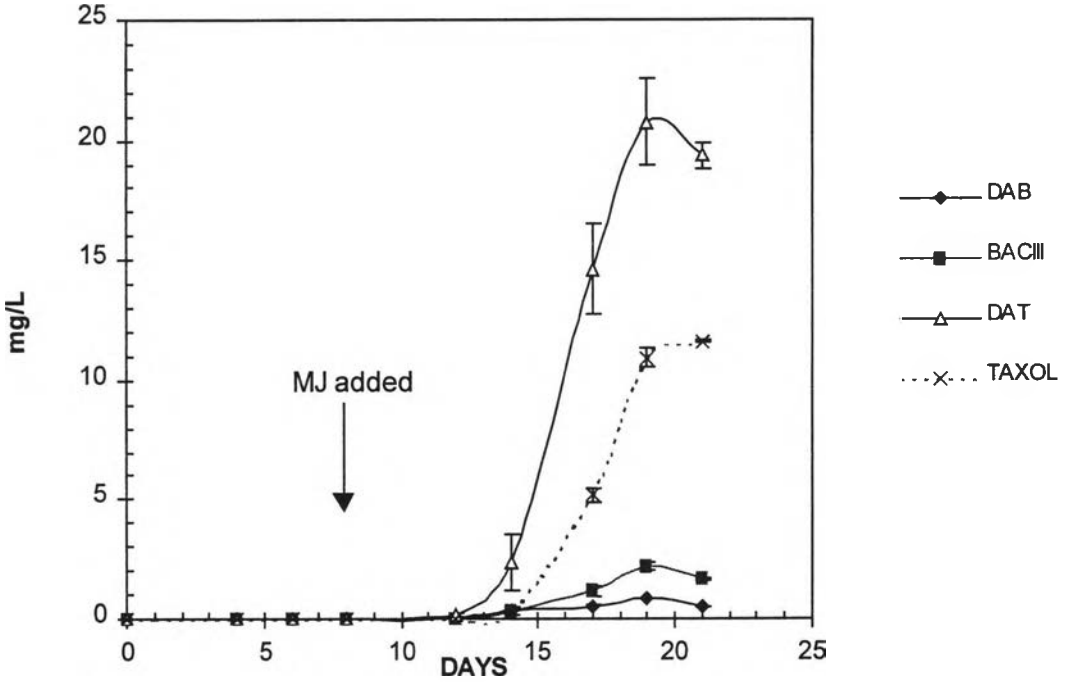


Figure 6.2 Kinetic profiles of taxanes in preliminary study at 200 µm MJ elicitation under mixture of headspace gas (7 ppm C2H4, 15%O2 , 0.5% CO2).

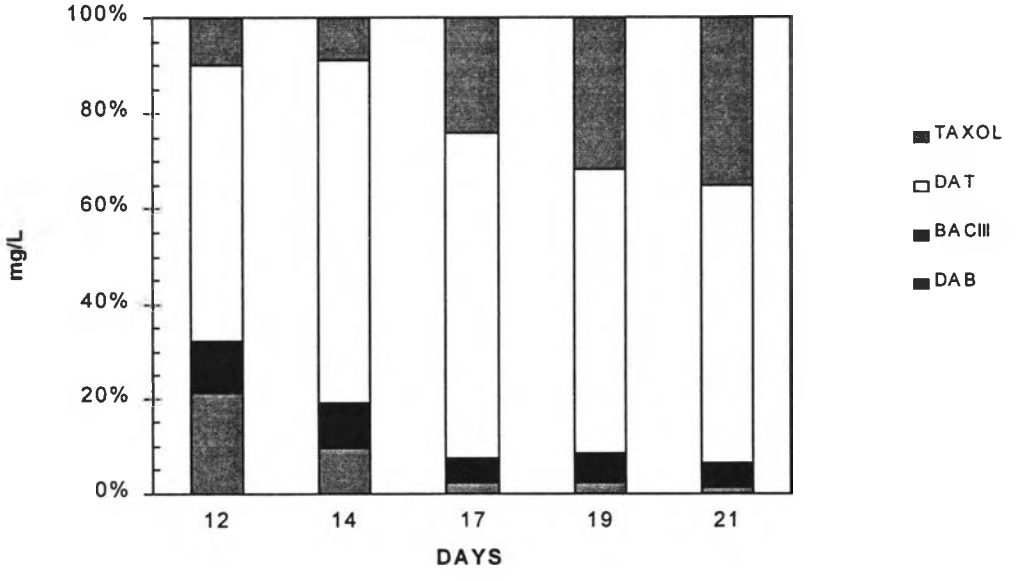


Figure 6.3 The relative proportion of individual taxanes to total observed taxane concentration (10-DAB, baccatin-III, 10-DAT, taxol) at various times in the preliminary study.

Sugar utilization

In all of experiments in this study, sugar utilization exhibited the similar kinetics regardless of MJ level. In general, sucrose was hydrolyzed by the cells to glucose and fructose by 15 days; glucose was consumed more than fructose as shown in Figure 6.4. The glucose and fructose in the medium were not depleted during 21 days of the experimentation.

The patterns of sugar utilization were similar to those from the previous studies; however, the total sugar profiles indicated a lag phase of sugar utilization. The cells began to consume sugar after 10-12 days, whereas in the preliminary study this lag phase was not observed. Subsequently, lower rates of taxol and 10-DAT production were obtained. After 18 days the release of fructose and glucose of the cells were also observed which might be a consequence of cell death.

In this experiment, there was no significant difference of sugar utilization profiles at various elicited MJ concentrations, which may imply no significant effect of MJ on cell growth in this study. Mirjalili and Linden (1995) reported increased culture growth rate when the concentration of MJ increased. Contrary to this, Yukimune et al. (1996) reported cell yield decreased as MJ concentration increased; Ketchum et al. (1998) observed 20% decrease in cell growth between control flasks and flasks elicited with 100 μM MJ.

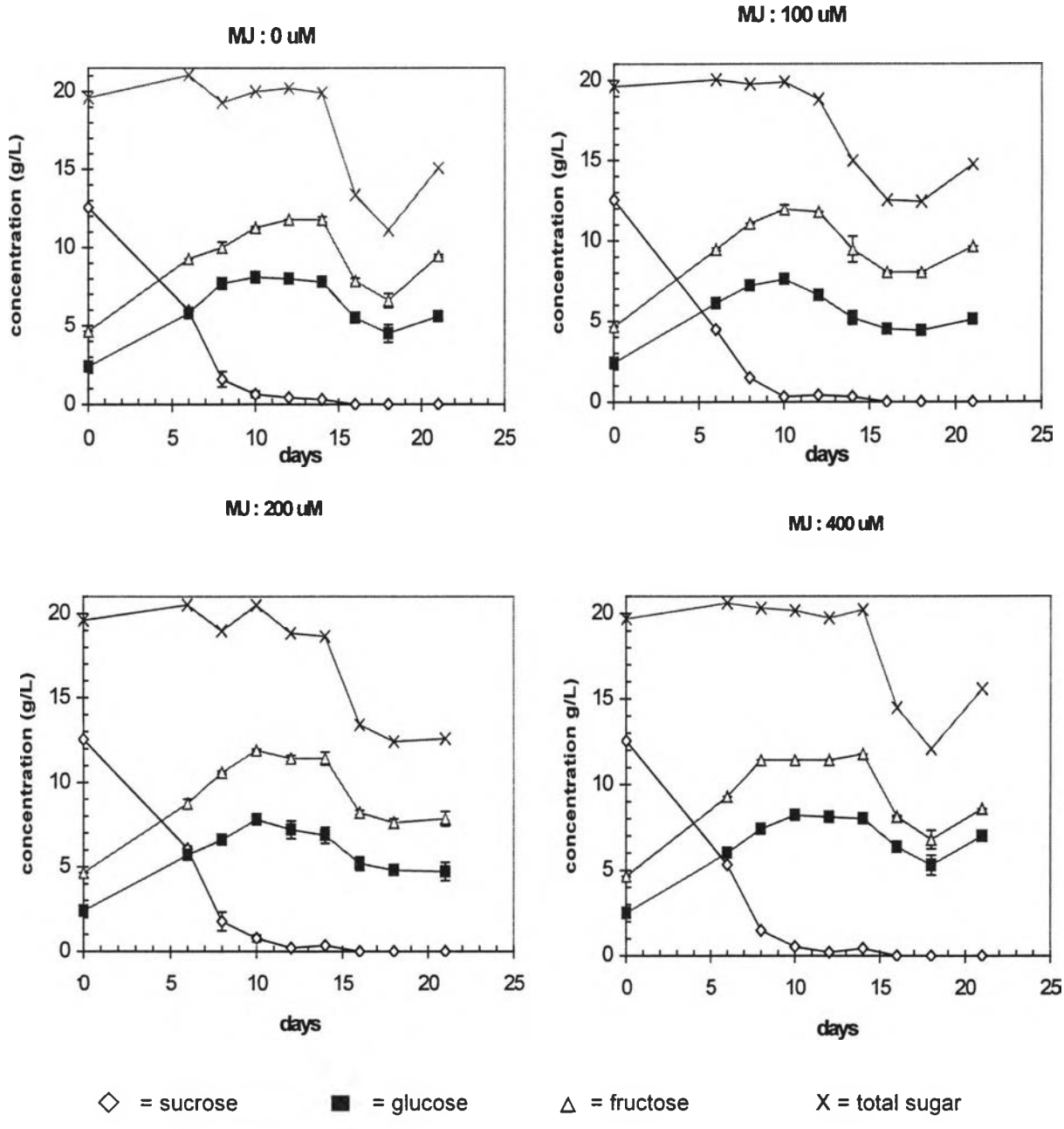


Figure 6.4 The effect of MJ concentration on sugar profiles in media of *T. canadensis*; MJ was added at day 8 of cell cycle.

Taxanes Analysis

The profiles and ratios of taxanes at 0, 100, 200 and 400 μM MJ elicitation are shown in Figure 6.5. Although the same lag phase of taxanes as in the preliminary study was observed, the taxane production rate was less than during the previous study (Section 6.2.1.) and taxanes still continued to accumulate until the last observed day (day 21). The taxanes obtained at day 21 from elicitation with 100 μM MJ maximized at about 17 mg/L 10-DAT, 4 mg/L taxol, 4 mg/L baccatin-III and 1 mg/L 10-DAB.

The relative proportion of individual taxanes to total observed taxanes (10-DAB, baccatin-III, 10-DAT, taxol) at various times in the system after elicitation with 100 μM and 200 μM MJ were similar to results of the preliminary study. The proportion of 10-DAT remained constant or decreased slightly between days 14 to 21 while the taxol ratio increased. Greater taxol ratios were observed when the system was exposed to higher concentrations of MJ (Figure 6.6). On the contrary, without MJ, the ratio of 10-DAT increased from day 14 to 21 whereas the ratio of taxol remained constant. The proportion of taxol increased with MJ concentration as shown in Figure 6.7. This observation was consistent with data of Yukimune et al. (1996) and our previous results in Chapter 3. MJ could promote the immediate pathway to taxol. The maximum ratio of taxol to total observed taxanes (10-DAB, baccatin-III, 10-DAT and taxol) was about 38% by wt. at 200 μM MJ, which was the same value observed in the preliminary study. However, this ratio was less than the ratios observed in *Taxus media* culture by Yukimune et al. (1996), who

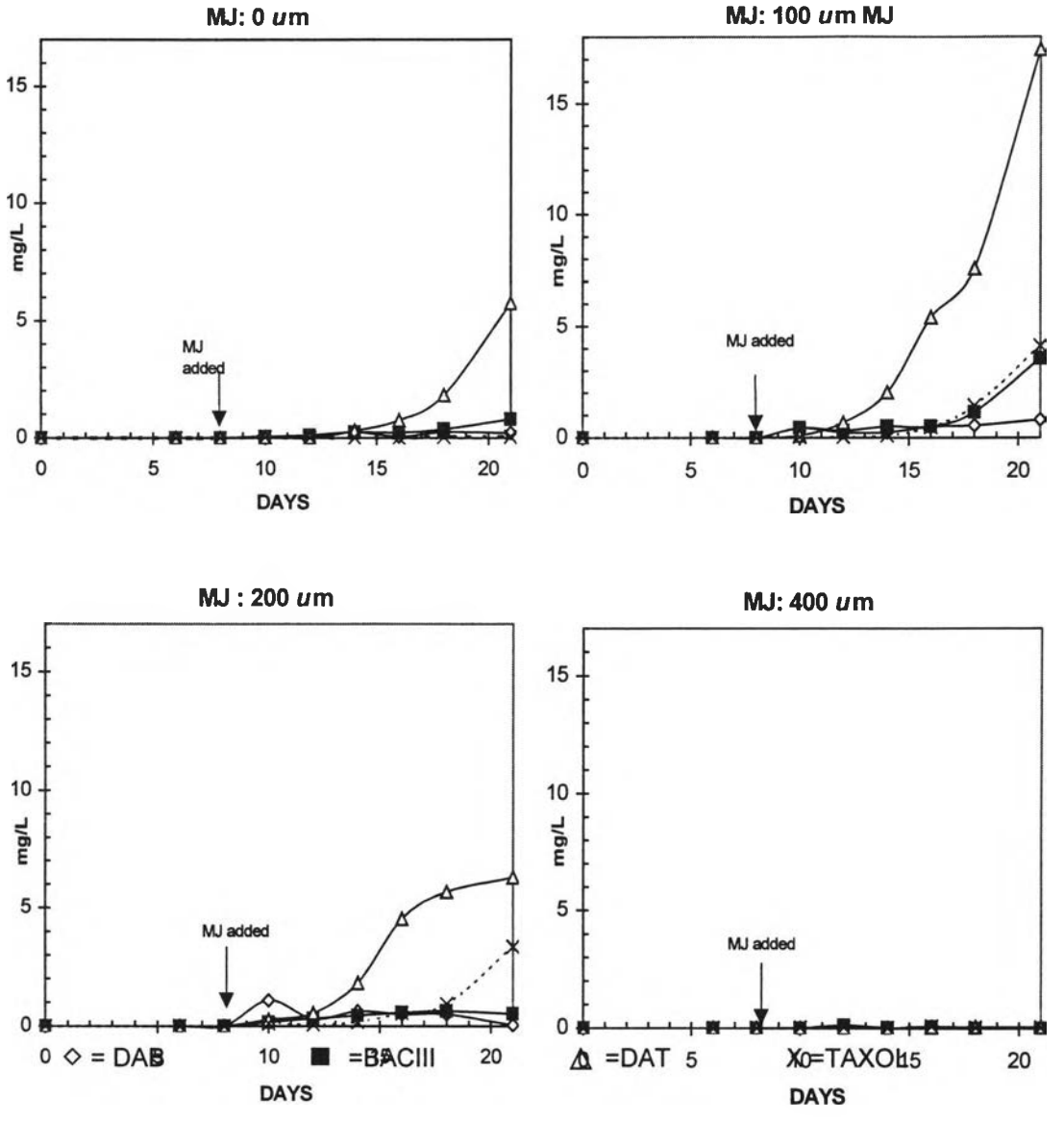


Figure 6.5 The effect of MJ concentration on kinetic profiles of taxanes in cell suspensions of *T.* canadensis under controlled mixture of head space gas; MJ was added at day 8 of cell cycle.

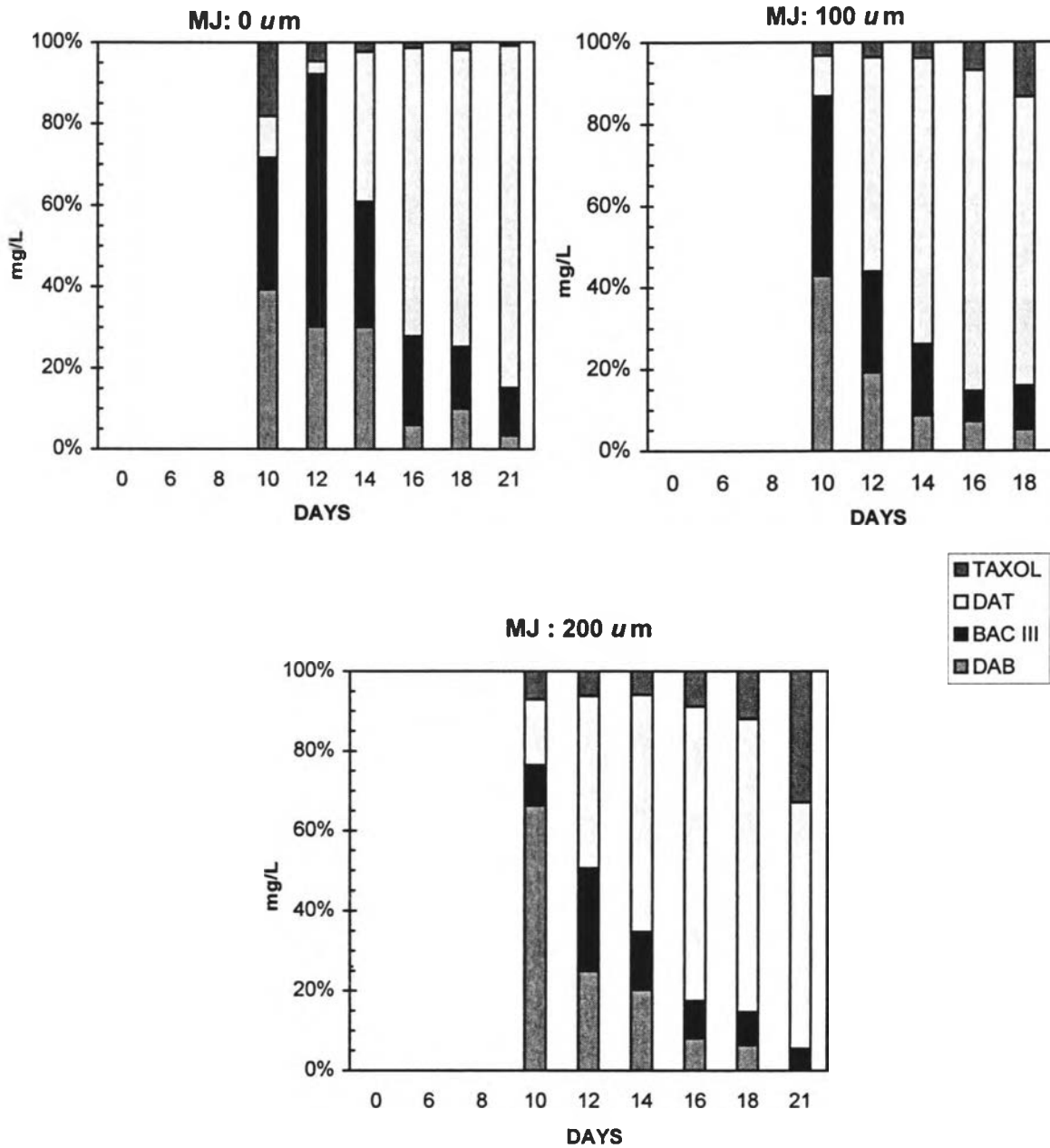


Figure 6.6 The effect of MJ concentration on the relative proportion of indicidual taxanes to total observed taxane concentration (DAB, baccatin-III, DAT, taxol) at various times of incubation.

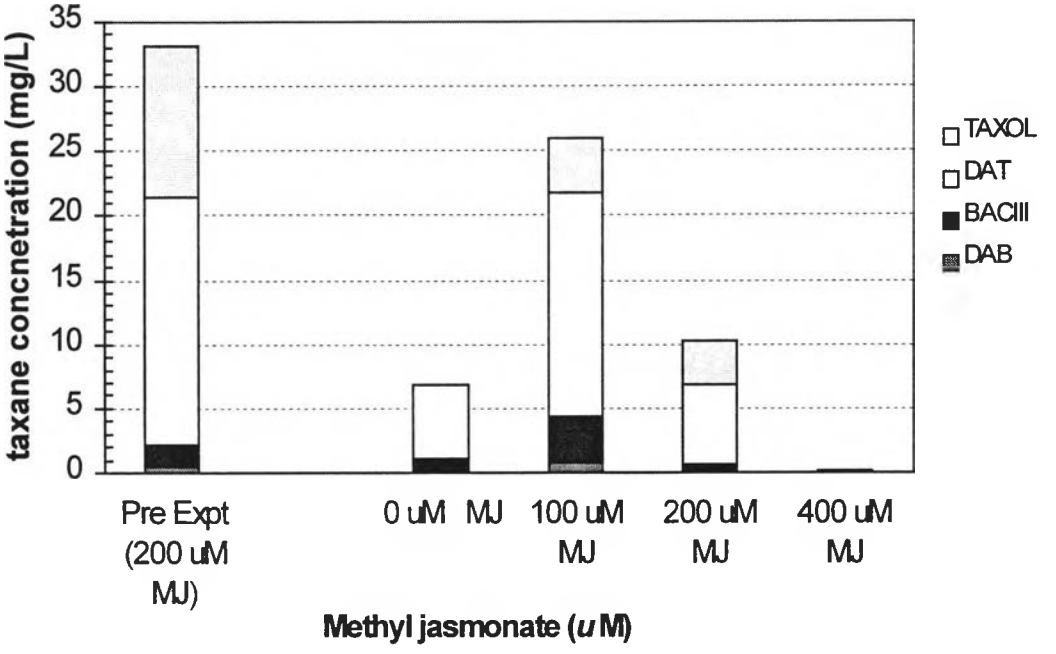


Figure 6.7 Effects of methyl jasmonate (MJ) on taxane amounts in cell suspension cultures of *T.canadensis* (C93AD) after 21 days of incubation under controlled mixture of headspace gas.

reported that taxol contributed about 68% (by MW) of the total taxanes. Part of the difference may come from the different of cell lines, culture conditions, and the source of product (we analyzed extracellular taxol, while Yukimune et al. analyzed intracellular taxol). Although the ratio of taxol at 200 μM MJ elicitation was almost same as in the preliminary study, the maximum concentration of extracellular taxol was only one-third of the previous one. The optimal concentration of MJ used for elicitation declined from 200 μM from the static study in Chapter 3 to 100 μM in this study. From the other reports, the optimal concentration of MJ for elicitation of taxol was 10 μM in *T.cuspidata* (Mirjalili and Linden, 1996), 100 μM in *T. media* (Yukimune et al., 1996) and 200 μM in *T. canadensis* (C93AD) (Ketchum et al., 1998).

The inhibition effect of MJ on cell production was observed at concentrations of 200 μM or more. At 400 μM MJ, the ability of the cells to produce taxol and 10-DAT declined dramatically, even to less than the values of the non-elicited system. The sugar utilization pattern was similar to that of other treatments. The results compared to the preliminary study showed obvious changes in cell sensitivity to MJ and the ability to produce taxanes. Both may be due to problems of inherent genetic or epigenetic instability of this cell line. Since 10-DAT accumulated in larger amounts relative to taxol and other taxanes; the ratio of 10-DAT to taxol was about six, and its conversion to taxol may be one of the rate limiting steps.

6.2.3 Kinetic Study of Precursor Supplementation with MJ and N- acetylchitohexaose Elicitation.

According to the studies in Chapter 5, taxol yield could be possibly enhanced by supplementation of side chain precursors together with the elicitation. The examination of taxane profiles in some cell lines (a larger amount of baccatin-III and 10-DAB compared to taxol and cephalomannine) indicated the occurrence of a metabolic block at the point of side chain addition (Fett-Neto and DiCosmo, 1996). This was also characteristic of our *T. canadensis* (C93AD) cultures that were not elicited with MJ. From our studies in the systems elicited with MJ, 10-DAT has been isolated from cell suspension medium at a larger amount relative to taxol or other taxanes. 10-DAT and taxol share the same basic structure with N-benzoyl-L-phenyl-isoserine (taxol side chain); the only difference is the degree with which hydroxyl groups have been acetylated (Figure 1.2). In parallel, 10-DAB and baccatin-III share the same basic structures without the side chain. The study by Zocher et al. (1996) demonstrated the ability to convert 10-DAB into baccatin-III using acetyl-CoA by extracts of crude cell homogenates of *T. cuspidata*. Analogous to this, the acetylation at the C-10 position of 10-DAT to be converted into taxol could possibly occur in a similar way. Therefore, we set up the kinetic studies to investigate the effects of N-benzoyl- L-phenylisoserine (taxol side chain) and acetyl CoA on elicited cultures of *T. canadensis* (C93AD). The experimental design is shown in Table 6.1.

Cell growth

Figures 6.8 and 6.9 represent the cell dry weight profiles and sugar uptake kinetics when the C93AD cell cultures were exposed to various treatments as given in Table 6.1.

The profiles of growth kinetics without MJ elicitation (expt. A and B) showed slight biphasic pattern. Slow growth rates (0.20-0.27 g dry wt./L day) occurred between 0 and 11 days of the cell culture cycle. Slow growth possibly was due to the stress of being transferred into fresh medium; the cultures might have had to adjust to the new environment during this period.

After the initial slow growth phase, the culture growth rate increased to 0.46-0.50 g dry wt./L day at least until day 16 where the growth rate decreased again due to the lack of some nutrients or the high concentration of toxic products in the system. This pattern of cell growth was also reported by Srinivasan et al. (1996) and Hezari et al. (1997). Dissimilar to the system without MJ, after day 11 the culture growth rate in the system with MJ (Figure 6.8C) dramatically declined to 0.04 g dry wt/ L day or about 1 tenth of the value observed in non-elicited systems. At day 23 of culture cycle, the cell dry weight in the system without MJ was about 10 g/L, while the cell concentration in the system exposed to MJ was about 4.5 g/L. No significant difference of cell growth rate was observed with the addition N-acetylchitohexaose, acetyl CoA or benzoyl-3-phenylisoserine (Figure 6.8 E-G).

According to the experimental results, there was a strong inhibition effect of 100 μM MJ on cell growth. Cell concentrations at day 23 decreased by 65-67% in all five systems elicited by 100 μM MJ. Mirjalili and Linden (1995) reported the increase of growth rate of the cultures *T. cuspidata* from 0.15 day^{-1} to about 0.22 day^{-1} at 100 μM MJ. Yukimune et al. (1996) and Ketchum et al. (1998) reported decreased of cell growth by 22% in *T. media* and 20% in *T. canadensis,* respectively. The differences of the effect of MJ on cell

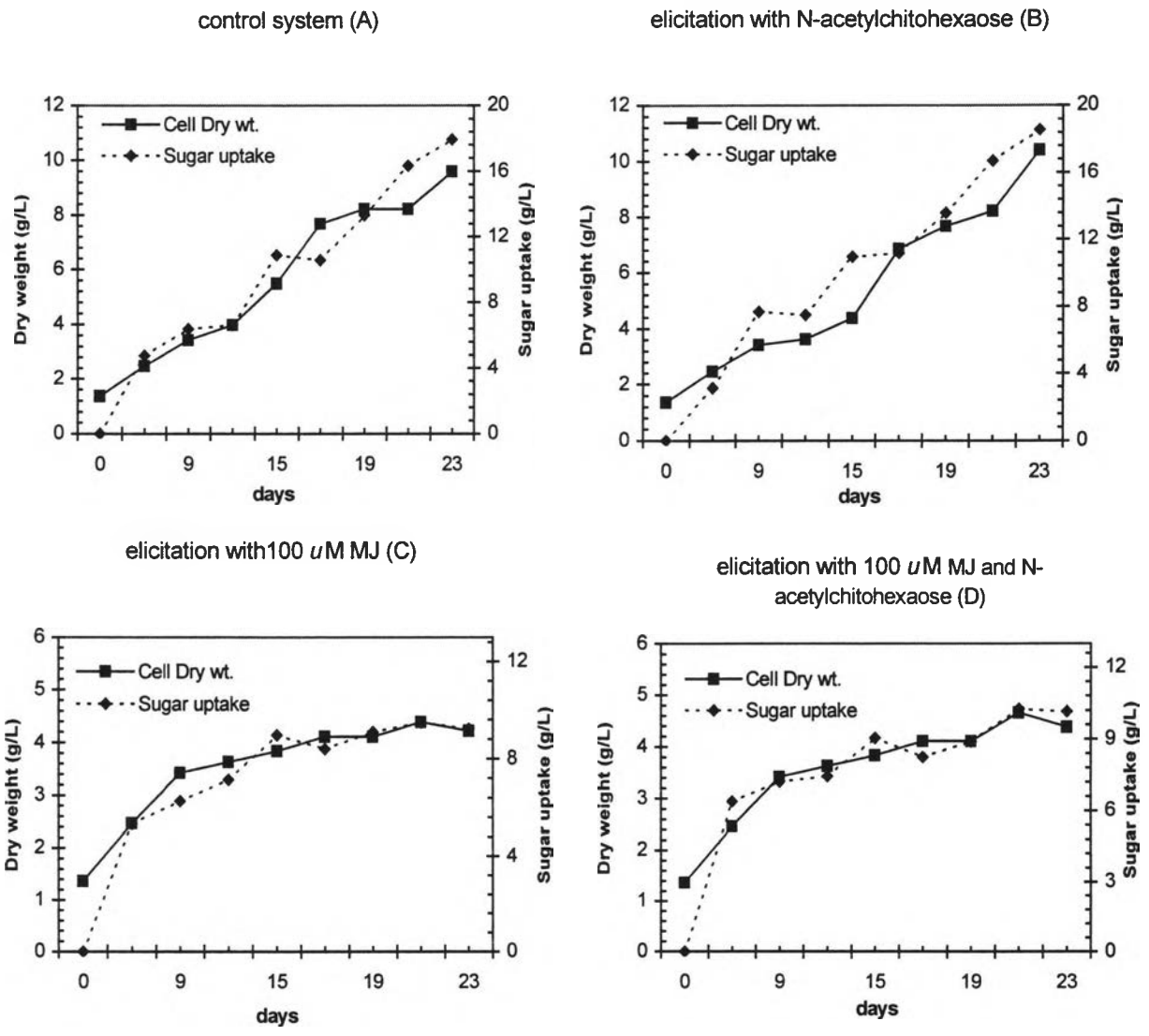


Figure 6.8 Effects of elicitors on kinetic profiles of growth and total sugar uptake of *T. canadensis* (C93AD); elicitors were added at day 9 of cell cycle.

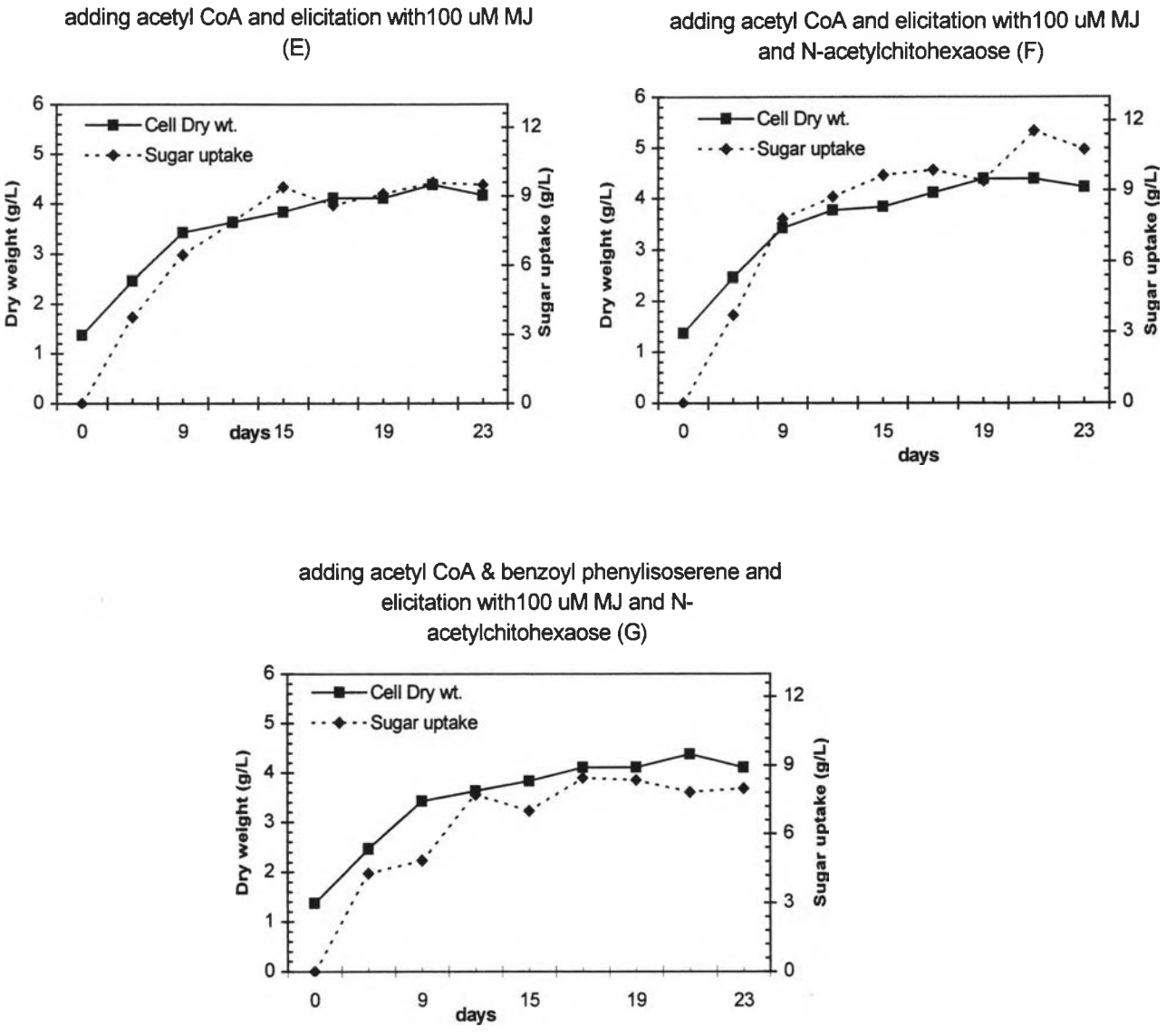


Figure 6.8 Effects of precursors and elicitors on kinetic profiles of growth and total sugar uptake of *T.canadensis* (C93AD); precursors and elicitors were added at day 9 of cell cycle.

growth may due to differences in plant species and/or culture conditions during the experiment.

Sugar Utilization

The patterns of sugar utilization in the studies above were similar to those in the preliminary study (6A). A more detailed inspection of the sugar utilization is seen in Figures 6.9(A-D) and Figures 6.10 (E-G). In the systems without MJ (Figures 6.10 A and B), sucrose was hydrolyzed by the cells to glucose and fructose by 12 days; glucose was consumed more than fructose. Glucose in the medium was depleted within 21 days while fructose still remained (about 2 g/L) during 23 days of the incubation. The total of sugar utilization in non-elicited cultures was about 19g in 23 days or 0.83 g day^{-1}.

In all systems exposed to 100μM MJ, the patterns of sugar utilization all resembled one another. In general, sucrose was hydrolyzed by the cells to glucose and fructose by 12 days; glucose was consumed more than fructose. However, glucose and fructose in the medium were not depleted during 23 days. In most cases (A to G), the sugar uptake patterns were similar until day 12 (3 day after MJ elicitation), after which the sugar uptake rate declined in all systems exposed to 100 μM MJ. The overall sugar uptake in MJ elicited systems was about 9 g/L in 23 days or 0.40 g day^{-1} (about 45% less than the non-elicited systems). The trends of total sugar utilization in both elicited and non-elicited systems were found analogous to cell dry weights as shown in Figure 6.8. Therefore, the sugar uptake pattern may represent the growth pattern in the system.

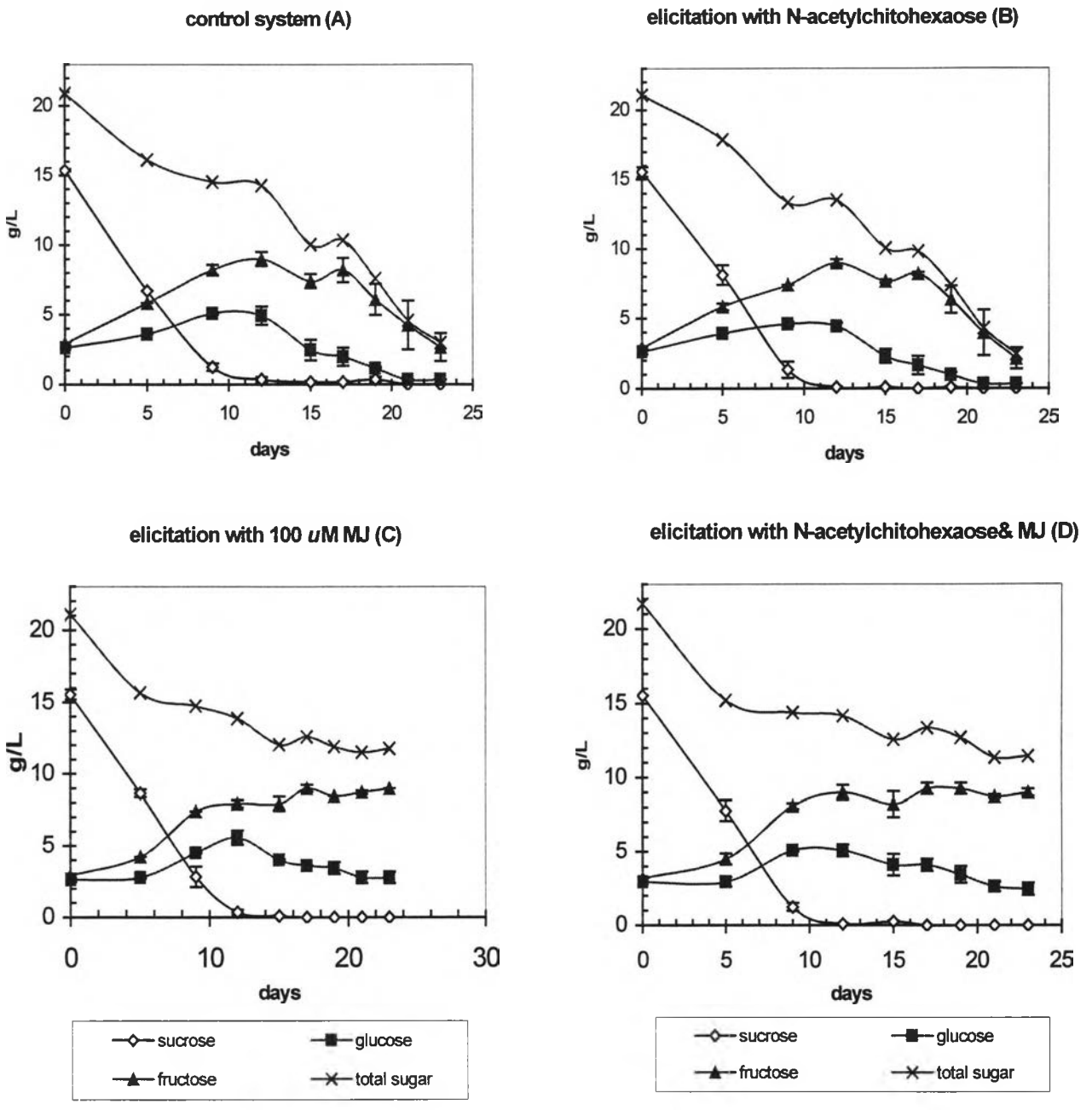


Figure 6.9 Effects of elicitors on sugar profiles of *T. canadensis* (C93AD); elicitors were added at day 9 of cell cycle.

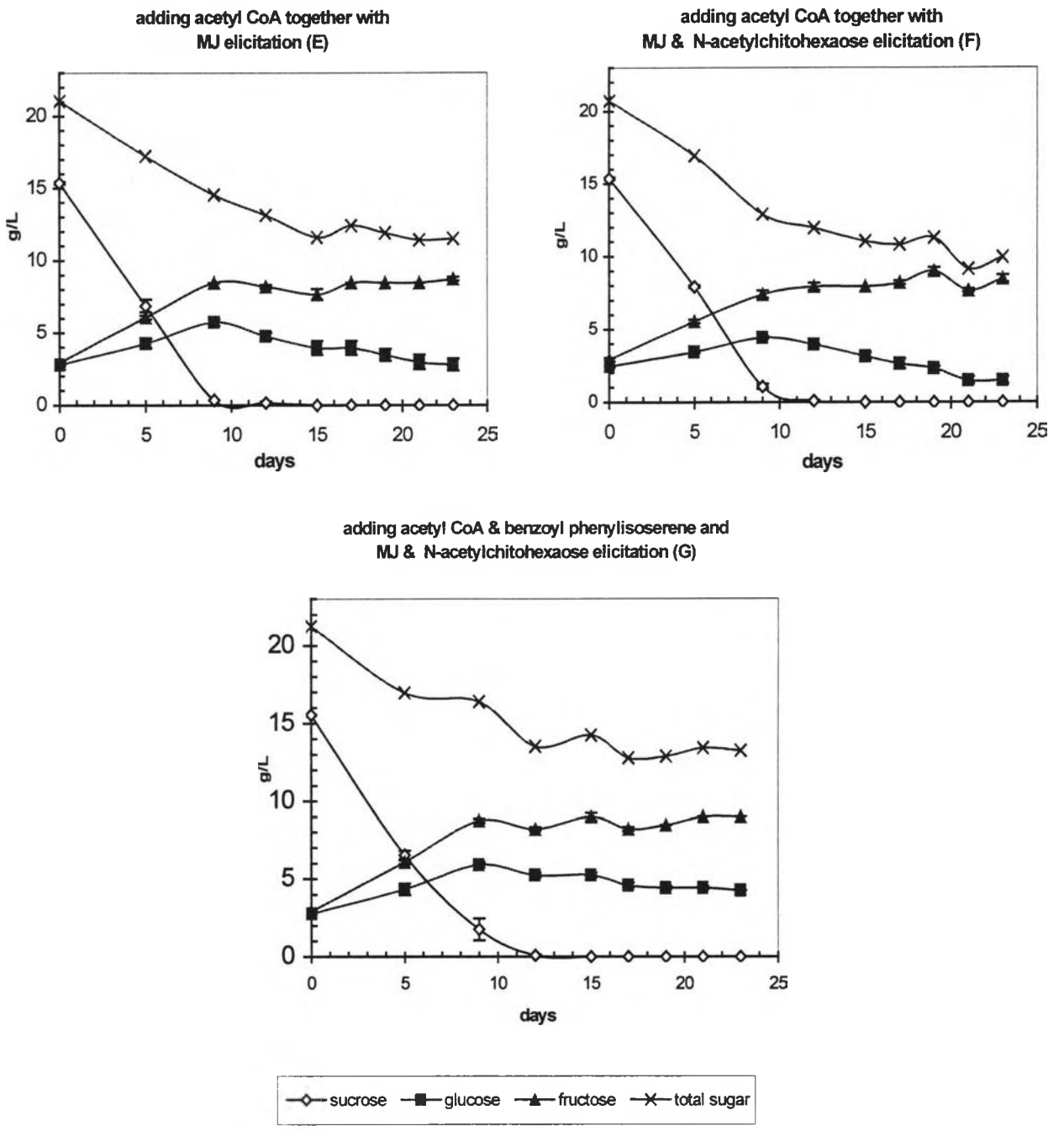


Figure 6.10 Effects of precursors and elicitors on sugar profiles of *T. canadensis* (C93AD); precursors and elicitors were added at day 9 of cell cycle.

Taxanes analysis

The kinetics of taxane production in the different treatments given in the experimental design (Table 6.1) are shown in Figure 6.11-6.17. Since the air compressor was down, elicitors and precursors were added and the control of headspace gas mixture was started on day 9 of the culture cycle instead of day 8 as in the previous studies. In the control experiment without any elicitor or precursor supplement (Figure 6.11), only slight taxane production was observed. 10-DAB (1.7 mg/L) was the only taxane, which was obtained at higher concentrations in non-elicited systems than in the elicited systems. The prominence of 10-DAB in non-MJ elicited system was also observed in the previous studies (Chapters 3 and 4).

Effect of N-acetylchitohexaose

Oligosaccharide elicitors have been found to be effective inducers of various defense responses in plants. Addition of oligosaccharides to plant cell cultures mimics the effects of elicitation from some pathogenic microbes. The addition of cell extracts and cultures filtrates of fungal cultures stimulated taxol and other taxanes in *Taxus sp. (*RO1-M28) (Srinivasan et al., 1995). According to our studies in Chapter 5, a dramatic increase of taxol and taxane products was obtained by the non-productive culture when the culture was elicited with 100 μM MJ and 0.5 μM N-acetylchitohexaose without supplementation of ethylene. The effects of the co-mediation between MJ and the oligosaccharide were reexamined in the kinetic studies.

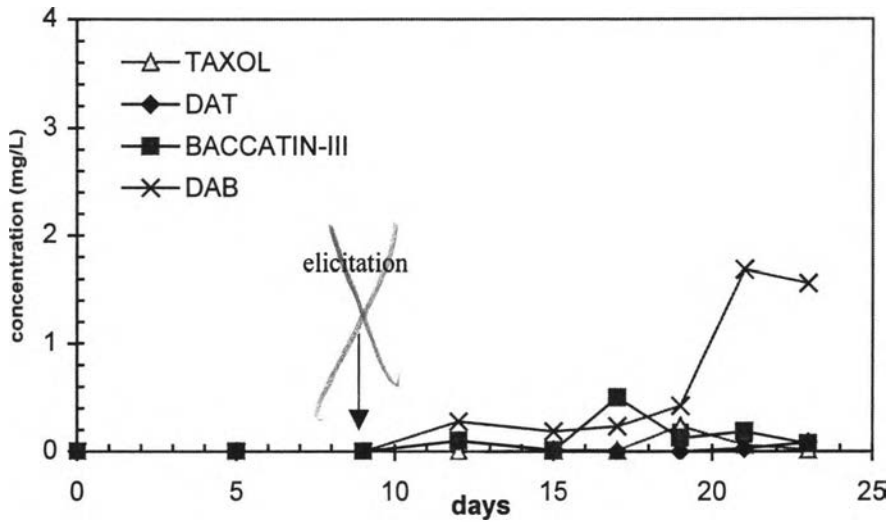


Figure 6.11 Kinetic profiles of taxane production of *T. canadensis* in the control system (expt. A).

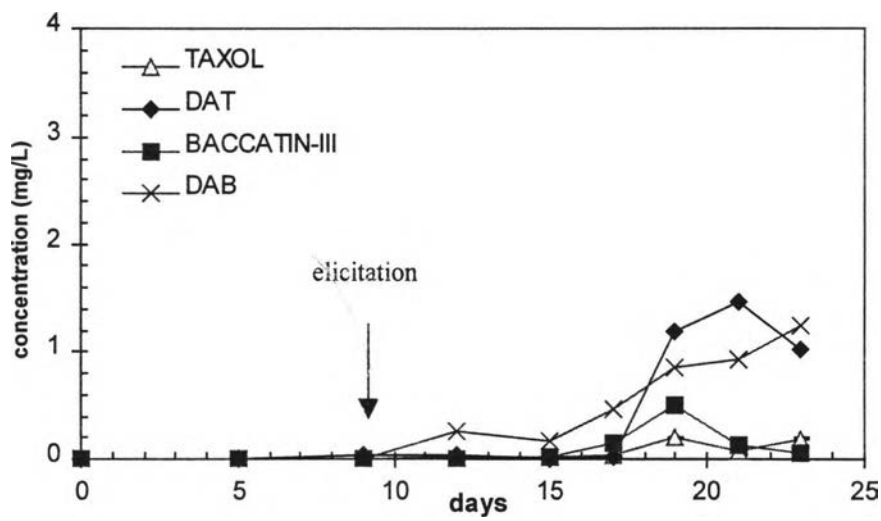


Figure 6.12 Kinetic profiles of taxane production of *T. canadensis* in the system elicited with N-acetylchitohexaose 0.5 μM (expt. B).

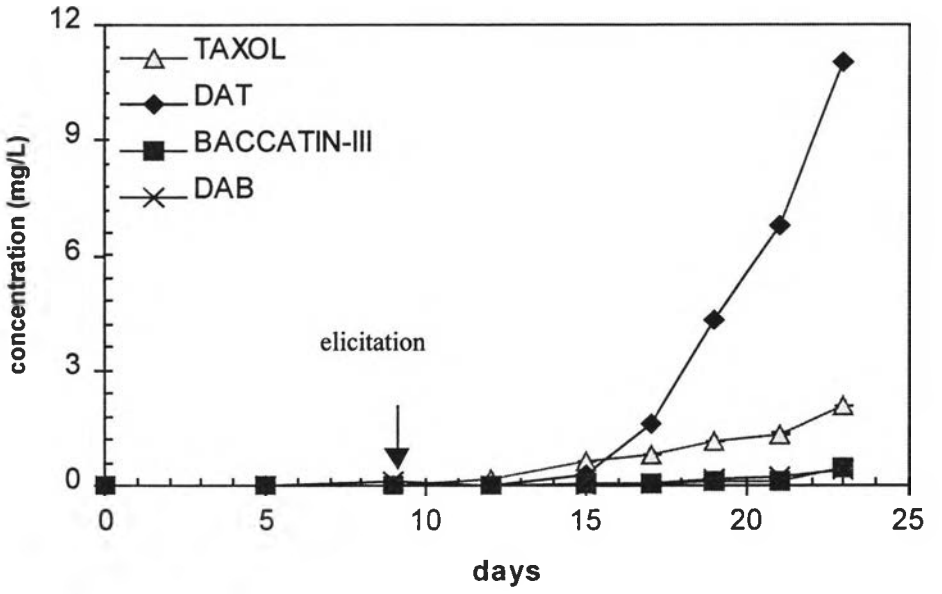


Figure 6.13 Kinetic profiles of taxane production of *T. canadensis* in the system elicited with 100 μM MJ (expt.C).

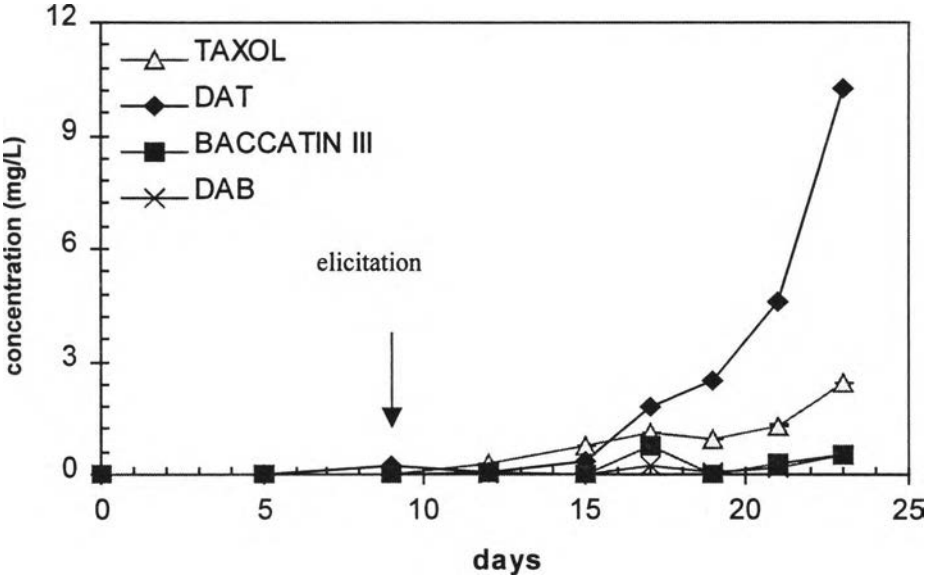


Figure 6.14 Kinetic profiles of taxane production of *T. canadensis* in the system elicited with 100 μM MJ and 0.5 μM N-acetylchito-hexaose (expt.D).

The results in the systems elicited with 0.5 μM N-acetylchitohexaose only (Figure 6.12) showed an increase of taxol and 10-DAT, and a decrease of 10-DAB relative to control. However, the concentrations of taxanes in systems without elicitation of MJ were very low. Elicitation with MJ promoted taxol and 10-DAT more than 30 fold compared to the control as shown in Figure 6.13. Slight increases of taxol (by 10-17%) and decreases of 10-DAT (by 10%) were observed in the systems elicited with MJ and N-acetylchitohexaose (Figure 6.14) compared to the system elicited with only MJ.

The role of co-mediation among MJ, N-acetylchitoohexaose and ethylene in stimulation of taxol production was difficult to understand, especially in the system using unstable cell line. Experiments using this oligosaccharide, in which ethylene was not supplied to the headspace and in which the closure on the growth vessels are not tight, demonstrate remarkably better co-mediated elicitation of taxane production relative to controls (Chapter 4). Therefore, ethylene appeared to result in efficient co-mediation of elicitation. However, the differences may also be from the instability of this cell line or the change of culture conditions.

Effect of acetyl CoA

In our elicitation studies, we always observed the predominance of deacetyltaxol (10-DAT) accumulation in culture medium compared to other taxanes. Assay of taxane compounds from *T. brevifolia* bark extract by Richheimer et al. (1992) also reported the highest amount of deacetyltaxol compared to other taxanes. However, acetylation of the C-10 position to convert from 10-DAT into taxol should occur intracellularly, probably

analogous to acetylation of 10-DAB to baccatin-III as demonstrated using acetyl-CoA by extract of crude cell homogenates of *T. cuspidata* (Fett-Neto and DiCosmo, 1996). Strobel et al. (1992) reported that radiolabeled acetate was incorporated uniformly in labeled taxol. Therefore, supplementation of acetyl-CoA together with MJ elicitation could be another way to improve taxol yields.

In the following kinetic studies, we added MJ, acetyl CoA, and N-acetylchitohexaose according to the design of experiments E, F, G in Table 6.1. Taxol was increased by 40-75% in all 3 systems treated with acetyl-CoA (Figure 6.15-6.17). The ratios of taxol to 10-DAT in the systems with 0.02 mM acetyl CoA supplementation were higher than in the systems without acetyl CoA, except for the system supplied with acetyl CoA together with the side chain precursor (Figure 6.17). According to the report by Hirasuna et. al. (1996), addition of 1.0 to10 mM acetate in non producing *T. brevifolia* (PC2) cultures increased the level of taxol from 0.082 mg/L to 0.12 and 0.17mg/L respectively, but addition of 50 mM acetate was detrimental to the cells and turned the cultures brown.

Again, we observed increases of ratios of taxol to 10-DAT when elicitation with N-acetylchitohexaose and MJ. Contrary to the previous studies, decline of taxol to 10-DAT ratios from day 17 to day 23 in all treatments and lower taxane production were observed as shown in Figure (6.18). The decline of taxol to 10-DAT ratios may be a consequence of the limitation of acetyl Co-enzymeA. The difference of the results may be due to the difference of time of adding MJ (day 9 instead of day 8) or/and the difference of culture conditions.

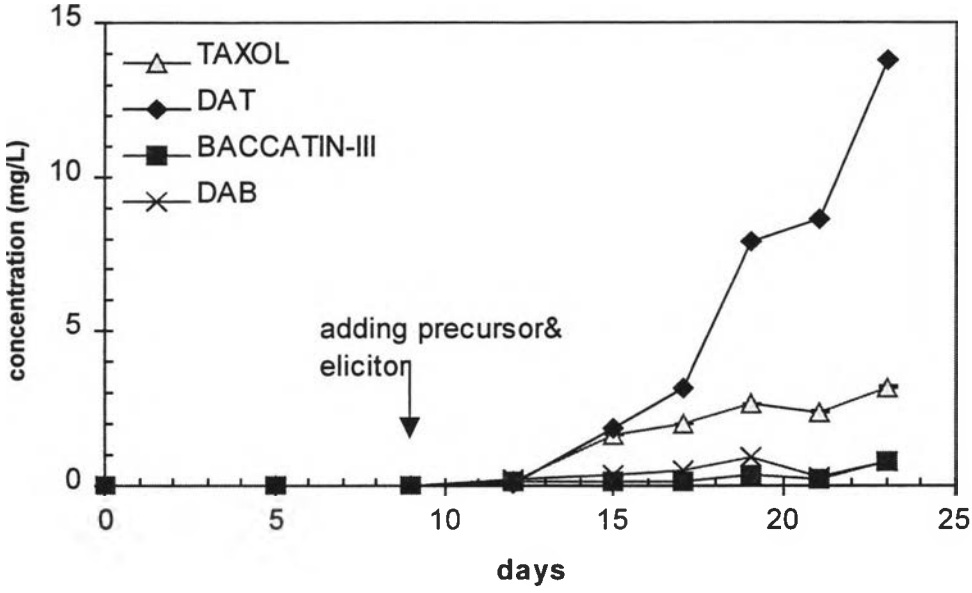


Figure 6.15 Kinetic profiles of taxane production of *T. canadensis* in the systemadding acetyl CoA and 100 μM MJ (expt.E).

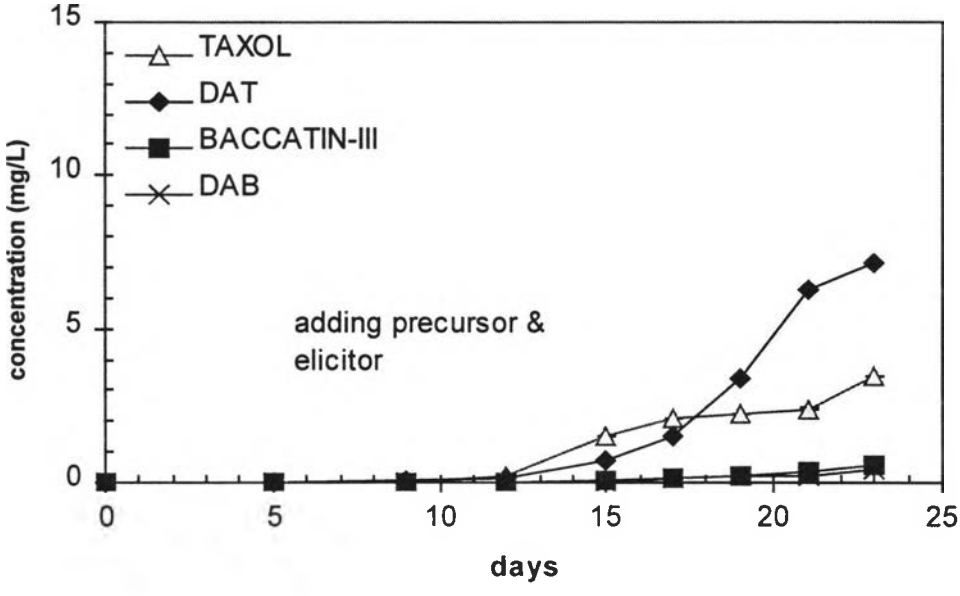


Figure 6.16 Kinetic profiles of taxane production of *T. canadensis* in the system adding acetyl CoA and elicitation with 100 μM MJ and 0.5 μM N-acetylchitohexaose (expt.F).

Effect of N-benzoyl-L-phenyl-isoserine

The maximum of taxol was observed in the system with supplementation of 0.02 mM acetyl CoA and 0.02 mM N-benzoyl-L-phenyl-isoserine together with MJ and N-acetylchitohexaose elicitation (Figure 6.17). In this system we obtained 3.5 mg/L of taxol (1.75 fold relative to MJ elicited system and more than 30 fold relative to non-elicited system) and 23 mg/L of 10-DAT (2.15 fold relative to MJ elicited system and more than 40 fold relative to non-elicited system).

Since we observed the distinct increase of 10-DAT compared to taxol by addition of the side chain precursor, N-benzoyl-L-phenyl-isoserine could involve in the pathway to 10-DAT. Combined with the results that the taxol level increased significantly by addition of acetyl CoA, the results supported our hypothesis regarding the taxol synthesis pathway. The non-side-chain taxanes (could be 10-DAB or another unknown intermediate) may be converted into 10-DAT by attaching the side-chain and further converted into taxol by acetylation at the C-10 position.

Different profiles of 10-DAB between unelicited systems and MJ elicited systems and high formation of 10-DAT in MJ elicited system implied MJ may involve in signal transduction to stimulate the pathway from 10-DAB or the unknown intermediate into 10-DAT and taxol respectively. The increase of baccatin-III with 10-DAB concentration and taxol concentration may imply parallel pathways between 10-DAB to baccatin-III and taxol to baccatin-III. As discussed in previous studies (Chapter 4) and according to the observation by Shuler's group (Srinivasan et al., 1996) and Ketchum et al. (1998), baccatin-III might not be the (only) immediate precursor of taxol; it may be a degradation product of taxol.

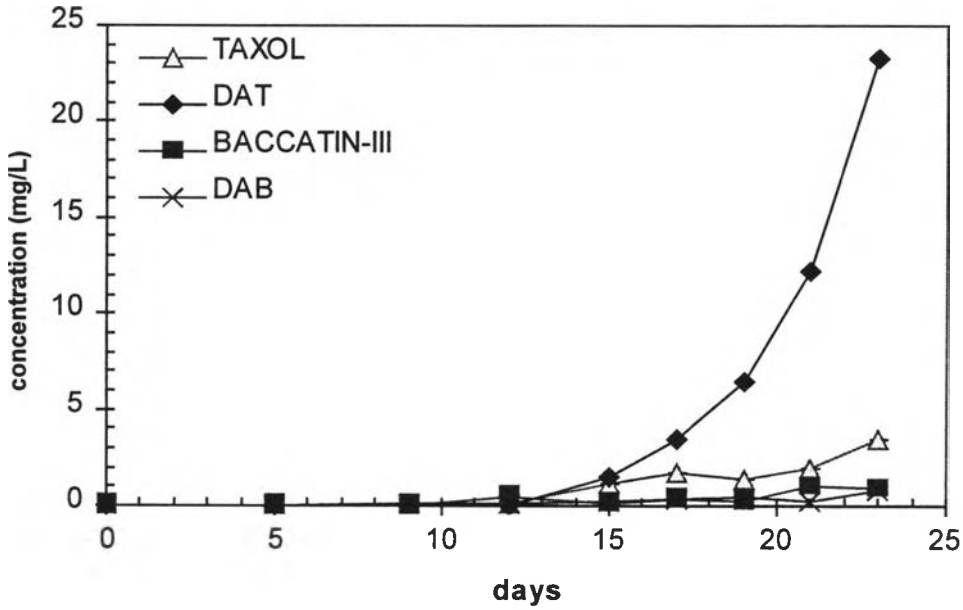


Figure 6.17 Kinetic profiles of taxane production of *T.canadensis* in the system adding acetyl CoA and N-benzoyl-L-phenylisoserine and elicitation with 100 μM MJ and 0.5 μM N-acetylchitohexaose (expt.G).

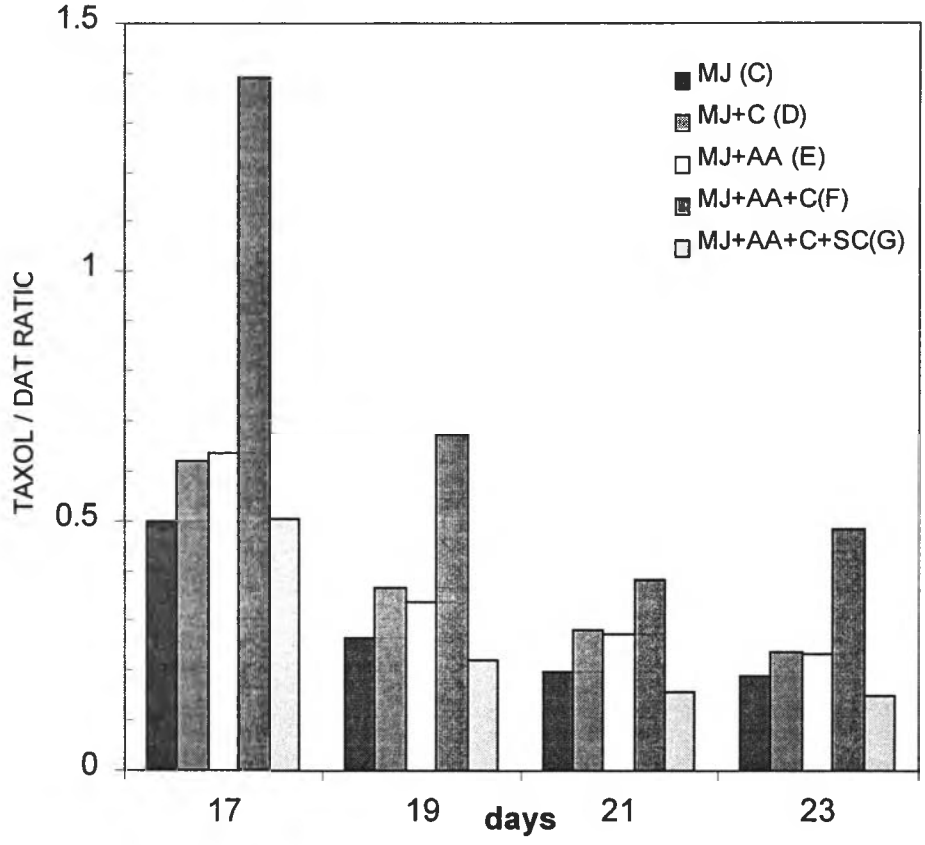


Figure 6.18 Effects of precursor supplementation and elicitation as in Table 6.1 on taxol to 10-DAT ratios in cell suspension cultures of *T. canadensis* (C93AD).

The comparison showing the effects of precursor supplementation and elicitation as given in Table 6.1 on taxane amounts after 23 days of incubation was presented in Figure 6.19. The overall results of cell growth, sugar uptake and formation of taxol and other taxanes in all treatments was summarized in Table 6.2. The growth yield dropped from approximate 0.5 in treatments without MJ to 0.3 in treatments with MJ. The precursors and intermediates did not improve Yx/s, but did significantly increase 10-DAT and taxol levels. 10-DAB decreased upon MJ elicitation. On the other hand, product yields Yp/x and Yp/s improved with MJ elicitation, and were better without chitohexaose elicitation. Since taxol/10-DAT ratios were better with chitohexaose elicitation, metabolic energy might be used to acetylate 10-DAT. Product yields approximately doubled when the taxol side chain, N-benzoyl-L-phenylisoserine, was included.

Table 6.2 The overall comparison of the kinetic activities at different treatments (as in Table 6.1) of *Taxus canadensis* (C93AD) suspension culture after 23 days of cell cycle.

TREATMENTS	Cell dry wt. (g/L)	Sugar uptake (g/L)	Yp/x (10^{-3})	Yp/s (10^{-3})	Yx/s	Taxane formation (mg/L)			
						10-DAB	BACIII	10-DAT	TAXOL
A (control)	9.59	17.93	0.21	0.10	0.46	1.56	0.08	0.09	0.02
B (C)	10.41	18.56	0.28	0.14	0.48	1.25	0.06	1.03	0.19
C(MJ)	4.21	9.22	4.85	1.50	0.31	0.37	0.44	11.00	2.06
D (MJ +C)	4.38	10.15	4.51	1.35	0.30	0.54	0.50	10.25	2.45
E (MJ+AA)	4.16	9.48	6.73	1.95	0.29	0.78	0.79	13.75	3.19
F (MJ+AA+C)	4.22	10.76	4.00	1.08	0.27	0.47	0.56	7.13	3.44
G(MJ+C+AA+SC)	4.11	7.98	10.49	3.57	0.34	0.78	0.93	23.25	3.50

* p = (10-DAB+BACIII+10-DAT+TAXOL), x =cell dry wt., s = sugar uptake

** C= N-acetylchitohexaose, MJ = methyl jasmonate, AA = acetyl CoA, SC= N-benzoyl-L-phenyl-isoserine (taxol side chain)

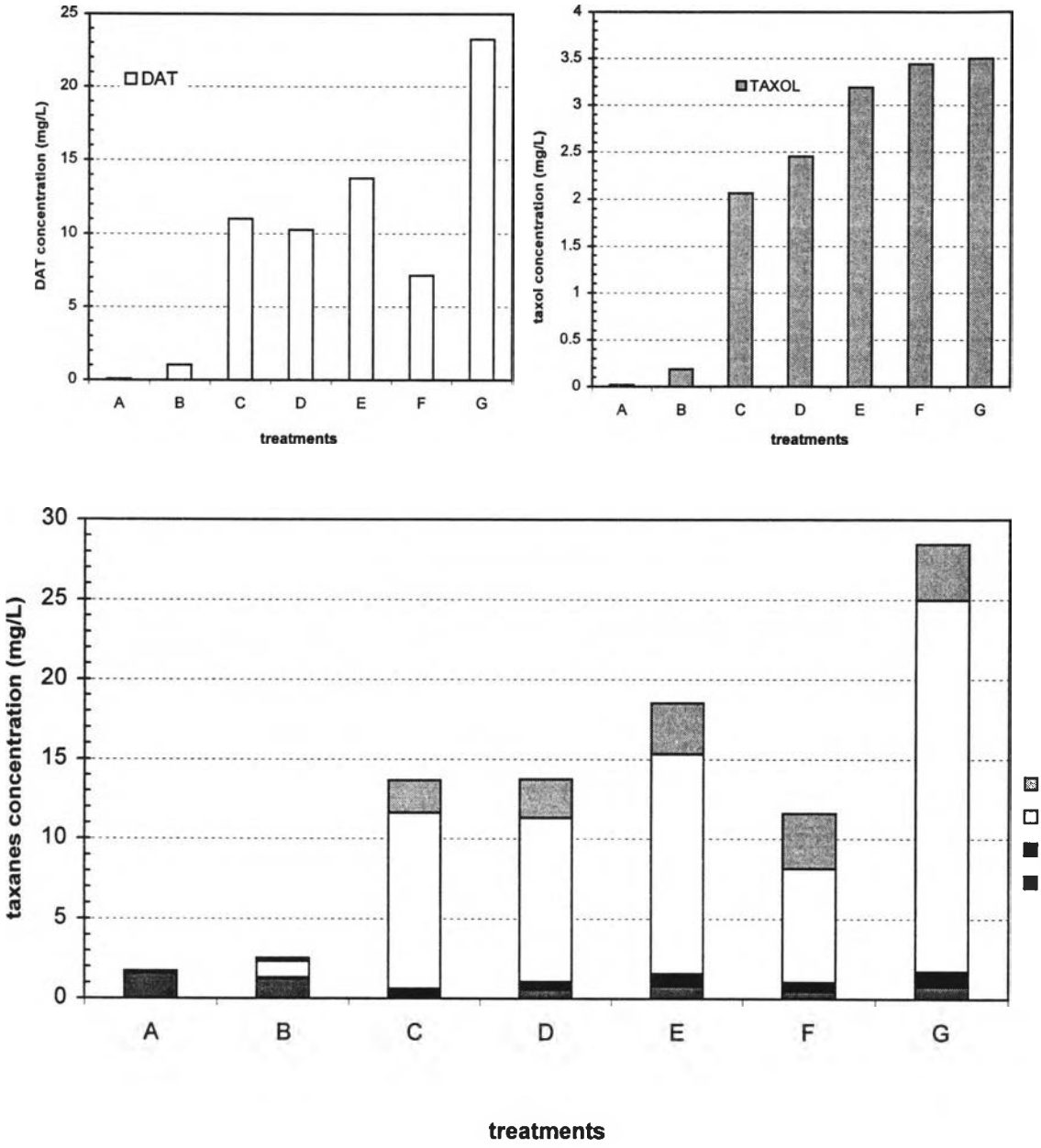


Figure 6.19 Effects of precursor supplements and elicitation as in table 6.1 on taxanes amounts in cell suspension cultures of *T.canadensis* (C93AD) after 23days of incubation; elicitor and precursor were added at day 9.

Stability of taxol production

From the taxane profiles, although the pattern of taxol production remained the same, we observed the drop in taxol production rate, which results in lower taxol concentrations compared to the previous study. The taxane production rate was less than those during the previous studies and taxane concentration still linearly related to time until day 23, the last observed day. Due to the ascending profiles (Figure 6.13), greater taxol levels could be obtained by further incubation. The ratio of taxol to other observed taxanes was also lower than those we observed at the same treatment (elicited with 100 μM MJ) in part 6.2.2 (Figure 6.5). The differences may from the one day later elicitation (from day-8 to day 9, as mentioned before) or the change of culture ability to produce taxol. The instability of taxol production in *Taxus sp.* was also reported in other studies (Hirasuna et al., 1996; Ketchum and Gibson, 1996; Ketchum et al., 1998). Due to the delicate mechanisms of regulation and channeling of carbon to subsequent pathways when geranylgeranyl diphosphate serves as a key branching point in plastid isoprenoid metabolism, these mechanisms are easily disturbed (Kleinig, 1989). The maintenance conditions, such as initial cell density, length of subculture interval, temperature etc. were found to strongly affect the ability of cells to produce taxol.

6.3 Conclusions

• MJ is an effective elicitor to stimulate taxane production in *Taxus canadensis* (C93AD). From the kinetic studies, the optimal concentration of methyl jasmonate, for elicitation of

taxol, was 100-200 μM depending on the cell conditions. Elicitation with 100 μM MJ at day 8 of culture cycle could promote taxol, 10-DAT and baccatin III formation up to 40, 40 and 10 fold relative to control. 10-DAB was the only observed taxane that decreased in the elicited system.

- No significant increase of taxanes occurred with addition of N-acetylchitohexaose in systems supplied ethylene. Slightly increased ratios of taxol to 10-DAT with elicitation of oligosaccharide were observed.
- Taxol was increased by 40-75% in all three systems by addition of acetyl CoA together with MJ elicitation. The ratios of taxol to 10-DAT in the system with acetyl CoA supplementation were greater than the system without it, except for the system supplemented with acetyl CoA and N-benzoyl-3-phenylisoserine.
- The maximum ratio of taxol to 10-DAT was obtained in the system adding acetyl CoA together with MJ and N-acetylchitohexaose elicitation.
- Distinct increases of 10-DAT concentrations and maximum of taxol production were obtained from the system supplemented N-benzoyl-L-phenyl-isoserine (side chain of taxol) and acetyl CoA, together with MJ and N-acetylchitohexaose elicitation.
- Results from examination of 10-DAB, baccatin-III, 10-DAT and taxol profiles when adding benzoyl-L-phenyl-isoserine and/or acetyl CoA in elicited cultures support our hypothesis of the proposed taxol pathway. The non-side-chain taxane (could be 10-DAB or another unknown) could be converted into 10-DAT by attachment of the side chain at C-13 position and further converted into taxol by acetylation at the C-10 position.

• Although in section 6.2.2, no significant effect of MJ was observed on cell growth, a strong inhibition of MJ on cell growth was observed in section 6.2.3. Cell concentrations at day 23 in all of the systems exposed to 100 μM MJ decreased by 65-67% and sugar uptake was about 45% less than the non-elicited systems.

• The variability of taxol production in the observed culture was the main problem in our studies. Different results using the same treatment were observed within the same cell line at different generations of the cultures. Part of the variability could be on account of the change of inherent genetic and epigenetic characteristics of the cell line and the differences of culture conditions, which imply the instability of this cell line (*Taxus canadensis* C93AD).

• The accumulation of taxol occurs in the stationary phase; the inverse relationship between taxol accumulation and their growth was observed. Therefore, taxol is a non-growth-related product. Precursor feeding at the stationary phase may be an effective way to increase taxol yield.

• Although the growth yield dropped with MJ elicitation, product yields (Yp/x and Yp/s) significantly improved. The precursors and intermediates did not improve Yx/s, but did improve production yields Production yields approximately doubled when the taxol side chain, N-benzoyl-L-phenylisoserine, was included. With MJ elicitation, production yields were better without chitohexaose elicitation. Since taxol/10-DAT ratios were better with chitohexaose elicitation, metabolic energy might be used to acetylate 10-DAT.

CHAPTER 7

PRELIMINARY KINETIC STUDY OF TAXANE PRODUCTION AND NUTRIENT UPTAKE IN SPINNER FLASKS WITH METHYL JASMONATE ELICITATION: BATCH VS SEMI-CONTINUOUS CULTURE WITH TOTAL CELL RECYCLE

From the engineering point of view, one barrier to the development of large scale fermentation for plant cells is the fragile nature of plant cells when coupled with hydrodynamic shear stress necessary for adequate mixing. Shear stress is one of the important factors that alter secondary metabolism of plant cells. Shear stress may affect plant cell growth and cellular metabolism negatively or positively depending on the level of applied shear stress, the properties of the cell line and its physiological state. Shear stress at 2.1 s^{-1} was found positive on taxol production (Sun and Linden, 1999), however shear stress at high levels resulted in physiology changes of the cells that eventually led to cell death.

Another consideration for production scale fermentation is the process strategy. The previous kinetic studies in Chapter 6 indicated taxol production was non-growth associated. In the stationary phase, when growth was minimal, conditions favored the production and accumulation of taxol and other taxanes. Therefore, prolonging the stationary phase of the cultures could increase the productivity. However, the long term

maintenance of the batch culture is not possible since nutrients will be depleted along with the accumulation of toxic products. Continuous processes can be used to maintain cell growth at steady state. However, the productivity of CSTR is limited due to the loss of cells with the outlet stream. Therefore, continuous cultures with cell recycle could be an alternative way to maintain higher productivity over a long period. Since a total cell recycle system is a dynamic system, as cells accumulation within the reactor the perfusion rate must be adjusted to satisfy the metabolic activity of the culture.

In this chapter, kinetic studies of *T. canadensis* (C93AD) with 100 μM MJ elicitation were performed in two 500 mL working volume Wheaton Celstir spinner flasks (Wheaton Science Products, Millville, NJ). The spinner flasks provided an ideal, totally inert cell growth environment with an addition of a concave dimple in the bottom to prevent the crushing of cells beneath the magnetic impeller and improve circulation and turbulence. Agitation was achieved by means of a magnetically driven flat bladed turbine rotating at 80 rpm, the lowest speed that still maintained a uniform distribution of cells throughout the liquid medium. A batch operation to observe the kinetic activities in the spinner flask was performed. The results were compared to those in shake flasks. Along with the batch culture, a semi-continuous culture with total cell recycle was conducted in order to check the possibility to improve taxane production.

7.1 Experimental Studies

In this chapter, batch and semi continuous cultures with total cell recycle of *T. canadensis* (C93AD) were performed in 500 mL (working volume) spinner flasks. A 100 mL inoculum was added to 400 mL of fresh medium in each flask, and incubated in the

dark at 23°C and 80 rpm agitation. Since we observed lower growth rates in the spinner flasks compared to shake flasks, elicitation with 100 μM MJ and control of the headspace gas mixture (7 ppm C_2H_4, 15% O_2 and 0.5% CO_2) were initiated after 16 days of transferring the cultures. Samples were taken every 2-3 days for analyses of pH, sugars and taxanes.

Generally, in perfusion systems, perfusion rate is based on cell concentration information. The growth rate was negligible in the production phase of this system after elicitation with 100 μM MJ as discussed in Section 6.2.3. Hence, fresh medium was fed based on the sugar utilization information in the semi-continuous operation instead. As sucrose was depleted and the remaining total sugar was reduced to about 15 g/L, 160 mL of free cell medium in the spinner flask was taken by pipetting, and 160 mL of fresh medium with 100 μM MJ was added. With this strategy, the total sugar in the semi-continuous system was maintained between 13-16 g/L. In Section 6.2.3, this concentration was the sum of glucose, fructose and sucrose concentrations in the early production phase.

7.2 Results and Discussion

Sugar utilization and cell growth

The patterns of sugar utilization in the spinner flask (500 mL working volume) (Figure 7.1) were similar to those in the shake flasks (50 mL working volume) (Figure 6.10). Sucrose in the culture medium was completely hydrolyzed to fructose and glucose within 12 days. Glucose was utilized at a faster rate, compared to fructose as shown in Figure 7.1 for the batch process and Figure 7.2 for the semi-continuous process. The average

sugar uptake rate in batch process was about 0.50 g/L day^{-1} during the first 12 days and declined to about 0.15 g/L day^{-1} after that. The total sugar uptake in batch operation elicited with 100 μM in the spinner flask was about 9.6 g/L, which was almost the same as the value observed in shake flask (9.2 g/L).

The total sugar profile represented in Figure 7.3 showed that with the strategy of feeding fresh medium, the total concentration of sugars in semi-continuous operation was between 13-16 g/L, which was almost the same level as in the batch operation. The patterns of sugar profiles in the semi-continuous culture (Figure 7.2) were the same as in the batch culture until the transfer of fresh medium was initiated (day 16). The cumulative sugar uptake in the semi-continuous culture continuously increased after transferring fresh medium into the system (Figure 7.4), while the cumulative of sugar uptake in the batch culture slightly increased after day 12. Over the entire 35 days of the experiment, the total sugar consumption in the semi-continuous culture was about 28 g/L; three times that observed in the batch culture.

From the analysis of cell concentration at the end of the experiments (day 35), the cell concentration was 2.68 g dry wt./ L in the batch culture and 3.11 g dry wt./L in the semi-continuous culture. In the previous kinetic studies (Chapter 6) and a report by Fett-Neto et al. (1994), the total sugar uptake was closely associated with the increase in culture biomass. The total sugar consumption in semi-continuous culture was 3 fold that of the batch culture, but the cell concentration was only 1.2 fold relative to the batch culture. From the comparison of the cultures in the shake flask and in the spinner flask, although the kinetics of sugar utilization in both systems were nearly the same, the cell growth differed. Cell growth in the spinner flask was only 0.63 of that observed in shake flasks.

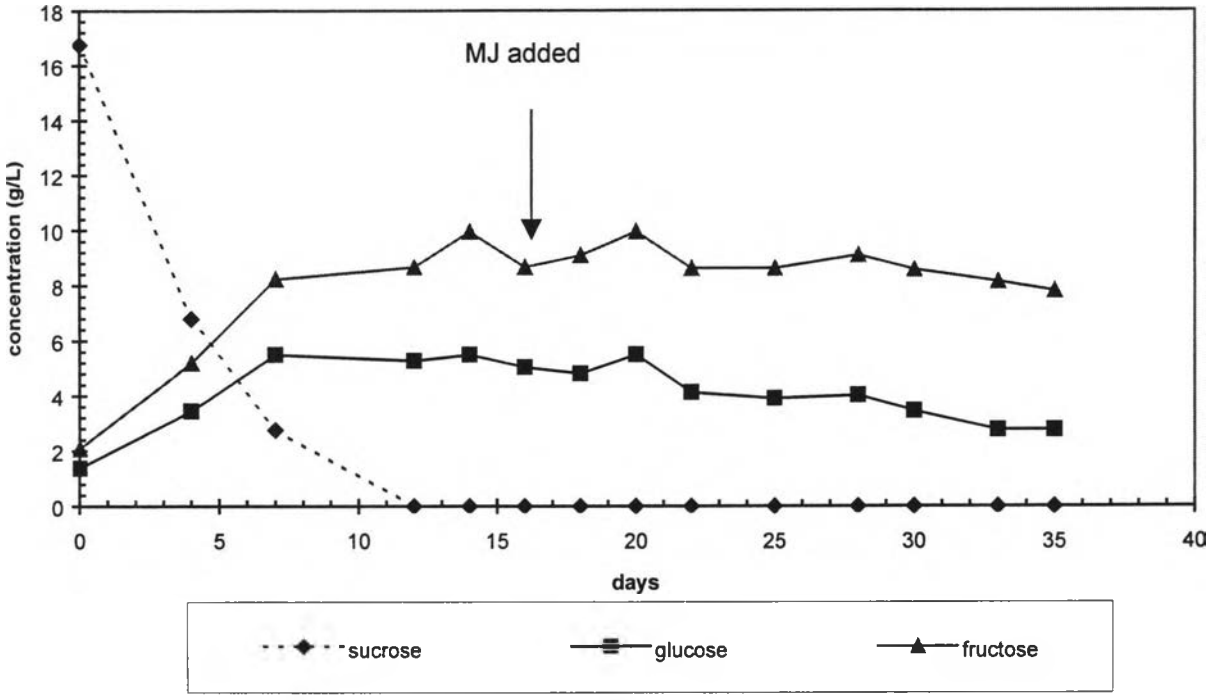


Figure 7.1 The sugar profiles of *T. canadensis* in a batch process in the spinner flask.

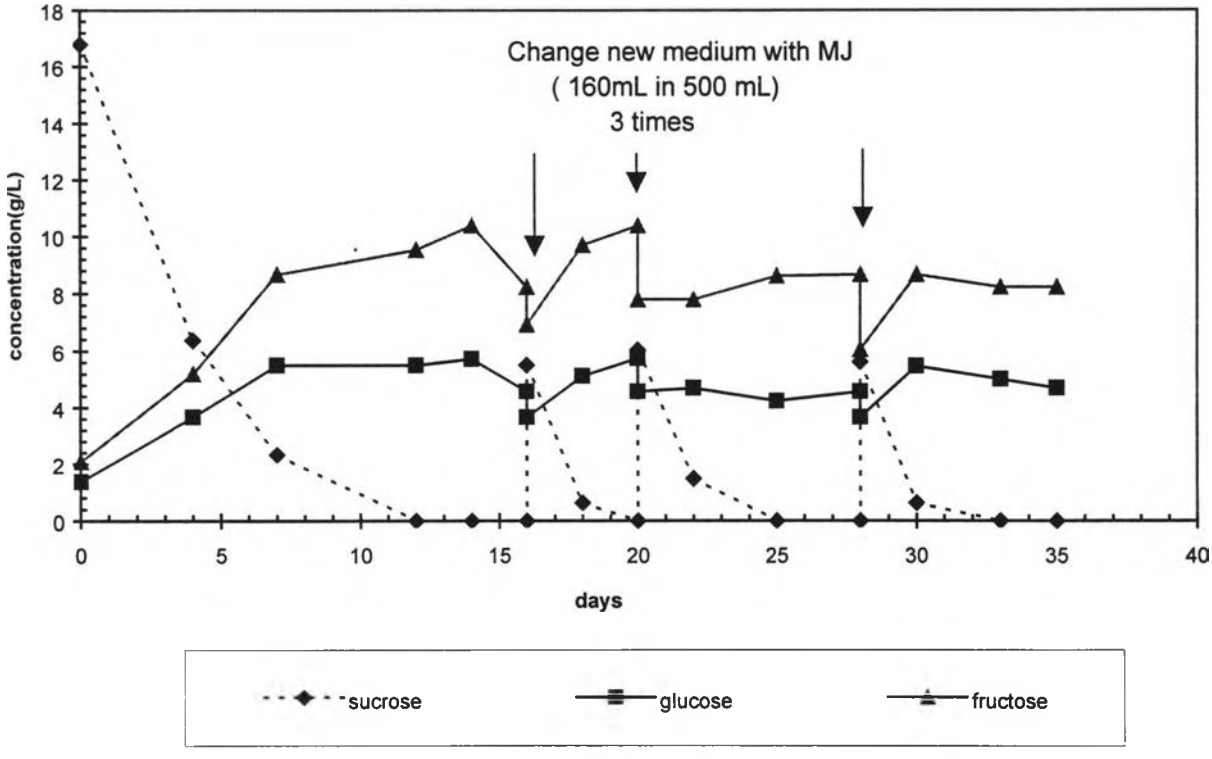


Figure 7.2 The sugar profile of *T. canadensis* in a semi-continuous process in the spinner flask.

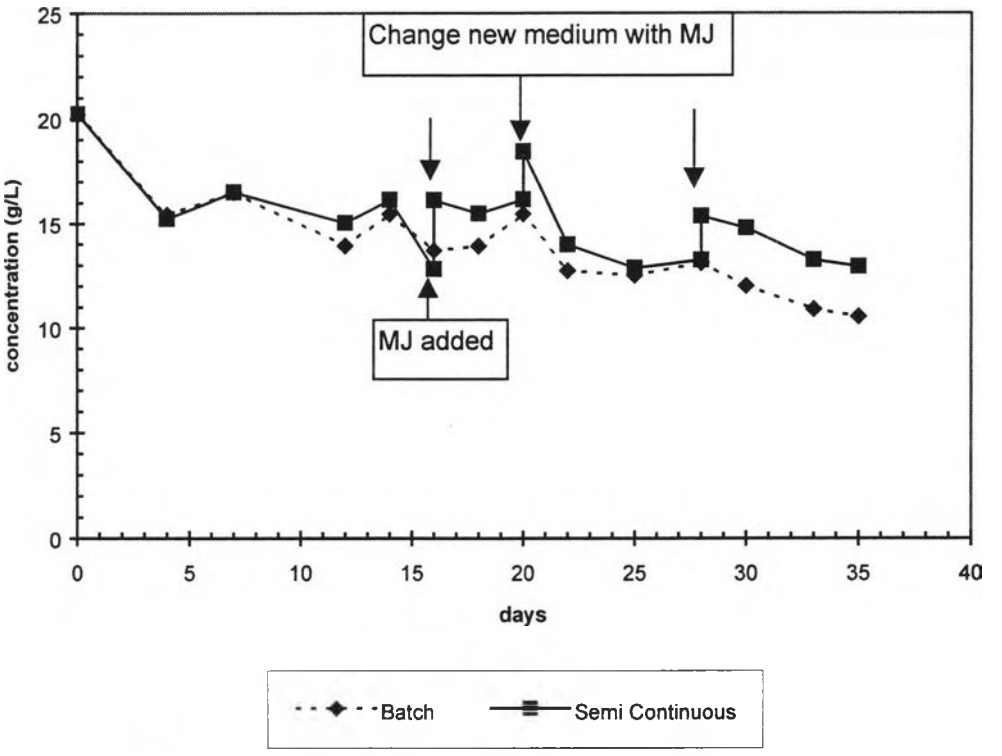


Figure 7.3 The comparison of total sugar concentration profiles of *T. canadensis* between batch and semi-continuous process.

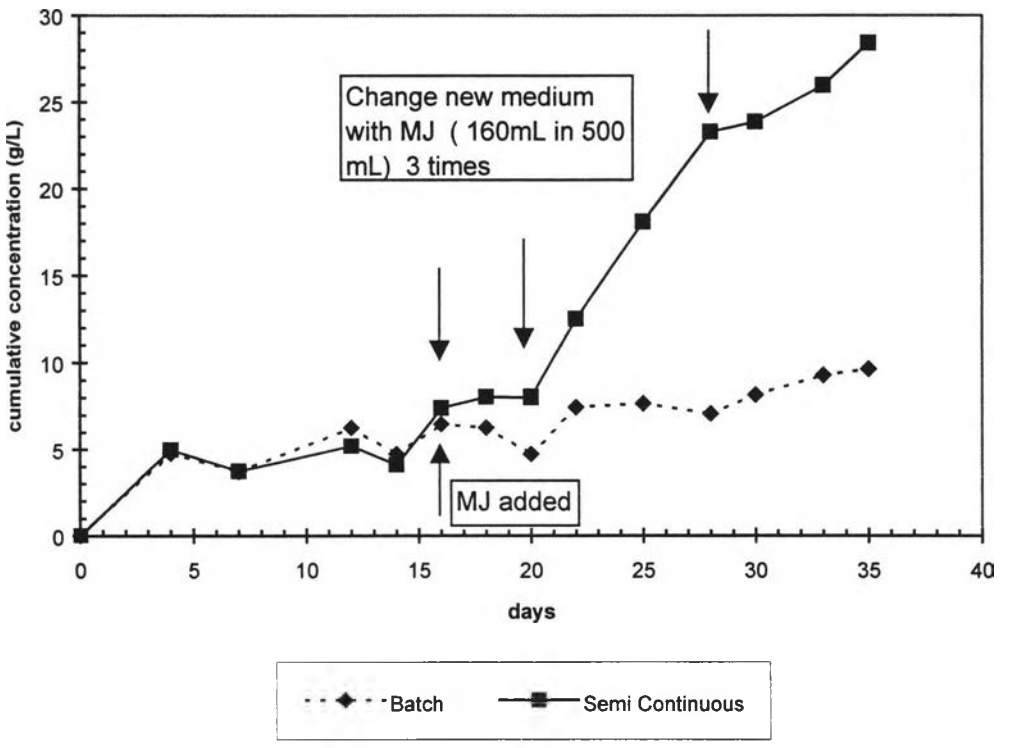


Figure 7.4. The comparison of cumulative sugar consumption of *T. canadensis* between batch and semi-continuous process.

During the kinetic studies in the spinner flasks, even though the agitation was at the low speed, a large amount of cell debris was observed. Some cell debris also flowed out with medium when transferring the fresh medium. These losses presumably had a partial effect on the apparent decrease in cell concentration and cell growth rate.

Cell debris, however, was not observed in shake flasks. The effects of shear stress on cell lysis and growth rate were reported in many plant cell lines. Zhong et al. (1994) reported a decrease of *Perilla frutescens* viability with both shearing time and magnitude of shear rate. Maijer et al. (1995) reported a strong inhibition of growth of *Catharanthus roseus* at agitation speeds of 4.2 $rev.s^{-1}$, although the metabolite activity was still observed. From the study of *T. cuspidata* in a rotary wall vessel system by Sun and Linden (1999), rotation at shear stress more than 5.2 s^{-1} damaged the plant cells and eventually lead to cell death. Seki et al. (1997) and Pestchanker et al. (1996) observed decreases of growth rate of *Taxus sp.* when scaled up from shake flasks into reactors.

pH profile

In batch culture, the medium pH increased rapidly from the initial pH 4 to pH 5.5 in 6 days and maintained constant around 5.5 ±0.2 after that (Figure 7.5). In the semi-continuous culture, after feeding fresh medium, the pH of the culture medium dropped to 4.6 – 4.8, and then rapidly increased to 5.5 within 2-3 days. Overall, the pH of the culture medium was maintained at 5.5 ± 0.2 in both batch and semi continuous cultures. This pattern of pH profiles was also observed in previous studies. From the report by Wickremesinhe and Arteca (1994) in suspension cultures of *Taxus media*, there were no substantial differences between the pH values of the medium after 21 days of growth with

initial medium pH adjusted between pH 3.0 and 7.0; the final pH at day 21 was always 5.4-5.6. Fett-Neto et al. (1994) suggested the transient increase of the internal pH of the cells was due to the uptake of NH_4^+. Ion flux may stimulate the initial release of taxol into the medium, as also previously proposed for the release of alkaloids by *Cinchona spp.* suspension (Parr et al., 1986).

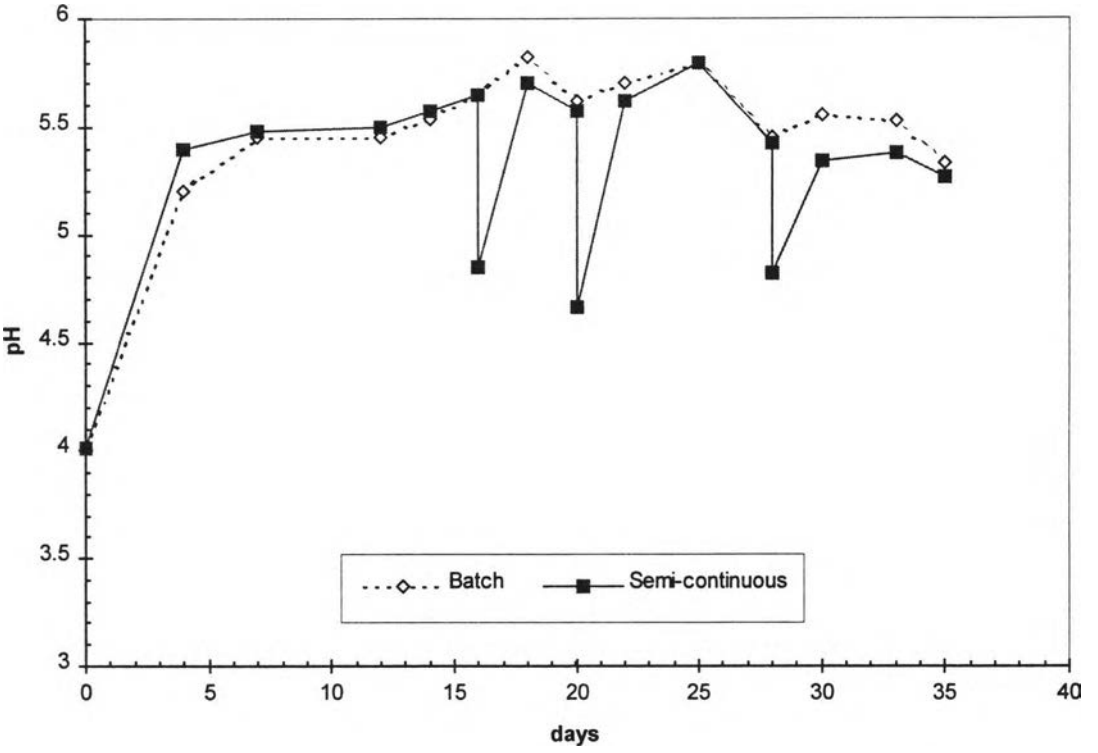


Figure7.5 Comparison of the pH profiles during the cultivation of *T. canadensis* between batch and semi-continuous cultures in the spinner flasks.

Taxane analysis

Growth rates in the spinner flasks were less than in the shake flasks. In the previous studies, elicitation using 100 μM MJ strongly inhibited cell growth. Therefore, elicitation with 100μM MJ and control of the headspace gas were initiated at day 16 of growth. As shown in Figure 7.6 - 7.8, before the transfer of fresh medium, the profiles of taxanes in batch and semi-continuous cultures were similar. 10-DAB accumulated after 4-7 days of growth, while baccatin III, 10-DAT and taxol were first observed at days 16-20 (0-4 days after elicitation).

In the batch operation (Figure 7.8), a sharp decrease of 10-DAT was observed after day 25, along with the increase of taxol accumulation. Two steps of baccatin III accumulation were observed at day 14-16 and after day 25 (Figures 7.6 and 7.8). The maximum of taxanes was obtained at day 33 with 4.5 mg/L of 10-DAT, 0.75 mg/L of taxol, 3 mg/L of 10-DAB and 0.87 mg/L of baccatin III. Referring to the proposed of taxol pathway discussed in Chapter 5, the profiles of each taxane observed in this study accommodate our hypothesis of the pathway from 10-DAT to taxol and the parallel pathway from 10-DAB to baccatin III and from taxol to baccatin III.

In semi-continuous culture with total cell recycle, 160 mL of fresh medium with 100 μM MJ was transferred into the flask at day 16, 20 and 28 according to the sugar information (Figure 7.2). Although the taxane concentrations were slightly diluted from the transfer of the medium, significantly increases of 10-DAT, taxol and baccatin III were observed (Figures 7.7 and 7.8). After day 20, the accumulation of 10-DAT, taxol and baccatin III linearly increased at average rates of 1.34, 0.20 and 0.16 mg/L day, respectively (Figure 7.8). Due to the profiles, higher levels of taxanes could be obtained

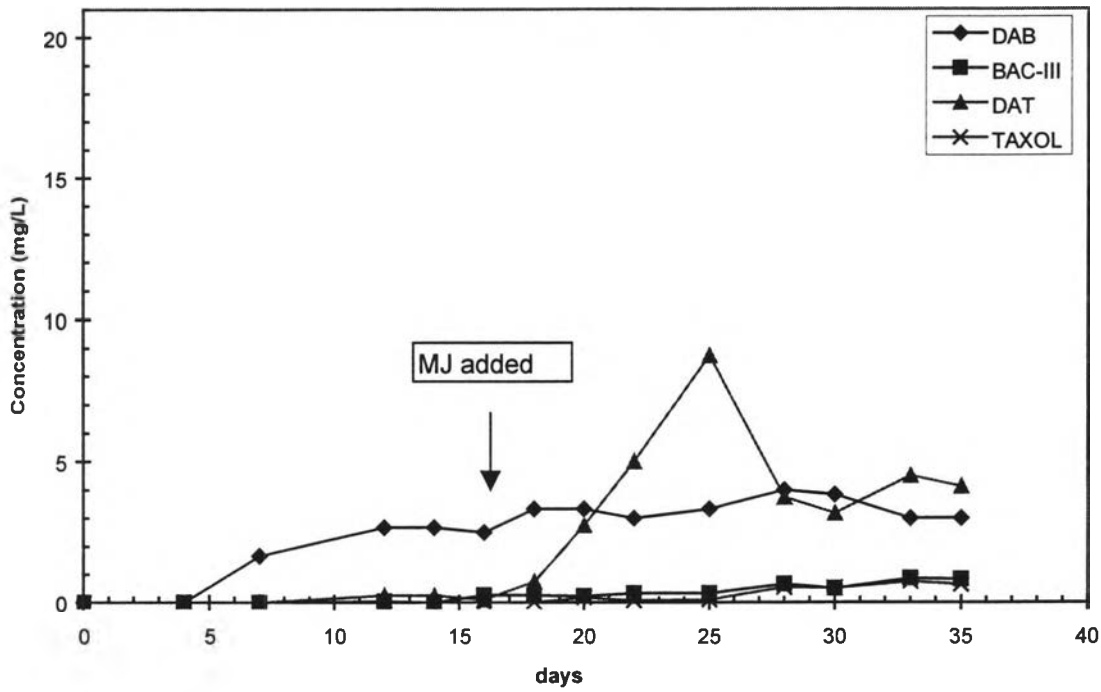


Figure 7.6 Kinetic profiles of taxane production by *T. canadensis* in the spinner flask elicited with 100 *u*M MJ in batch operation.

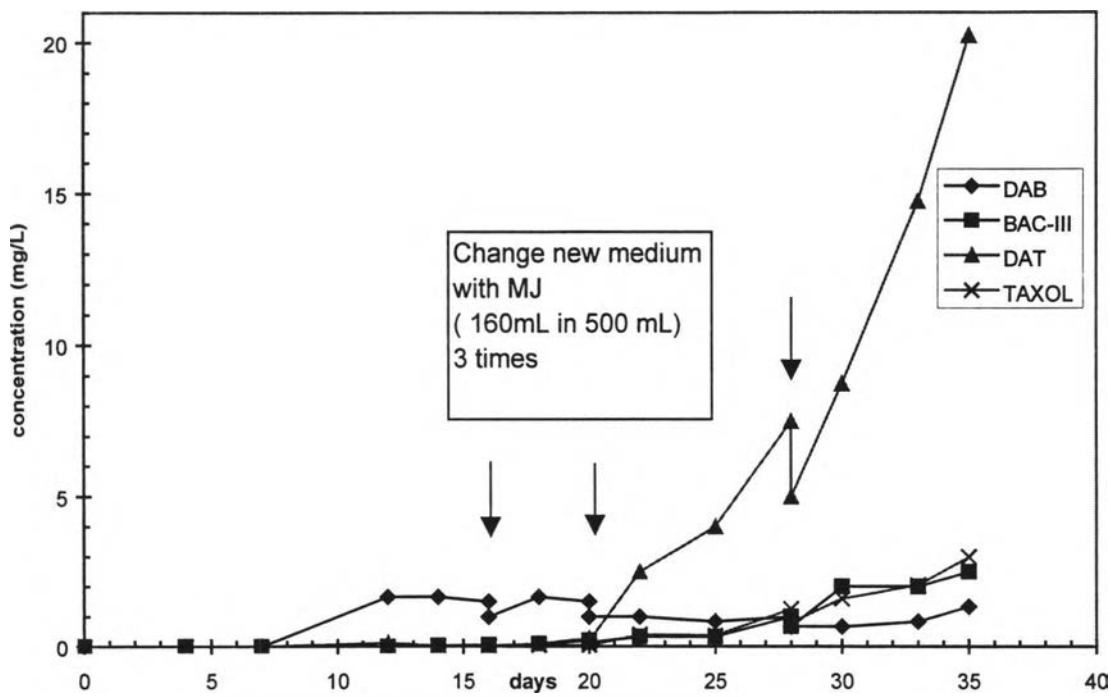


Figure 7.7. Kinetic profiles of taxane production by *T. canadensis* in the spinner flask elicited with 100 *u*M MJ in semi-continuous operation.

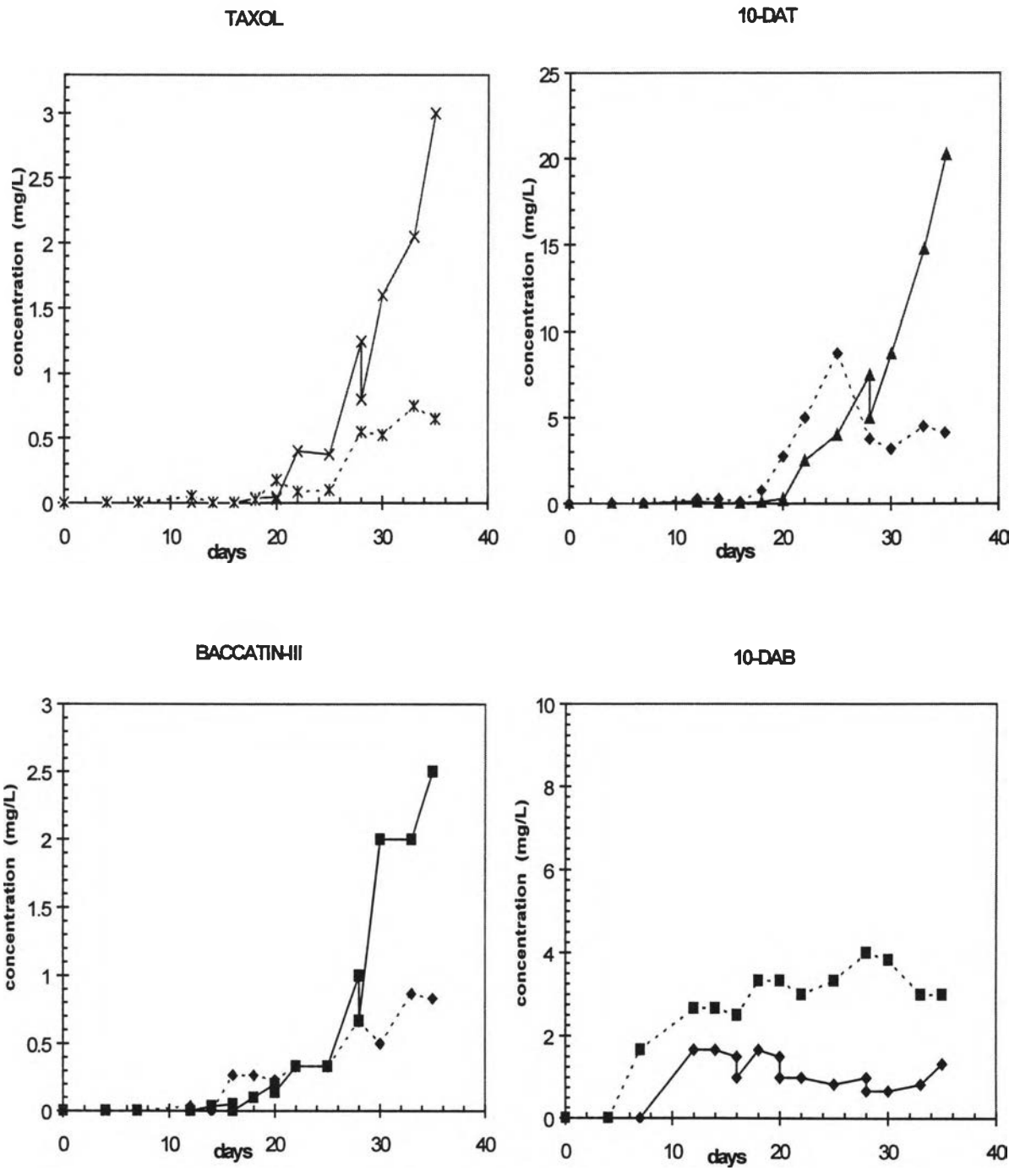


Figure 7.8 The comparison of taxane profiles by *T. canadensis* between batch and semi-continuous operations.

---------- Batch ———— Semi-continuous

by further incubation using the same process strategy. As always observed in elicited *Taxus* cultures in our studies, 10-DAT accumulated to concentrations greater than other taxanes. In semi-continuous culture with cell recycle, the total amount of 10-DAT, taxol and baccatin III secreted into the medium during 35 days of cultivation reached respectively 20.3, 3.0 and 2.5 mg/L or respectively 4.5-, 4.0- and 3.0- fold compared to the maximum observed in the batch operation. 10-DAB was the only observed taxane that decreased by semi-continuous operation (1.3 mg/L in semi-continuous culture versus 3.0 mg/L in batch culture).

It has been reported by Seki et al. (1996) that with the perfusion culture of *T. cuspidata* using suspension or immobilized cells in 300 mL shake flasks (100 mL working volume) without elicitation, the taxol productivity increased by a factor of ten at 0.9 d^{-1} (0.3 mg/ gDCW.d) compared to batch operation, however lower concentrations were obtained (0.3 mg/L in perfusion operation versus 1.8 mg/L in batch).

In our studies, although the profiles and the amounts of sugar uptake in the spinner flask and shake flask cultures are nearly the same, the total taxane production and cell growth rate differed. The lower cell concentrations, which resulted in lower taxane production in the spinner flasks, could be related to differences in hydrodynamic shear. From the studies by Pestchanker et al. (1996) in suspension cultures of *T. cuspidata*, although the production of taxol in a Wilson-type bioreactor (WR) and shake flasks were the same (22 mg/L), cell growth in WR was only 0.43 of that observed in shake flasks (136 mg L^{-1} vs. 310 mg L^{-1}).

The overall comparisons of batch culture in the shake flask, spinner flask and semi-continuous culture with cell recycle in the spinner flasks of our studies are summarized in

Table 7.1. In batch processes, although no significant difference of sugar uptake and $Y_{P/X}$ between cultivation in the shaking flask or spinner flask, but growth yield (Yx/s) and production yield ($Y_{P/S}$) of the cultures in the spinner flask were only 60% of those observed in the shaking flask. From the comparison of cultures in the spinner flasks, growth yield (Yx/s) in semi-continuous process was less than 50% of the batch process. On the other hand, total sugar uptake and Yp/x in semi-continuous process increased about 3 fold compared the batch process. No significant difference of $Y_{P/S}$ was observed between batch and semi-continuous cultures in the spinner flasks.

Table 7.1 The overall comparison of kinetic activities of *T. canadensis* (C93AD) at in the operation of (1) batch in shake flask: day 23, (2) batch in spinner flask: day 33 and (3) semi-continuous in spinner flask: day 35.

TREATMENTS	Cell dry wt. (g/L)	Sugar Uptake (g/L)	$Y_{P/X}$ (10^{-3})	$Y_{P/S}$ (10^{-3})	$Y_{X/S}$	Taxane formation (mg/L) 10-DAB BACIII 10-DAT TAXOL
Batch in Shake Flask (from expt 6.III. B) MJ elicitation at day 9	4.21	9.22	4.85	1.50	0.31	0.37 0.44 11.00 2.06
Batch in Spinner Flask MJ elicitation at day 16	2.68	9.67	5.12	0.94	0.18	3.00 0.87 4.50 0.75
Semi Continuous in Spinner Flask MJ elicitation at day 16	3.11	28.42	14.33	1.11	0.08	1.33 2.50 20.25 3.00

- p = (10-DAB+BACIII+10-DAT+TAXOL), x =cell dry wt., s = total sugar uptake.
- Accumulation of taxane (p) loss during medium transfer = 4.59 mg/L.

During the cultivation in spinner flasks, dark pigmentation in the medium increased progressively, particularly during the stationary phase. We also observed the older

brown-colored cell suspension in previous studies particularly, when cells produced high levels of taxanes. This observation also reported in *T. cuspidata* by Fett-Neto and DiCosmo (1996) and in callus cultures of *T. baccata, T.cuspidata,* and *T. media* (Wickremesinhe and Arteca, 1993 a). Darkening of the medium was probably due to the release and oxidation of phenolic compounds produced by the cell cultures (Fett-Neto et. al., 1992).

7.3 Conclusions

- A considerably improved taxane production was observed using semi-continuous culture with total cell recycle. 10-DAT, taxol and baccatin III increased by a factor of 4.5, 4.0 and 3.0, respectively, relative to the batch culture during 35 days of cultivation.
- Although the kinetic profiles of sugar uptake in the spinner flask and shake flask cultures are almost the same, the rates of total taxanes production and cell growth differed. The lower growth and production rate observed in the spinner flask cultures should be related to differences in hydrodynamic shear.
- Since 10-DAT accumulates to high concentrations in the culture medium, precursor feeding with fresh medium together with MJ elicitation in semi-continuous culture with cell recycle or perfusion culture could be an alternative way to enhance taxol production.
- Growth yield (Yx/s) and production yield ($Y_{P/S}$) of the cultures in the spinner flask dropped approximately 40% relative to those in shaking flask. Production yield (Yp/x) in semi-continuous process increased about 3 fold compared the batch process. No significant difference of $Y_{P/S}$ was observed between batch and semi-continuous cultures in the spinner flasks.

CHAPTER 8

STATISTICS ASSOCIATED WITH PRINCIPAL COMPONENTS ANALYSIS (PCA) MODEL: APPICATION TO TAXANE PRODUCTION IN *TAXUS CANADENSIS* (C93AD) SUSPENSION CULTURES

In order to enhance taxol production in suspension cultures of *Taxus sp.*, the regulation and signal transduction of biosynthetic pathways of the secondary metabolites have been investigated. The studies on elicitation and signal transduction showed interdependence of ethylene and the methyl jasmonate (MJ) action in affecting taxol biosynthesis reactions. Reproducible results from independent experiments demonstrate complex changes in taxol and 10-deacetyl taxol, which increase in a manner proportional to MJ and ethylene concentrations. A complex model is proposed to explain the action and effects of both ethylene and MJ on production of taxol and other taxanes. In order to monitor quality and detect fault of the system and determine the relevant variables for optimization, chemometric methods are applied.

Chemometrics is the science of relating measurements made on a chemical system to the state of the system via application of mathematical or statistical methods in order to design optimal experiments for investigation of chemical systems (Wise and Gallagher, 1996). Most chemometric methods fall under the statistical techniques known as

multivariate analysis. One of the most frequently applied techniques is Principal Component Analysis (PCA).

Principal Component Analysis (PCA) is a widely used technique for data compression and information extraction, which relies upon an eigenvector decomposition of the covariance matrix of process variables (Wise and Gallagher, 1998; MacGregor and Kourti, 1995). By this approach, redundant information in process measurement can be compressed in a manner that retains the essential information and can be more easily displayed than each of the process variables individually. Also, often essential information lies not in any individual process variable but in how the variables change with respect to one another, i.e. how they co-vary.

In this study, statistics associated with the PCA model are applied to taxane data obtained in the experiment presented in Chapter 3. Once the PCA model is established, outlier data can be detected from a measure of variation in each sample within the model (Hotelling's T^2 statistic) together with the information of how well each sample conforms to the model (Q-statistic). A cross correlation matrix from the correlation coefficients for each pair of variables was developed in order to elucidate a relationship between the observed variables.

8.1 Principal Components Analysis (PCA)

Principle Component Analysis is a tool to find combinations of variables or factors that describe major trends in a data set. PCA accounts for correlation among process variables and significantly reduces the number of variables.

PCA relies on an eigenvector decomposition of the covariance matrix of the process variables. PCA is scale dependent, thus numerically larger variables appear more important. A data matrix was generally autoscaled to zero mean columns and unit variance prior to analysis. For a given data matrix X with m rows (samples) and n columns (variables), the PCA decomposition is:

$$X_{m\,x\,n} = t_1 p_1^T + t_2 p_2^T + t_3 p_3^T + \ldots\ldots + t_k p_k^T + \ldots\ldots + t_q p_q^T + E \qquad (1)$$

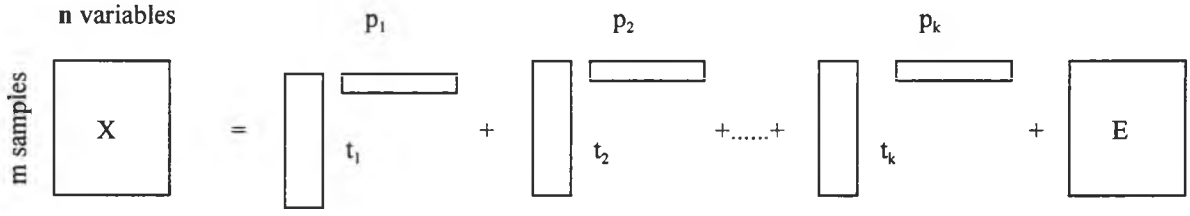


Figure 8.1 The PCA decomposition (Wise and Gallagher, 1998).

Where, q = min {m,n}, the $t_i p_i^T$ pairs are ordered by the amount of variance captured.

E = a residual matrix, and

p_i = the eigenvectors of the covariance matrix

Covariance matrix of X is defined as

$$\mathrm{cov}(X) = \frac{X^T X}{m-1} \qquad (2)$$

$$\mathrm{cov}(X) p_i = \lambda_i p_i \qquad (3)$$

Where,

p_i are eigenvectors of the convariance matrix, and

λ_i are the eigenvalues associated with the eigenvector p_i.

In PCA, the p_i are known as loading and contain information on how variables relate to each other.

$$t_i = Xp_i \qquad (4)$$

t_i are score vectors in linear combination of the original X data defined by p_i; t_i are the projections of X onto the p_i . In PCA, scores show relationships among samples. The t_i form an orthogonal set ($t_i^T t_j = 0$ for $i \neq j$), while the p_i are orthonormal ($p_i^T p_j = 0$ for $i \neq j$, $p_i^T p_j = 1$ for $i=j$).

This decomposition is performed in order to find the linear combinations that maximize the variance of X on each orthogonal direction. t_i p_i pairs are arranged in descending order of λ_i, where the corresponding eigenvalues (λ_i) are the variances of the PC's described by the t_i p_i pairs.

Therefore, the first principal component (PC) of X is a linear combination $t_i = Xp_i$, which captures the maximum variance subject to $|p_1|=1$. The second PC is the linear combination $t_i = Xp_i$, which captures the next greatest variance subject to $|p_2|=1$, and subject to the condition that it be uncorrelated with (orthogonal to) the first PC (t_i). Additional PC's up to q are similarly defined (MacGregor and Kourti, 1995). Generally it is found that the most of the variability in the data can be captured in the first few PC's; 2 or 3 PC's are often sufficient to explain most of the predictable variations in a process (MacGregor, and Kourti, 1995; Puente and Karim, 1999). PCA also produces linear combinations of variables that are useful descriptions, or even predictors, of particular process events (Wise, et al., 1995).

A rule of thumb for choosing the minimum number of PCs, that are necessary to be retained in the model is to choose those PCs for which eigenvalues are greater than 1 or to look for a "knee" or a sharp change in the slope of the plot of eigenvalues and PC number (Wise and Gallagher, 1998).

8.2 Quality Monitoring and Fault Detection

Once the model is developed, new data can be viewed as projections onto PCA-space. Scores from PCA models, t has zero mean and variance equal to their associated eigenvalue. If it is assumed that the scores are normally distributed, confidence limits for the scores can be calculated by applying the student's t-distribution. The limits on the scores for the i^{th} PC for confidence limits, 1-α, is given by:

$$t_{i,\alpha} = \pm \text{sqrt}(\lambda_I) * t_{m-1,\alpha/2} \qquad (5)$$

Where, m = number of samples,

$t_{m-1,\alpha/2}$ = the probability point on the (single sided) t-distribution with m-1 degrees of freedom and area is $\alpha/2$.

Hotelling's T^2, sum of normalized squared scores, is a measure of the squared distance between an observation sample and its average.

In general form,

$$T^2 = \left(y - \bar{y}\right)^T s^{-2} \left(y - \bar{y}\right) \qquad (6)$$

or

$$T_i^2 = t_i \lambda^{-1} t_i^T = x_i \mathrm{P} \lambda^{-1} \mathrm{P}^T x_i^T \qquad (7)$$

for PCA model $s_i^2 = \lambda_i$.

Where t_i refers to the i^{th} row of T_k, the matrix of k scores vectors from the PCA model. The matrix λ^{-1} is a diagonal matrix containing the inverse eigenvalues associated with the k eigenvectors (Wise and Gallagher, 1996).

Hotelling's T^2 statistic indicates the variation in each sample within the PCA model. The confidence limit, $(1-\alpha)$, for T^2 can be calculated from the F-distribution by

$$T^2_{k,m,\alpha} = \frac{k(m-1)}{m-k} F_{k,m-k,\alpha} \qquad (8)$$

for k variables and m samples.

A lack of a model fit can be calculated from the Q-statistic. Q, a residual between a sample and its projection into the k principal components retained in the model, indicates how well each sample conforms to the PCA model (Wise and Gallagher, 1998). Q can be calculated from the sum of squares of each row error (E from equation (1)) or sum of squares of the individual residuals.

$$E = X - T_k P_k^T = X - X P_k P_k^T = X(I - P_k P_k^T) \qquad (9)$$

Therefore,

$$Q_i = e_i e_i^T = x_i (I - P_k P_k^T) x_i^T \qquad (10)$$

Where e_i is the i^{th} row of E and P_k is the matrix of k loadings vectors.

The upper control limit $(1-\alpha)$ of the $Q_{residual}$ can be calculated by the following relations (Jackson, 1980).

$$Q_\alpha = \Theta_1 \left[\frac{c_\alpha \sqrt{2\Theta_2 h_0^2}}{\Theta_1} + 1 + \frac{\Theta_2 h_0 (h_0 - 1)}{\Theta_1^2} \right]^{\frac{1}{h_0}} \qquad (11)$$

Where, $$\Theta_i = \sum_{j=k+1}^{n} \lambda_j^i \quad \text{for i = 1, 2, 3} \qquad (12)$$

$$h_0 = 1 - \frac{2\Theta_1\Theta_3}{3\Theta_2^2} \qquad (13)$$

and c_α is the standard normal deviation cutting off an area of α under the upper tail of the distribution if h_0 is positive and under the lower tail if h_0 is negative. In equation (12), k is the number of principal components retained in the process model and n is the total number of principal components. A more detailed explanation of the $Q_{residual}$ analysis can be obtained from Jackson (1991), MacGregor and Kourti (1995) and Wise and Gallagher (1998).

By setting the scores plot of sample number against Hotelling's T^2, and Q residuals, outlier samples (samples with very high Q, T^2 or both) can be easily detected (Figure 8.2). The $Q_{residual}$ is a measure of the distance of a data point off the plane, which represents the variation of the data outside of observed PCA model. T^2 is a quantity of the distance from the multivariate mean. Large Q indicates unusual variation outside the model; large T^2 indicates unusual variation inside the model.

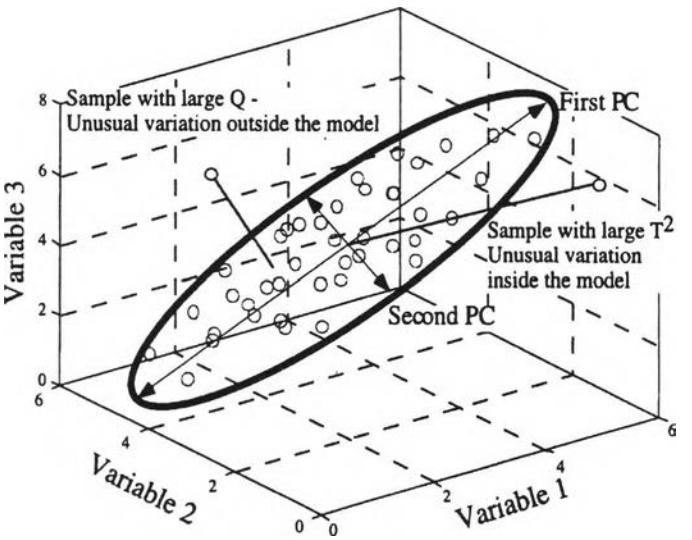


Figure 8.2 Graphical representation of PCA (Wise and Gallagher, 1998).

8.3 PCA: Application to Taxane Data

Our past work has identified approaches that enhance taxol productivity in cell culture, which established complex interdependence of ethylene and methyl jasmonate (MJ) in affecting taxol biosynthesis. Concentrations of taxol increase in a manner roughly proportional to MJ concentration. The level of enhancement is also dependent on ethylene concentration on the headspace. A binding and inhibition model is proposed to explain the action and effects of both ethylene and MJ on production of taxol and other taxanes (Chapter 3).

To analyze the effect of methyl jasmonate and ethylene on taxane production and to apply quality monitoring and fault detection to taxane data in our experiments, a PCA model based on the taxane data set (Figure 8.3) was developed using PLS_Toolbox 2.0 (Eigenvector Research, Inc, Manson WA, 1998). In Figure 8.4, the plot of the eigenvalues shows a sharp change of eigenvalue between PC 2 and PC 3, thus 2 PCs were recommended for the model. PCA was performed after auto-scaling to zero mean columns and unit variance of the data. It was found that two factors could capture over 96% (71.02 % for PC1 and 25.08% for PC2) of the variation as shown in Table 8.1. Therefore the two new variables, which are linear combinations of the 4 variables, can replace the original variables, with no significant loss of information.

The loading plots show relationships among variables. The corresponding loading in Figure 8.5 indicates the similar contribution of three variables; variable 1 (methyl jasmonate (MJ)), variable 3 (10-deacetyl taxol (10-DAT)) and variable 4 (taxol) on PC1 and PC2. Variable 2 (Ethylene) is the only observed variable with different contribution than the others.

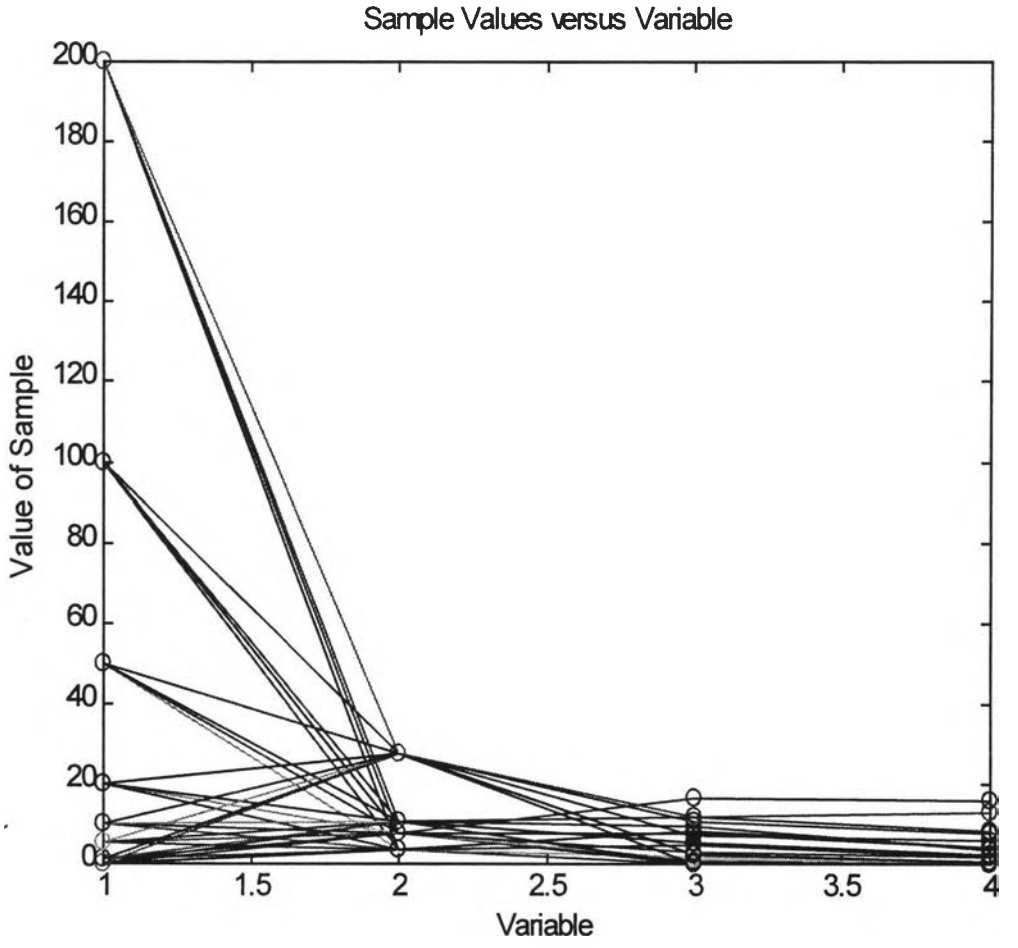


Figure 8.3 Taxol data before auto-scaling.

Var: data Data: modeled (calibration set) Size: 32 rows x 4 cols Samp Lbls: Var Lbls:	Model: calibrated on loaded data PC(s): 2 Data: 32 sams x 4 vars Scaling: autoscaled

Number of PCs Selected: 2

Percent Variance Captured by PCA Model

Principal Component	Eigenvalue of Cov(X)	% Variance This PC	% Variance Cumulative
1	2.84e+000	71.02	71.02
2	1.00e+000	25.08	96.11
3	1.09e-001	2.74	98.84
4	4.63e-002	1.16	100.00

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Table 8.1 Percent variance captured by PCA model of taxol data

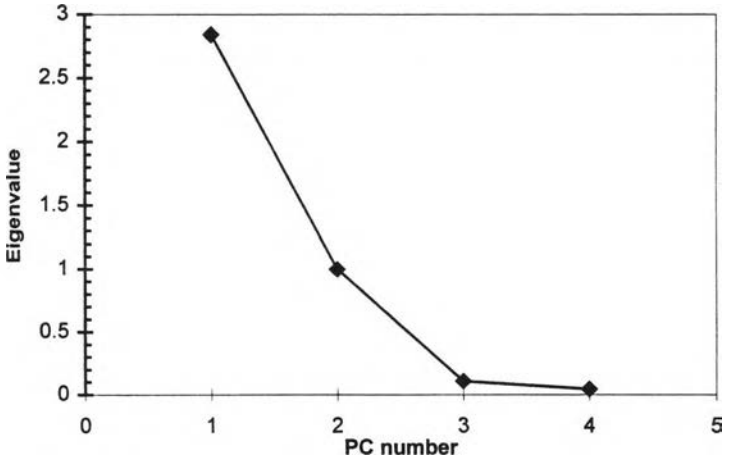


Figure 8.4 The plot between eigenvalues and PC number

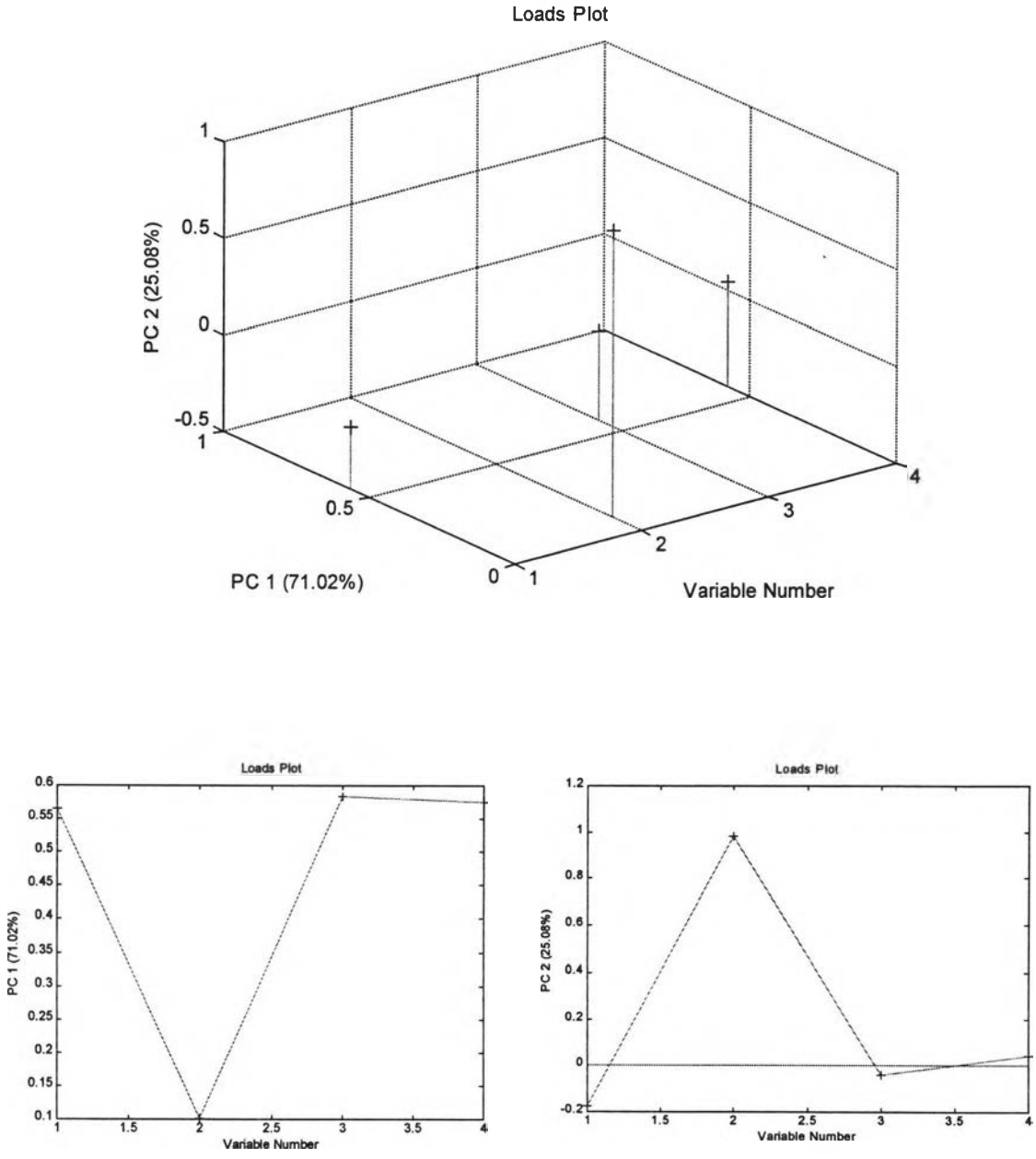


Figure 8.5 Loadings for PC1 and PC2 of the taxol data.

PC1 accounted for 71% of total variation, is strongly influenced by variables 1 (MJ), 3 (10-DAT) and 4 (taxol), but is slightly influenced by variable 2 (ethylene). PC2, which accounts for 25% of total variation, is strongly influenced by variable 2 (ethylene) but does not show much contribution due to variable 1 (MJ), 3 (10-DAT) or 4 (taxol). The results imply that MJ is the important parameter on taxane production; 10-DATand taxol are strongly linear proportional to MJ concentration. The effect of ethylene on taxane production was observed but not as strongly as MJ; relationships between ethylene and taxol and 10-DATproduction may be nonlinear.

The score plots with the 99% and 95% confidence limits were used to detect the outlier sample as shown in Figure 8.6 and Figure 8.7. According to the score plot, for 32 data points, two points (number 32 and 16) lie outside the 95% confidence zone, and one point (number 16) lies outside the 99% confidence zone. Figure 8.8 and 8.9 show the $Q_{residual}$ and T^2 values at 95% confidence limits. $Q_{residual}$ of sample number 8 and 16 are outside the 95% limit; T^2 of sample number 16 and 32 are outside the limit. Incorporation of the analysis of $Q_{residual}$, T^2 and scores plots indicated a unique sample at number 16. The contribution plot of $Q_{residual}$ and T^2 of variables at sample 16 (Figure 8.10) diagnosed an abnormal contribution of variable number 4 (taxol) compared to other samples near by (not shown). According to the fit of this data set with our binding and inhibition models (Chapter 3) as shown in Figure 8.11, sample 16 is the only point for which the taxol concentration varied from the prediction of the hypothetical model. Replacement of taxol data in sample 16 with the value obtained from our model shows that $Q_{residual}$ of the replacement data falls within the statistical limits (not shown).

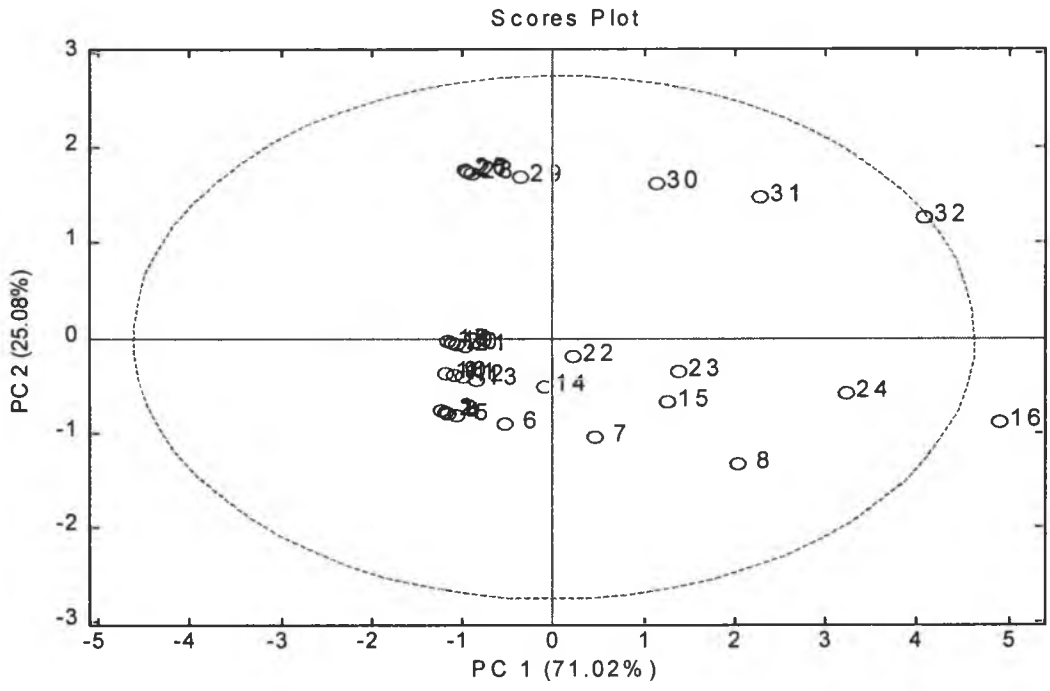


Figure 8.6Graphical representation of principal components analysis of taxol data with 99 % limits.

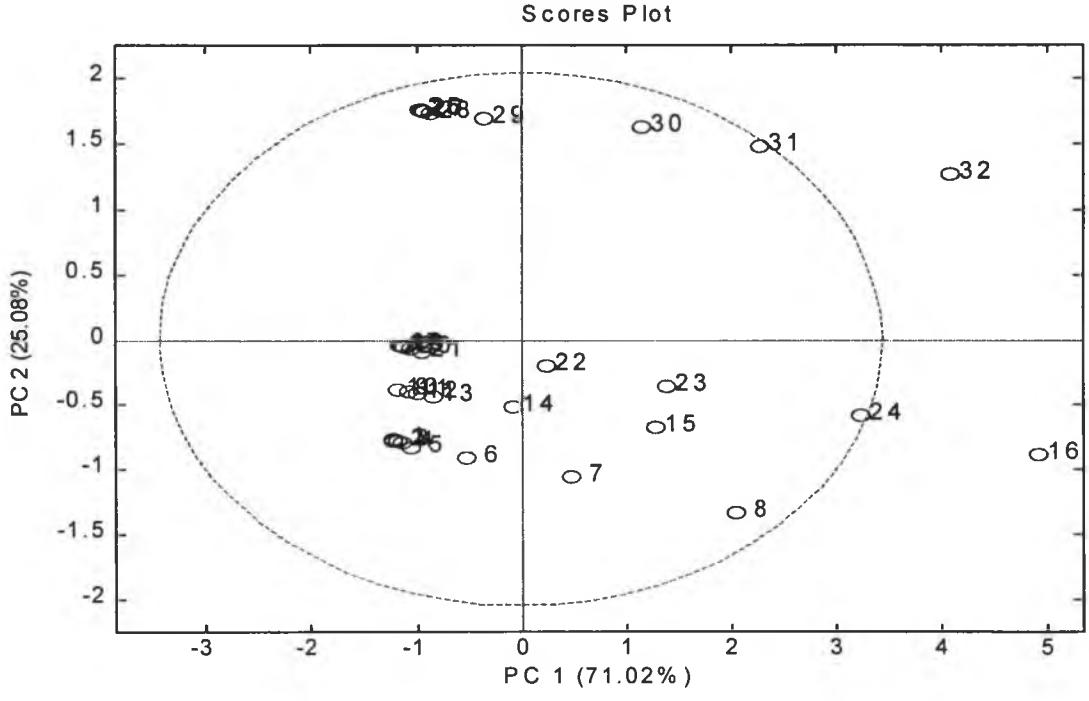


Figure 8.7Graphical representation of principal components analysis of taxol data with 95 % limits.

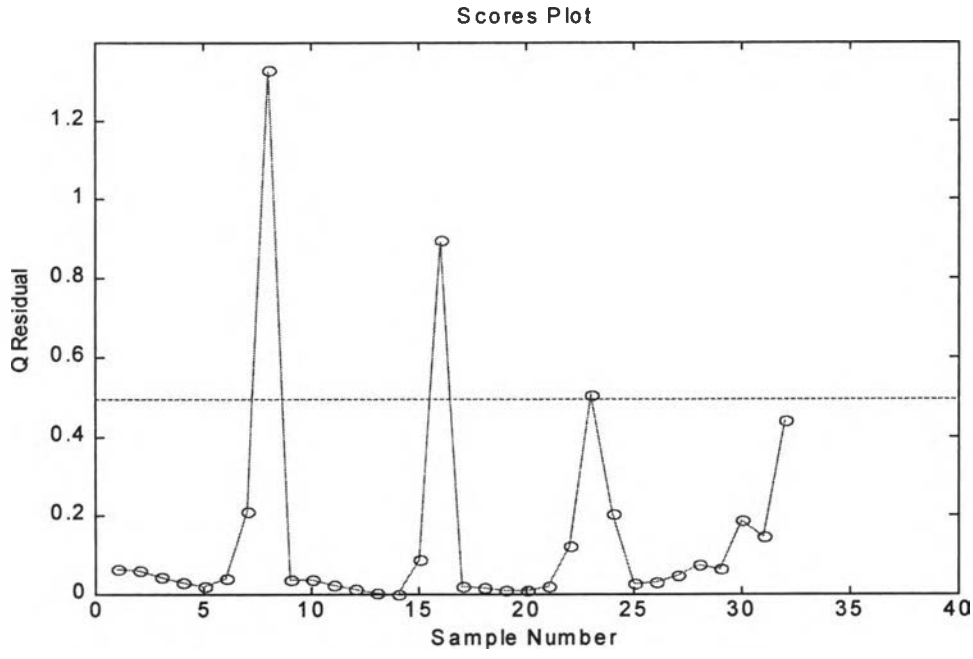


Figure 8.8 Sample Q residuals of taxol data for 2 PC model with 95% limits

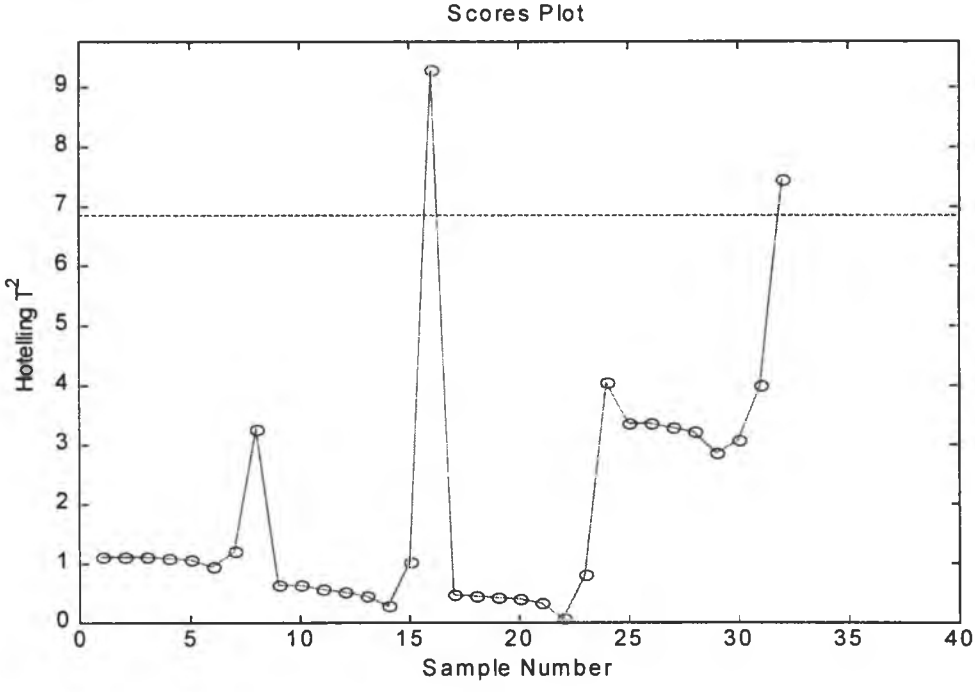


Figure 8.9 Hotelling's T^2 of Taxol data for 2 PC Model with 95% Limits

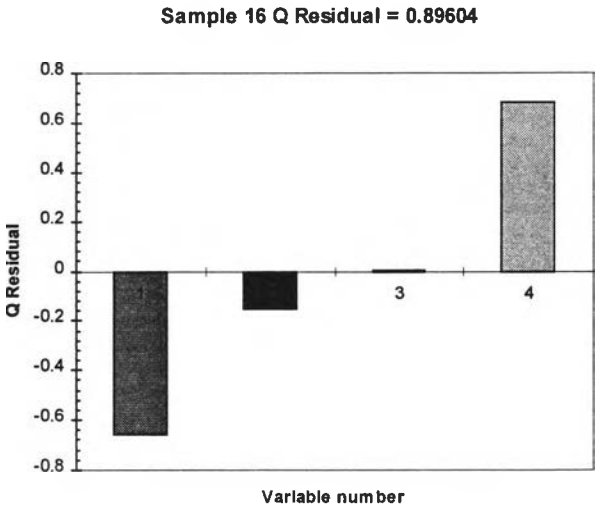


Figure 8.10 The contribution plot of $Q_{residuual}$ of variables at sample 16.
1= MJ, 2= C_2H_4, 3= DAT, 4= Taxol

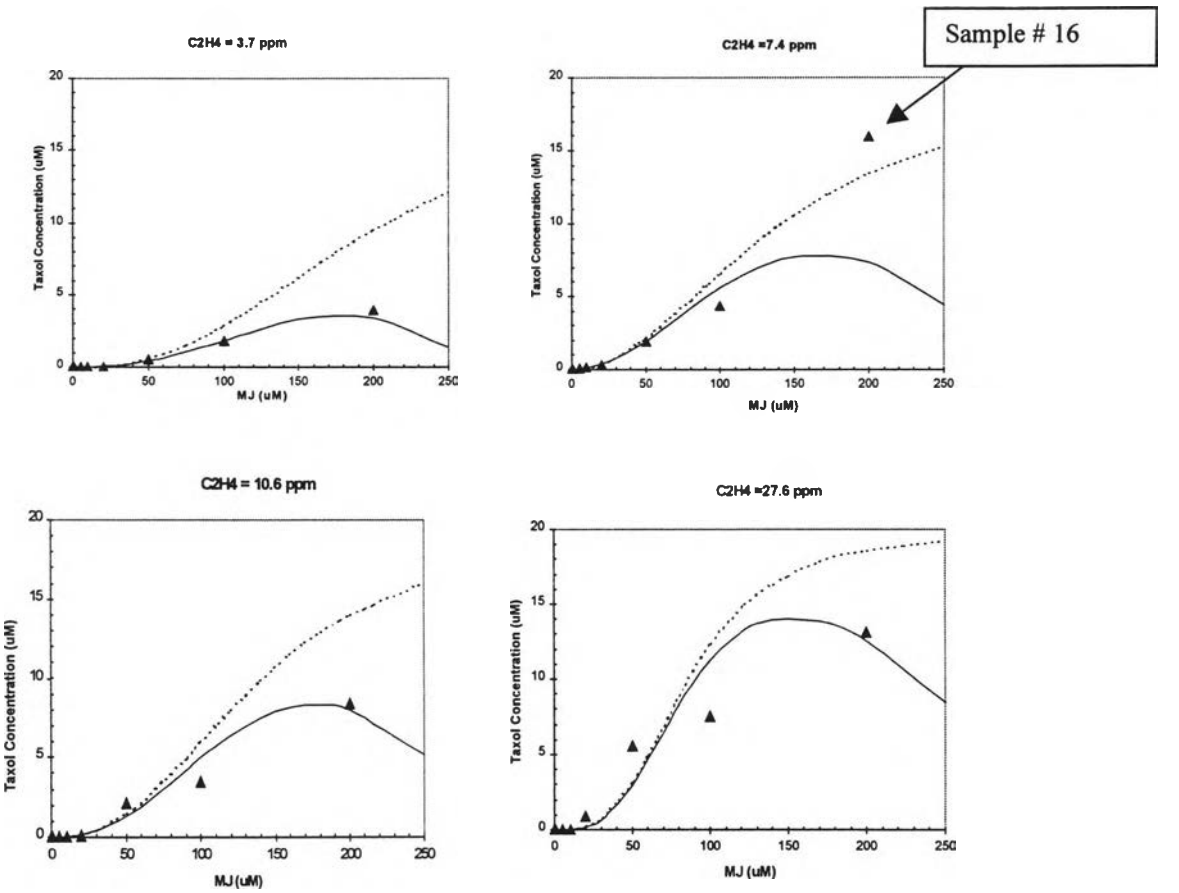


Figure 8.11 The comparison of data from experiments and from hypothetical model.

▲ = Experimental data, —— = Binding model, ----- = Binding + Inhibition model

8.4 Cross Correlation

A measure of the dependence of the variables in a dimensionless quantity can be obtained from the correlation coefficient (r). Correlation shows the amount of correlation between variables in a data set.

$$r = \frac{S_{XY}}{S_X S_Y} \tag{14}$$

Where sample variances and covariance of x and y are given by

$$S_X^2 = \frac{\sum (x-\bar{x})^2}{n}, \qquad S_Y^2 = \frac{\sum (y-\bar{y})^2}{n}, \qquad S_{XY} = \frac{\sum (x-\bar{x})(y-\bar{y})}{n} \tag{15}$$

n = number of sample.

Therefore,

$$r = \frac{\sum (x-\bar{x})(y-\bar{y})}{\sqrt{\sum (x-\bar{x})^2}\sqrt{\sum (y-\bar{y})^2}} \tag{16}$$

or

$$r = \frac{n\sum xy - (\sum x)(\sum y)}{\sqrt{[n\sum x^2 - (\sum x)^2][n\sum y^2 - (\sum y)^2]}} \tag{17}$$

Since $S_{XY} \le S_X S_Y$,

Therefore $-1 \le r \le 1$

If x and y are independent, then Cov(x,y) = S_{xy} = 0, or r = 0. If x and y are completely dependent, Cov (x, y) = S_{xy} =$S_x S_y$, or r = 1. The value of r^2 can be interpreted as the fraction of the explained variation (obtained from a linear regression between x and y) to the total variation. If all of the total variation is explained by the regression line, then r^2 = 1 or r ± 1, which means a perfect linear correlation. If r is positive, y tends to increase with x (the slope of the least-squares line is positive). If r is negative, y tends to decrease

with x (the slope of the least-squares line is negative). In case of r =0, all of the total variation is unexplained, which means variables x and y are uncorrelated. A correlation coefficient measures how well a given regression curve fits sample data. Therefore, for nonlinear data, $|r| < 1$ may not mean an insignificant correlation, but means small linear correlation, which may be a large nonlinear correlation.

8.5 Cross Correlation: Application to Taxane Data

Suspension cultures of *Taxus canadensis* (C93AD) were challenged with various concentrations of ethylene and methyl jasmonate (MJ). The concentrations of taxol and other taxanes were determined after 21 days of cell culture transfer. Eight MJ concentrations (0-200 μM) were studied at four constant headspace ethylene concentrations (3.7, 7.4, 10.6 and 27.6 ppm), one oxygen concentration (15% (v/v)) and one carbon dioxide concentration (0.5 %(v/v)). The results of taxol, 10-deacetyl taxol (10-DAT), baccatin III, and 10-deacetyl baccatin III production responses to concentration of MJ and ethylene are discussed in Chapter 3.

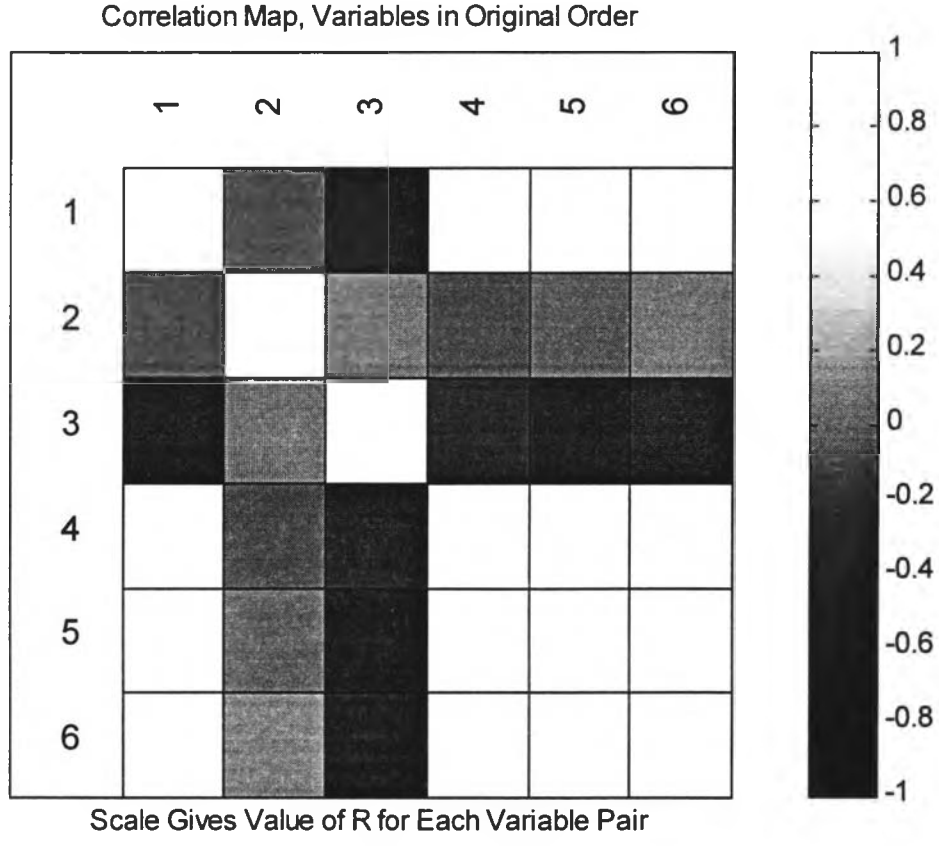
To elucidate the correlation between MJ or ethylene on taxane production and the relationship among the observed taxanes, a correlation matrix analysis was performed. A map of the correlation (Figure 8.12) and the correlation coefficient (Table 8.2) were performed using the PLS_Toolbox 2.0. The correlation coefficients between each pair of observed variables show:

1) Strong positive linear relationships between MJ and 10-DAT, taxol and baccatin-III concentration. The correlation coefficient between a pair of MJ and 10-DAT, MJ and taxol, and MJ and baccatin III is 0.923, 0.870 and 0.828, respectively.

1) Strong positive linear relationships between MJ and 10-DAT, taxol and baccatin-III concentration. The correlation coefficient between a pair of MJ and 10-DAT, MJ and taxol, and MJ and baccatin III is 0.923, 0.870 and 0.828, respectively.
2) Strong positive linear relationships between 10-DAT and taxol, 10-DAT and baccatin III and taxol and baccatin III: the correlation coefficients are 0.940, 0.928 and 0.832, respectively.
3) Positive relationships between ethylene and taxane concentrations. The correlation coefficients between ethylene and 10-DAB, 10-DAT, taxol, and baccatin III are 0.172, 0.125, 0.196 and 0.009, respectively.
4) Negative linear relationship between MJ and 10-DAB; the correlation coefficient is – 0.394.

The interpretations of the data using cross correlation accommodate the results observed in our kinetics studies with and without supplementation of taxol side chain (N-benzoyl-L-phenylisoserine) and acetyl-CoenzymeA discussed elsewhere (Chapter 6). The correlation coefficients between MJ and taxanes show the strong effect of MJ on the accumulation of taxane with side chain (10-DAT, taxol). Therefore, synthesis or attachment of the N-benzoyl-3-phenylisoserine ester to C-13 of 10-deacetylbaccatin III to form taxoids appears under the control of MJ.

These observations also provide hints about the order by which these compounds appear in the final assembly of taxol. The cross correlations among the taxanes support our postulated pathways from 10-DAT to taxol and from taxol to baccatin III. The biosynthesis pathway of taxol from geranylgeranyl pyrophosphate was proposed (Chapter



1= MJ , 2= C2H4, 3= 10-DAB, 4=BACCATIN-III, 5= 10-DAT, 6=TAXOL

Figure 8.12 The correlation map shows the amount of correlation of between variables in the taxol data set.

	1	2	3	4	5	6
1	1.0000	0.0000	-0.3935	0.8275	0.9232	0.8695
2	0.0000	1.0000	0.1717	0.0086	0.1249	0.1959
3	-0.3935	0.1717	1.0000	-0.2393	-0.3856	-0.3080
4	0.8275	0.0086	-0.2393	1.0000	0.9277	0.8316
5	0.9232	0.1249	-0.3856	0.9277	1.0000	0.9400
6	0.8695	0.1959	-0.3080	0.8316	0.9400	1.0000

Table 8.2 The correlation coefficients of between variables in the taxol data set.

5). The separated pathway of taxanes with side chain (10-DAT, taxol) from taxanes without side chain (10-DAB and baccatin III) was suggested. 10-DAT could be a direct precursor of taxol. Baccatin III may be either a degradation product of taxol or product from 10-DAB.

8.6 Conclusions

PCA is performed on the experimental data. Control limits then can be established using Hotelling's T^2 and Q residuals. From the analysis of T^2 and Q residuals, unusual samples can be detected by comparison with the control limits. The loading plot then can be used to identify the specific cause of "fault" in that sample. The lack of fit of the detected sample from PCA model agrees well with the fitting between experimental data and the values obtained from a binding model (Chapter 3).

The interpretations of the data using cross correlation techniques accommodate the results observed in previous studies. The results support the understanding of the effect of MJ and ethylene on taxane production and a hypothetical biosynthetic pathway for taxol.

The results of the PC analysis are encouraging and suggest that the method could be used for quality monitoring and fault detection. However, since the method is based on the assumption of normality, one of the concerns is whether the scored data is normally distributed. Additionally, the cross correlation matrix that is determined by calculating the correlation coefficients for each pair of variables can be a useful tool to measure the dependence of the variables in a dimensionless quantity.

CHAPTER 9

CONCLUSIONS AND RECOMMENDATIONS

MJ is an effective elicitor to stimulate taxane production in *Taxus canadensis* (C93AD). Taxol production was enhanced about 30 fold over unelicited conditions using 0.5% CO_2, 15% O_2 and 7 ppm ethylene with 200 μM MJ injection after 8 days of cell transfer. In Chapter 3, the mechanism of MJ and ethylene action on the taxol biosynthesis pathway is discussed. We postulate that specific biosynthetic steps for taxane production in plant cell culture are regulated by allosteric regulation of ethylene binding, based on our interaction and binding modeling of taxol formation in elicited suspension cell cultures of *T. canadensis*. This allosteric behavior could, but does not necessarily, indicates modulation of ethylene binding by MJ. Inhibition effects of MJ concentration on taxol production, especially at concentrations greater than 100 μM, is observed and expressed in mathematical terms in the developed model.

The interdependence of methyl jasmonate with chitin and chitosan derived elicitors is studied using plant cell suspension cultures of *T. canadensis*. The optimal elicitor concentration using N-acetylchitohexaose was 450 nM, but only in the presence of methyl jasmonate. The optimal MJ for co-mediation with colloidal chitin or oligosaccharides of chitosan as elicitors was 100-200 μM. Since the relative stimulation in experiments with headspace gas regulation was not as striking as in the system without headspace gas regulation, elicitation of chitin or chitosan oligosaccharides may induce

ethylene production by the plant cell cultures. Ethylene results in accumulation of taxanes when applied together with methyl jasmonate.

Taxol yields are improved by feeding potential taxol side chain precursors and acetylCoA together with MJ elicitation. The precursor studies with/without MJ elicitation provide hints about the order by which these compounds appear in the final assembly of taxol. Synthesis or attachment of the N-benzoyl-L-phenyl-isoserine ester to C-13 of 10-deacetylbaccatin III to form taxoids appears under the control of MJ. Since 10-deacetyltaxol accumulates to high concentrations in the culture medium, its conversion to taxol may be a rate limiting step. Acetylation of the C-10 position should occur intracellularly, probably analogous to acetylation of 10-deacetylbaccatin III as demonstrated using acetyl-CoA by crude cell homogenates of *T. cuspidata* (Zocher et al., 1996). Similarly, since the accumulation of baccatin III is linked to MJ as is 10-deacetyltaxol, these may also have an acetyl-CoA transesterification relationship.

Through this work, the biosynthesis pathway of taxol from geranylgeranyl pyrophosphate was proposed as shown in Figure 9.1 following the examination of taxol and other taxane profiles in our studies combined with the information from studies of other groups as described in Chapter 5. The separated pathway of taxanes with side chain (10-DAT, taxol and others) from taxanes without side chain (10-DAB and baccatin III) was suggested. 10-DAT could be a direct precursor of taxol. Baccatin III may be either a degradation product of taxol or a product of 10-DAB.

The kinetic studies indicated taxol production was not growth-associated. In the stationary phase, when growth was minimal, conditions favor the production of taxol and other taxanes. Therefore, prolonging the stationary phase of the culture could increase

productivity. A considerably larger amount of taxanes is observed using semi-continuous culture with total cell recycle. 10-DAT, taxol and baccatin III increased by a factor of 4.5, 4.0 and 3.0, respectively, relative to the batch culture during 35 days of cultivation. Overall sugar uptake in the semi-continuous culture increased by 3.0 times relative to the batch culture. Since 10-deacetyl taxol accumulates to high concentrations in the culture medium, feeding precursors together with MJ elicitation in semi-continuous culture with cell recycle or perfusion culture could be an alternative way to enhance taxol production.

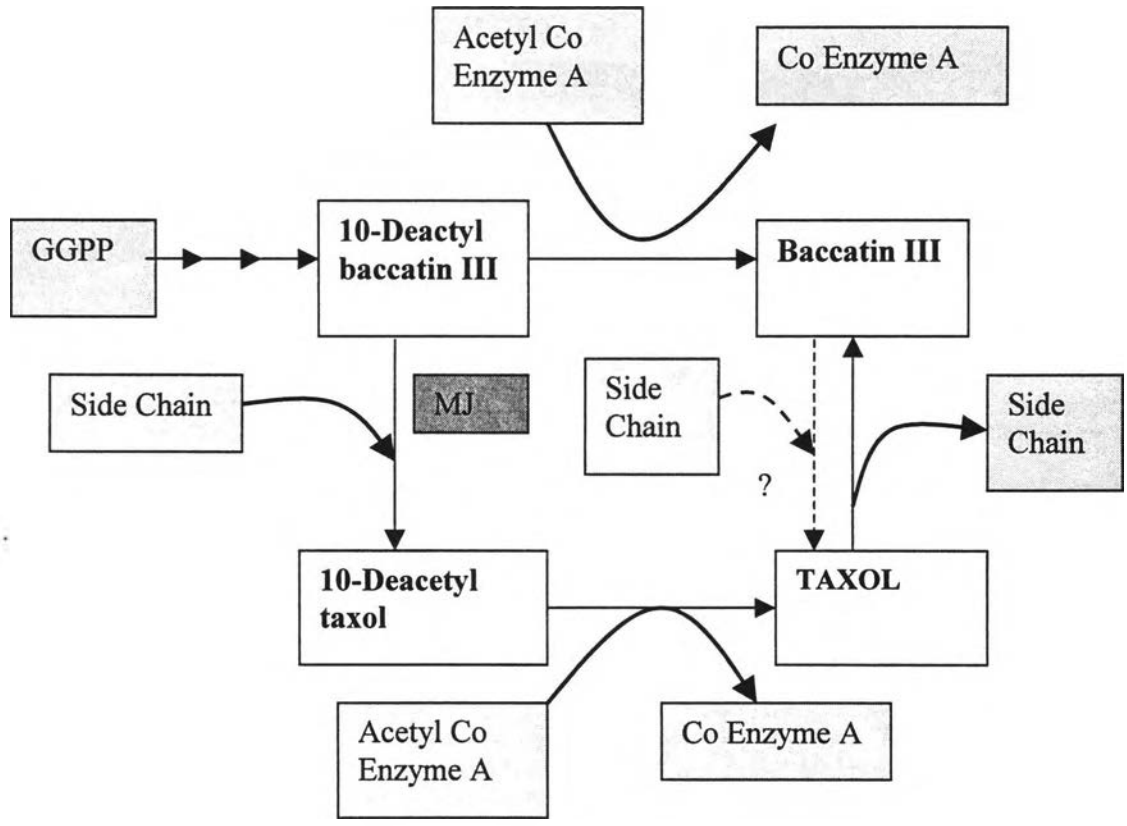


Figure 9.1 The proposed biosynthesis pathway of taxol from geranylgeranyl pyrophosphate (GGPP).

Although the amounts and profiles of sugar uptake in kinetic studies in the spinner flask and shake flask cultures are similar, the rates of production of total taxanes and growth of cells differed. During the kinetic studies in the spinner flasks, although low speed agitation was performed, a large amount of cell debris was observed. The lower cell growth and taxane production rates observed in the spinner flask cultures could be partial related to the effect of hydrodynamic shear stress.

In this work, some applications of statistics on experimental data are demonstrated. The statistical regression analysis and the linear regression model were applied to the data of Mirjalili and Linden (1995). The sign and magnitude of the coefficients of each factor allowed comparison of relative important parameters. The statistical model demonstrates the optimal headspace gas combinations was 17-15% (v/v) O_2, 0.5-1% (v/v) CO_2 and 6-8 ppm ethylene. Statistics associated with Principal Components Analysis (PCA) model are applied to determine the relevant variables in order to optimize taxol production and to use for quality monitoring and fault detection of the experimental data in Chapter 3. From the analysis of Hotelling's T^2 and Q residuals, a unique sample is detected by comparison to the control limits. The loading plot is then used to identify the specific cause of that sample. The lack of fit of the detected sample from the PCA model agreed well with the fitting between experimental data and the values obtained from the hypothetical model discussed in Chapter 3. The interpretations of the data using cross correlation techniques accommodate the results observed in previous studies (Chapter 3-7). The results support the understanding of the effect of MJ and ethylene on taxane production and the biosynthetic pathway for taxol as discussed in Chapter 5.

The results of the PC analysis are encouraging and suggest that this method could be used for quality monitoring and fault detection. However, since the method is based on the assumption of normality, one of the concerns is whether the score data are distributed normally. The cross correlation matrix determined by calculating the correlation coefficients for each pair of variables can be a useful tool to measure the dependence of the variables in a dimensionless quantity.

Further Directions

As mentioned before, variability is the most problematic issue in using cell cultures of *Taxus sp.* for taxol production. The instability of taxol production in *Taxus sp.* has also been reported in other studies (Mirjalili and Linden, 1995; Hirasuna et al., 1996; Ketchum and Gibson, 1996; Ketchum et al., 1998). Variability may be due to genetic changes by mutation in the culture or epigenetic changes due to physiologic conditions. Cell maintenance conditions such as initial cell density, length of subculture interval, temperature etc. were found strongly to affect secondary metabolism of the plant cells. The understanding of the causes of the variability in the productivity and how to control them are important, especially for production in the commercial scale. The development of techniques to maintain the cell lines for a long term, such as freezing or freeze-drying, is necessary.

Genetic engineering of metabolic pathways based on fundamental understanding of the biosynthetic pathways, especially for the rate limiting steps, will be a promising tool to enhance taxol productivity of the cell line. The detailed knowledge of the biosynthetic pathway can be obtained from many approaches. Elicitation followed by characterization of the induced enzymes in the pathway and the genes encoding them can be one of the

potential approaches. More understanding of the detailed pathways could be obtained by in vitro studies for a specific reaction using a radiolabeled precursor. Mathematical simulation of kinetic profiles of taxanes and feeding precursors with and without elicitation should be an alternative way to predict rate limiting steps and will provide more understanding of the function of applied elicitors.

From the engineering point of view, one barrier of large scale development for plant cells is the fragile nature of the cells when coupled with the hydrodynamic shear stress in large reactors. Further improvement of reactor agitation systems or of special techniques such as addition of shear protective agents to protect the cell against shear damage with no negative effect on the production and allow adequate mixing is required.

Another consideration for production scale is the process strategy. Since taxol is a non-growth associated product; several operation approaches with elicitation and potential precursor feeding could be an effective way to improve the productivity, such as by semi-continuous culture with cell recycle, perfusion, immobilization, and two-stage systems. Specific process designs can be developed based on the nature of the product accumulation and their production kinetics.

Gas phase composition and the concentration of elicitors in the system are the important keys to stimulate taxol production. The on-line monitoring and control of the head space gas and medium composition could result in greater taxol production. A complex neural network could be used as a dynamic predictor of the behavior of the system and could be useful for application within control and optimization schemes in the large scale production. Statistical multivariable analyses, such as PCA, PLS and MPCA,

could be useful techniques to determine the relevant variables of the system for optimization of production and for process monitoring and fault detection.

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APPENDIX A

STATISTICAL REGRESSION MODEL

The data of the effect of controlled headspace gas on taxol productivity in suspension cultures of *Taxus cuspidata* from the dissertation of Mirjalili (1995) was analyzed using statistical program (SAS) to identify the optimum of gas combination and the interaction of each parameters. A linear regression technique was used to fit the data on quadratic equations containing variables in the system. The sign and magnitude of the coefficients of each factor allowed comparison of important parameters. The optimization was obtained from the derivative of the quadratic equation (A1).

$$\begin{aligned}\text{Taxol productivity} = {} & -2.0734 + 0.3068[O_2] + 0.0755[\log (CO_2)] + 0.3656[\log (C_2H_4+1)] \\ & - 0.0087 [(O_2)^2] + 0.00125 [O_2 \times \log (CO_2)] - 0.1197[(\log (CO_2))^2] \\ & - 0.01164 [\log (C_2H_4+1) \times O_2] - 0.0757 [\log(C_2H_4+1) \times \log (CO_2)] \\ & - 0.0914 [(\log(C_2H_4+1))^2] \qquad \text{(A1)}\end{aligned}$$

At the maximum point, ethylene (C_2H_4), oxygen (O_2) and carbon dioxide (CO_2) are 6.29 ppm, 17.00 % (v/v) and 1.35 % (v/v) respectively, with 0.697 mg/L day of taxol productivity. The statistical analysis shows that $\log(C_2H_4+1)$, (O_2), $(O_2)^2$, $(\log CO_2)^2$, $[\log(C_2H_4+1) \times (O_2)]$, $[(\log(C_2H_4+1))^2]$ terms have effects at the 95% significant level on taxol production. The effects of $\log(C_2H_4+1)$, (O_2) are positive, while the effects of $(O_2)^2$,

$(\log CO_2)^2$, $[\log(C_2H_4+1) \times (O_2)]$, $[(\log(C_2H_4+1))^2]$ are negative. The effects of ethylene, oxygen and carbon dioxide on taxol production are shown in Figure A.1.

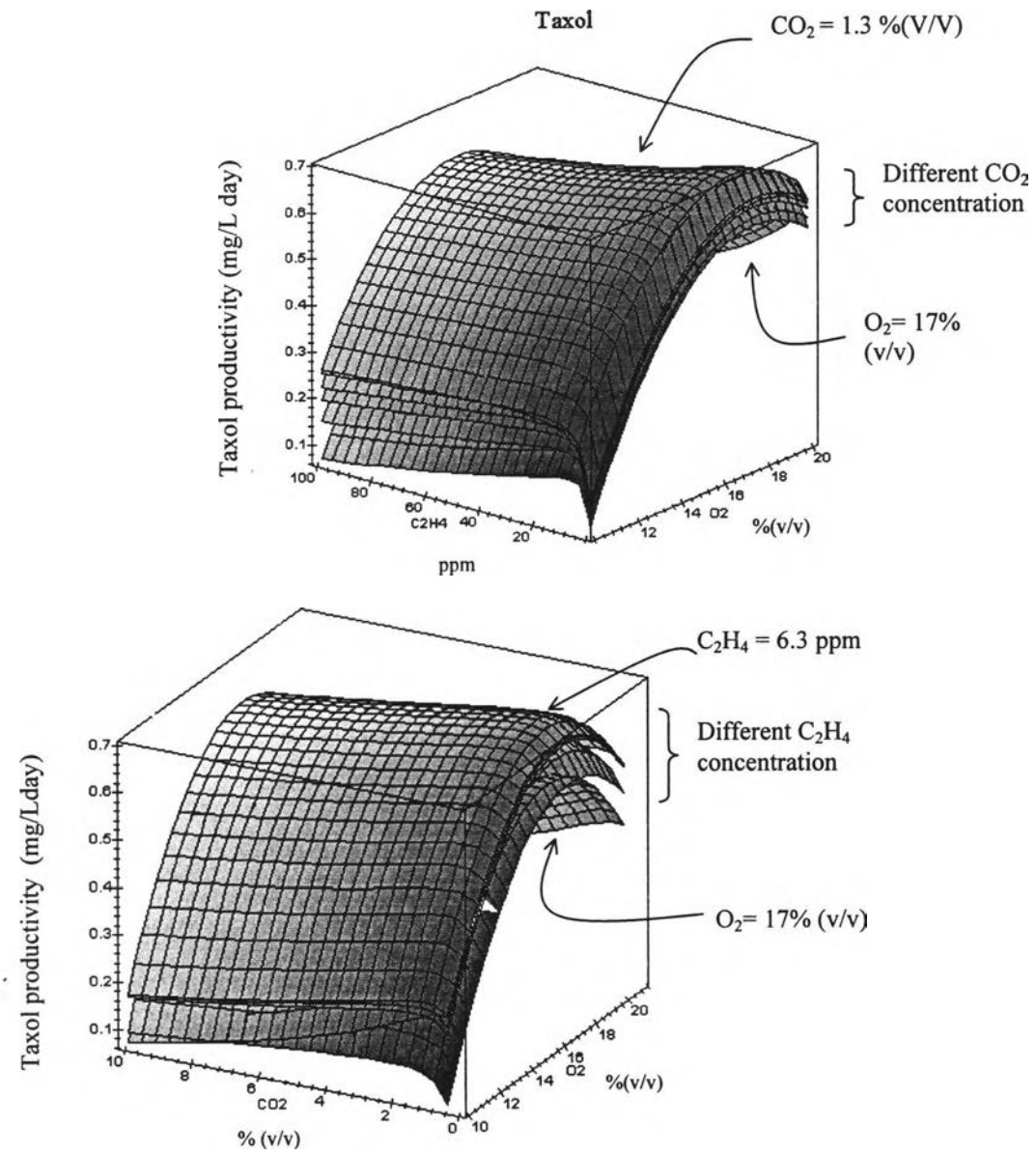

Figure A.1 Dependence of taxol production on headspace gas concentration of ethylene, carbon dioxide and oxygen according to the linear regression model.

APPENDIX B

IN VITRO STUDIES

The biosynthesis of taxol is a multistep process. One intermediate reaction was thought to be the acetylation of 10-deacetylbaccatin III (10-DAB) to baccatin III, an assumed precursor of taxol (Zocher, 1996). Fett-Neto and DiCosmo (1996) detected the activity of acetyl-transferases converting 10-deacetylbaccatin III to baccatin III and 10-deacetyltaxol to taxol, both using acetyl-CoA as acetyl donor in cell-free protein extracts from needles and cell suspensions of *Taxus sp.* However, the activities observed were relatively low. The studies by Zocher et al. (1996) demonstrated the cell free acetylation of 10-DAB to baccatin III in crude extracts from roots of *Taxus baccata* saplings using ^{14}C-or ^{3}H-labeled acetyl-coenzyme A as the acetyl donor. Free taxanes and the release of bound compounds having taxane antibody reactivity by xylanase in female, haploid-derived cell suspension cultures of *Taxus brevifolia* was reported (Durzan and Ventimiglia, 1994).

The examination of taxane profiles in our studies indicated a large amount 7-xylosyl-10-deacetyl taxol (7-xylo-10-DAT) compared to taxol and others in the system elicitation with methyl jasmonate (MJ). In Chapter 5 and Chapter 6, improved taxol yield and increase of ratios of taxol to 7-xylo-10-DAT following addition of acetyl-Coenzyme A in suspension cultures of *T. canadensis* (C93AD). Therefore, deacetyl taxol (10-DAT) may be a direct precursor of taxol (discussed in Chapter 5).

To confirm the proposed pathway from 10-DAT to taxol, in vitro studies were performed using crude extracts from suspension cell of *T. canadensis* (C93AD). The experimental procedures of the experiment was followed the procedures described by Zocher et al. (1996) with acetyl Coenzyme A as acetyl donor.

Materials and Methods

Preparation of cell free extracts: The suspension cell cultures used in the experiment part B.1 were obtained from the 23 day old cells in shaking flasks with and without 100 μM MJ elicitation. The suspension cell cultures used in the experiment part B.2 (kinetic study) were obtained from frozen cells of a 35 day old spinner flask culture with 100 μM MJ elicitation. The suspension cell cultures were centrifuged and washed three times with distilled water before were frozen and pulverized in a mortar. The resulting power was extracted with buffer (1:1 v/v). The buffer was formulated according to Zocher et al. (1996) (Table B.1). The homogenate was centrifuged at 15,000 rpm for 60 min. The supernatant was then desalted by passage through a Sephadex G 25 column, which was previously equilibrated with the same buffer without dithiothreitol.

Acetylation experiments: For experiment part B.1, 2 mL of crude extract were mixed with 5 mM $MgCl_2$, 0.02 mL of ethanolic solution of taxane, and 0.10 mL of acetyl CoA solution (25 mg/mL). For experiment part B.2, 5 mL of crude extract were mixed with 5 mM $MgCl_2$, 0.03 mL of ethanolic solution of taxane, and 0.12 mL of acetyl CoA solution (25 mg/mL). Experiment part B.1 and B.2 were conducted in 5 mL and 10 mL vials respectively, and then incubated at 25°C in a shaking incubator. Samples were taken after 21 hr of incubation (Expt.B.1) and after 2, 3, 12 and 21hr of incubation (Expt. B.2) for analysis of taxol and other taxanes followed the procedures described in Chapter 2.

Components	Concentration
$Na_2S_2O_5$	10 mM
Sodium ascorbate	10 mM
Dithiothreitol	5 mM
Polyvinylpyrrolidone ($M_r \approx 10,000$)	1% (w/v)
Tris/HCl (pH = 7.8)	100 mM
Glycerol	10% (v/v)

Table B.1 Buffer preparation.

Results and Disscusion

The results of enzymatic acetylation of 10-DAB, 10-DAT and 7-xylo-10-DAT were summarized in Table C1. The summarized of the results are described below:

1) With 10-deacetyl baccatin III (10-DAB) as a substrate, we observed baccatin III and unknown peak at retention time 6.4 min (Table B.1) where, the accumulations of baccatin III after 21 hours in the treatments with acetyl CoA supplementation are greater than in the treatments without acetyl CoA supplementation. No significant difference of baccatin III accumulation was observed between using crude enzyme preparations from suspension cell cultures treated with or without MJ.
2) With 10-deacetyl taxol (10-DAT) as a substrate, we observed a striking peak at retention time =18 min (taxol retention time = 19 min); the concentration of this unknown taxane is about 16-20% of 10-DAT concentration. Taxol and 10-DAB peak were observed at very low concentrations (less than 0.2 % and 0.8% 10-DAT, respectively). We also observed unknown peaks at 9 and 25 min. No significant

difference was observed between using crude enzyme preparation from suspension cell cultures treated with or without MJ and with or without acetyl CoA supplementations.

3) With 7-xylosyl-10-deacetyl taxol (7-x-10-DAT) as a substrate, we observed an increase in the taxol peak (< 0.06% of 7-x-10-DAT concentration) and the 10-DAT peak (< 3% of 7-x-10-DAT concentration). No significant difference was observed between using crude enzyme preparation from suspension cell cultures treated with or without MJ and with or without acetyl CoA supplementations.
4) From kinetic study (at 2, 3, 12 and 21 hr); taxane concentrations obtained from the reactions described in 1), 2) and 3) increased with time (Part B.2).

Although we have detected the activity of acetyl-transferase converting 10-DAB to baccatin III and 10-DAT or 7-xylosyl-10-DAT to taxol in crude enzymes extracts from cell suspensions of *T. canadensis* (C93AD), the activities observed were relatively low. Overall, no significant difference between using crude enzyme preparation from suspension cell cultures treated with or without MJ and with or without acetylCoA supplementation was observed. The results show MJ elicitation may not be involved in regulation of the acetyl-transferase activities. However, in our in vivo studies the ratio of taxol to 10-DAT was greater when the cells were elicited with MJ (Chapter 5). The question is why no significant difference was observed between the treatments with and without acetylCoA supplementation. Could another source of the acetyl group exist in the prepared enzyme solution or buffer than in vivo? Additional experiments, however, are recommended to confirm the existence of these activities using radiolabeled acetyl-CoA.

The verification of taxane peaks observed from the in vitro studies was kindly provided by Dr.D.M. Gibson (USDA/ARS at Ithaca, NY) based on absorption spectra.

Table B.2 The HPLC results from in vitro studies.

PART B.1 Enzymatic Acetylation of 10-DAB, 10-DAT & 7x 10-DAT :
: Enzyme obtained from the crude extracts of 23 day old suspension cell *T. canadensis* in shaking flask w& w/o MJ

Substrate	Culture	Inc Time hr)	TAXANE CONCENTRATION (mg/L)								
			DAB 5 min	6.4* min	Baccatin III 7 min	7 x- DAT 8.8 min	9 ** Min	DAT 11.5min	18*** Min	Taxol 19 min	25*** min
DAB	MJ elicited	21	**19.046**	0.476	0.019		0.075		0.044		
DAT	MJ elicited	21	0.762				1.447	**125.786**	22.012	0.188	0.629
7x DAT	MJ elicited	21				**52.767**		1.258		.031	
DAB+AA	MJ elicited	21	**28.571**	0.476	0.381		0.063	0.283	0.025		
DAT+AA	MJ elicited	21	0.476				1.447	**125.786**	20.755	0.252	0.629
7x DAT+AA	MJ elicited	21				**51.572**		1.572		0.019	
DAB	unelicited	21	**23.810**	0.762	0.029			0.023	0.022		
DAT	unelicited	21	0.952				1.886	**125.786**	25.157	0.256	0.755
7x DAT	unelicited	21				**44.025**		1.447	0.038		
DAB+AA	unelicited	21	**20.952**	0.476	0.286	0.063		0.252	0.016		0.031
DAT+AA	unelicited	21	0.952				1.258	**125.786**	18.868	0.252	0.629
7x DAT+AA	unelicited	21				**44.025**		0.943	0.038	0.019	

PART B.2 Kinetic Study: Enzyme obtained from the crude extracts from frozen cell of 35 day old suspension cell *T. canadensis* (C93AD) from spinner flasks.

Substrate	Culture	Inc Time (hr)	DAB 5 min	6.4 * min	Baccatin III 7 min	7 xylo DAT 8.8 min	9** min	DAT 11.5min	18*** Min	Taxol 19 min	25*** min
DAB+AA	MJ elicited	2	**19.048**	0.095	0.048	0.063	0.126	0.075		0.006	
DAT+AA	MJ elicited	2	0.476					**125.786**	1.887	0.063	0.126
7x DAT+AA	MJ elicited	2				**30.818**				0.006	
DAB+AA	MJ elicited	3	**16.190**	0.143	0.058	0.063	0.126	0.082		0.013	
DAT+AA	MJ elicited	3	0.429		0.067			**125.786**	3.428	0.176	0.189
7x DAT+AA	MJ elicited	3				**16.981**		0.251	0.006		
DAB+AA	MJ elicited	12	**23.809**	0.238	0.120	0.145	0.220	0.157		0.013	
DAT+AA	MJ elicited	12	0.333		0.067	0.157	1.070	**125.786**	6.289	0.157	0.283
7x DAT+AA	MJ elicited	12				**21.384**		0.283	0.008		
DAB+AA	MJ elicited	21	**23.809**	0.238	0.143	0.440	0.534	0.535		0.063	
DAT+AA	MJ elicited	21	0.457		0.095	0.314	1.572	**125.786**	12.578	0.314	0.471
7x DAT+AA	MJ elicited	21				**44.025**		0.755	0.019	0.019	

DAB =10-DAB III, DAT = 10-DAT, 7xDAT = 7-xylosyl-10-DAT, AA= acetyl CoA
*, **, *** estimated concentration based on 10-DAB, 10-DAT and Taxol peak, respectively.

SAMPLE	OBSERVED TAXANE PEAK			
	Retention time (min)	(name)	Area (uV*sec)	Match Spectrum
*DAB/MJ elicited/ AA/ 21hr	6.34	Baccatin 3		
(sample from part B.1)	14.23	Taxol		
	4.38		1169627	Baccatin III
	6.18	Baccatin 3	921537	7-epi-10-DAT
	7.4		185110	7-epi-10-DAT
	7.93		1750010	
DAT/MJ elicited/ AA/ 21hr	8.82		74299437	
(sample from part B.1)	13.08	Caphalo-	13981110	Taxol
	14.23	taxol		
	18.67		508415	7-epi-10-DAT
	4.42		98054	
	5.42		41355	Cephalomannine
	6.03	Baccatin 3	10546294	Taxol
7xDAT/MJ elicited/ AA/21 hr	6.8		374341	10-D-7-xylo-taxol c
(sample from part B.1)	7.9		1479645	10-D-7-xylo-taxol c
	8.8		203527	Taxol
	12.09	Cephalo-	59417	
	14.23	Taxol		
	4.32		188309	Baccatin 3
DAB /MJ elicited/ AA/ 21hr	6.34			
(sample from spinner flask)	7.9		102310	10-D-7-xylo taxol c
	12.27	Cephalo-		
	14.23	Taxol		
	4.34		170360	Baccatin III
	4.77		48344	
	6.17	Baccatin 3	228452	7-epi-10-DAT
	7.37		112347	10-DAT
	7.91		942858	
DAT/MJ elicited/ AA/ 21hr	8.79		63105319	
(sample from spinner flask)	12.39	Cephalo-	70653	10-D-7-xylo taxol c
	13.06		4381181	Taxol
	14.23	Taxol		
	18.64		121499	10-DAT
*7xDAT/MJ elicited/ AA/ 21hr	6.34	Baccatin 3		
(sample from spinner flask	14.23	Taxol		

Table B.3 Verification of taxane peaks based on adsorption spectra.
* Too dilute solution.

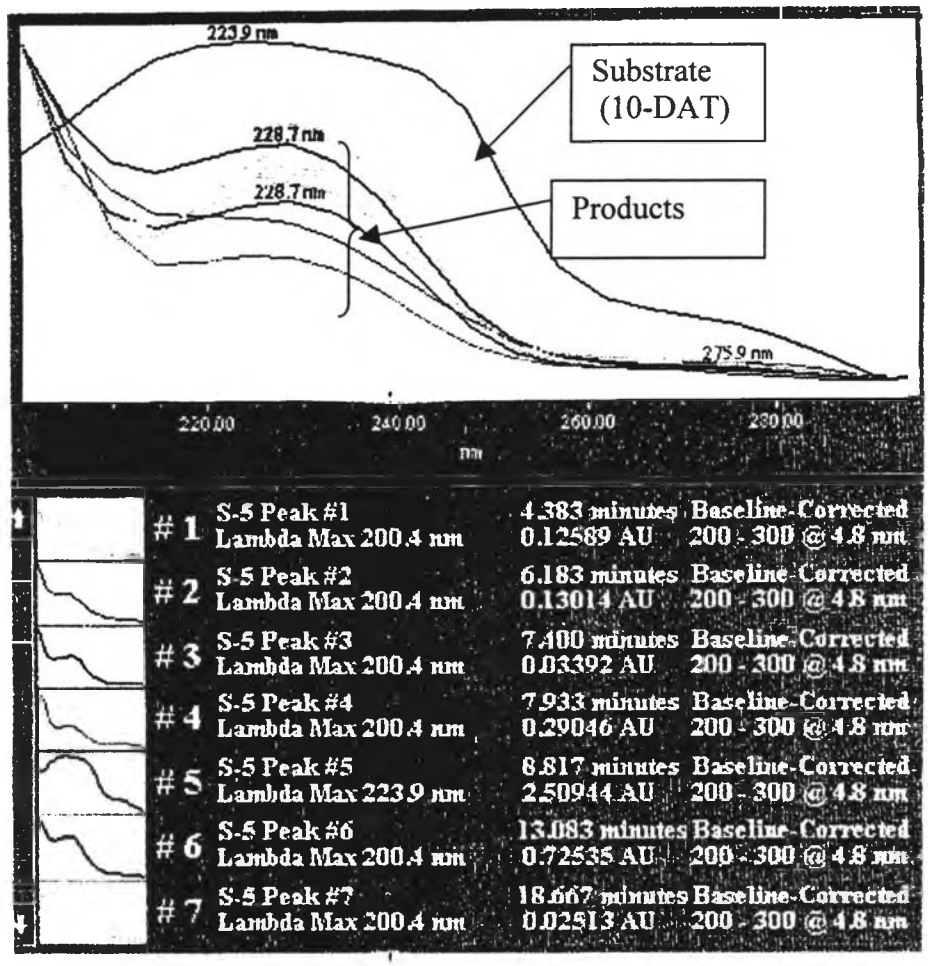

Figure B.1 The spectra of the sample obtained from the cell free acetylation of 10-DAT in crude extracts from suspension culture of *Taxus canadensis* (C93AD) using acetyl-Coenzyme A as the acetyl donor.

The results are summarized in Table B.3. From the verification, spectra of many taxanes were observed during acetylation using crude extracts from suspension cell of *T. canadensis* (C93AD). The interesting observation is the verification of a spectrum of the striking peak with retention time =18 min next to the taxol peak (retention time = 19 min) that formed by acetylation using 10-deacetyl taxol as the substrate. From the UV scan, electronic structure of this compound is very similar to taxol (Table B.2 and Figure B.1). There was no observation of this peak (18 min), when baccatin III or 7-xylosyl –10-DAT

was used as a substrate. Mass spectrometer parent ion analysis is recommended to identify this compound.

APPENDIX C

VERIFICATION OF THE UNKNOWN PEAK

The attempts to verify the unknown peak observed at retention time about 9 min from HPLC analysis as we referred as 10-DAT in this dissertation is discussed in this appendix. Since the retention time of this peak from HPLC analysis is in the range of 7-xylosyl-10-DAT (Figure C.1), the identification of this peak was based on 7-xylosyl-10-DAT. The absorption spectra showed similar trends with the standard 7-xylosyl-DAT (Figure C.2). However, the identification can not be confirmed since the trend is not clear due to the very diluted concentration of the sample. The identification of the compound by mass spectrometer has been conducted, however we did not observe the significant peak of 7-xylosyl-10-DAT or any taxane compound from the examined sample. The main problems are due to the diluted concentration and the purification of the examined sample. The analysis of pure and concentrated sample of this unknown compound by electron-spray mass spectrometer parent ion analysis is recommended to identify the structure and verification of the compound. However, if this compound is not 10-deacetyl taxol, it still might be a precursor of taxol. From the study in Chapter 3 to 7, we always observed the similar trend of this compound to taxol, and if the incubation time was long enough, at the end we observed the declined of this compound and the slightly increase of taxol (Chapter 7). In the precurssor study (Chapter 6) with supplementation of taxol

side chain we observed the increase of this unknown compound even greater than the increase of taxol. Supplementation of acetyl-CoA increased ratio of taxol to this compound. Also in the vitro studies (Appendix B), the acetylation of 10-DAT to taxol is promising. In the statistic analysis (Chapter 8), we observed the very strong linear correlation of taxol and this compound (correlation coefficient is always more than 0.8). The correlation of taxol and baccatin III is also in the positive direction but not as strong as the correlation of taxol and this unknown compound, whereas the correlation of taxol and 10-DAB is in the negative direction.

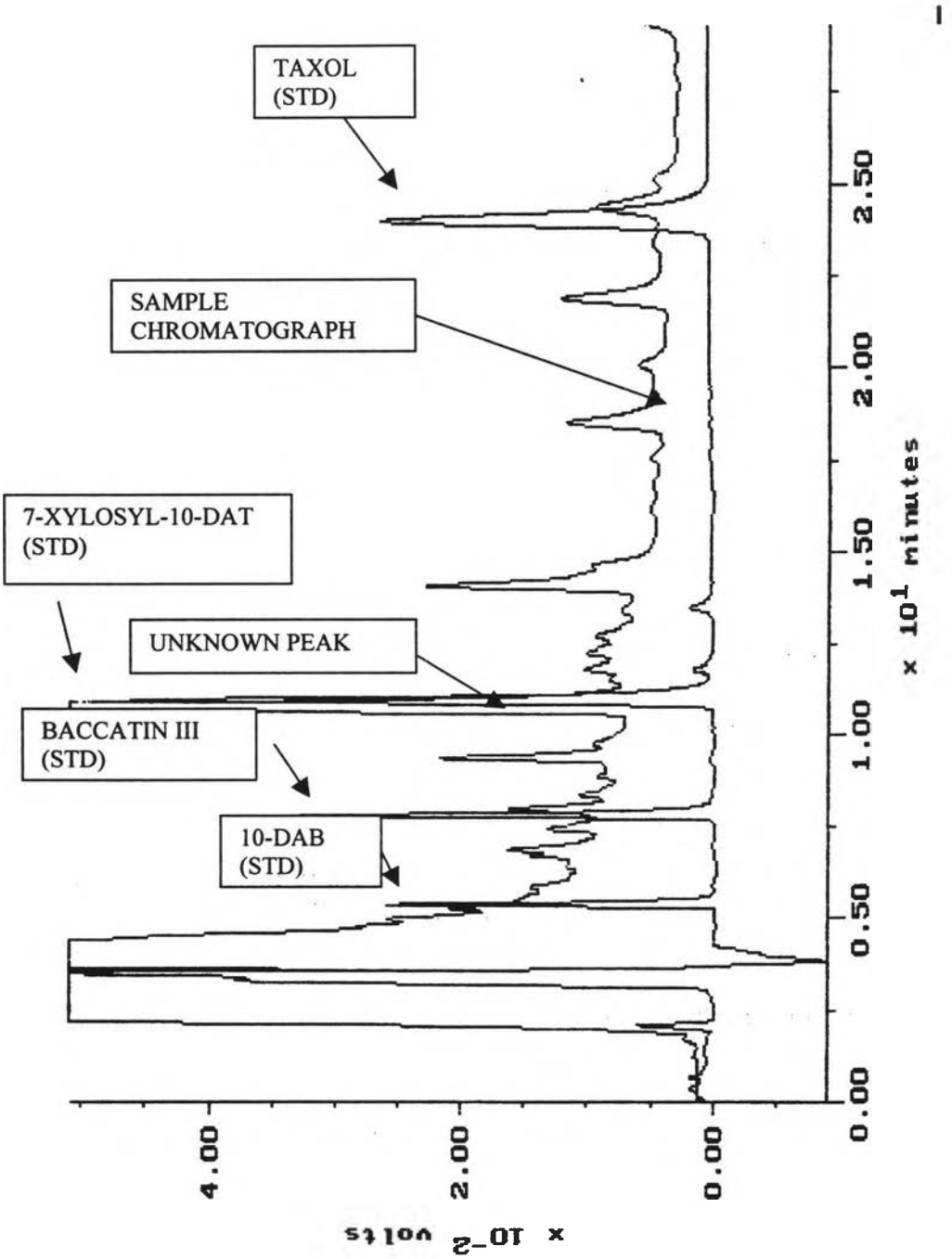

Figure C.1 Overlay profiles from HPLC analysis of a sample obtained from medium culture of *Taxus canadensis* (C93AD) and standard compounds (10-deactyl baccatin III, baccatin III, taxol and 7-xylosyl-10 deacetyl taxol).

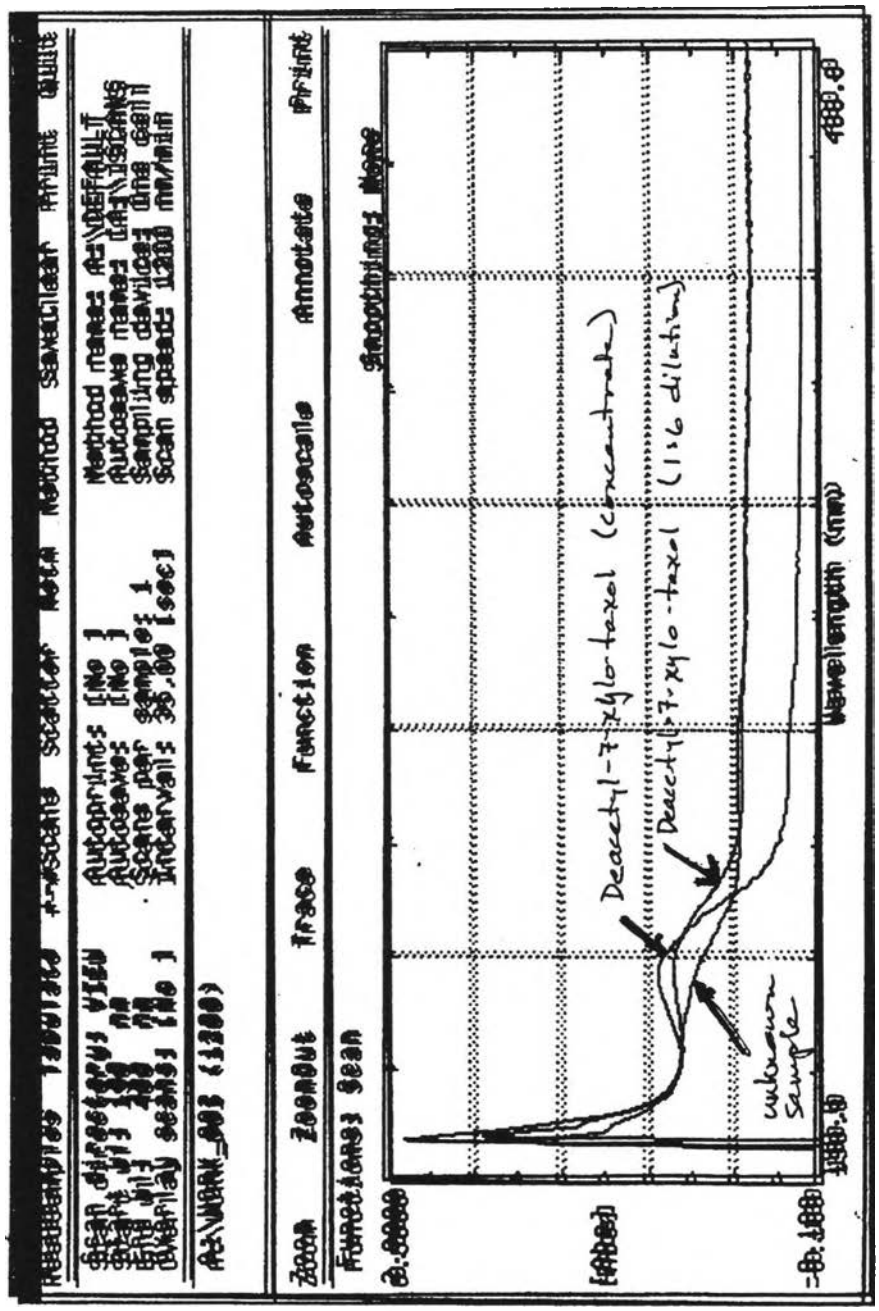

Figure C.2 Overlay spectra of absorption spectra from the unknown peak and 7-xylosyl-10-deacetyl taxol (standard sample obtained from Hauser Chemical Research, Inc.).

APPENDIX D

ELICITATION WITH FATTY ACIDS

Some carbohydrates and fatty acids were found effective as signal induction mediators for regulation of secondary metabolism of plant cells. In preview studies, we screened potential elicitors co-mediated with or without methyl jasmonate (MJ) for their ability to induce greater taxol production. In Chapter 3 and Chapter 4, effects of ethylene and MJ interaction and effects of chitin- and chitosan- oligosaccharide co-mediated with methyl jasmonate on taxane production had been discussed. The experimental results of elicitation with some other potential compounds than MJ and oligosaccharide in systems provided 6 ppm of ethylene, 15% oxygen, and 0.5% carbon dioxide on the headspace were summarized as shown in Figure D.1 and D.2. In experiment D.1, effects of elicitation with 10 μM fatty acids (1 = linolenic acid, 2 = linoleic acid, 3 = cis 5, 8, 11-eicosatrienoic acid, 4 = cis 8, 11, 14- eicosatrienoic, 5 = cis 5, 8, 11, 14, 17-eicosapentaenoic acid, 6 = arachidonoc acid, 7 = methyl jasmonate, and 8 = control (pure ethanol) on accumulation of taxol and baccatin in suspension cell cultures *Taxus cuspidata* after 21 days of incubation were observed. No significantly increase of taxanes was observed when elicited with eicosatrienoic acid, eicosatrienoic, eicosapentaenoic acid, or arachidonic acid. Improved taxol and baccatin-III approximately 2 fold relative to control were observed when the cultures were elicited with linolenic or linoleic acid. However, the

elicitation effect was not as striking as with MJ. Taxol and baccatin III increased 7 and 2 fold respectively, with MJ elicitation.

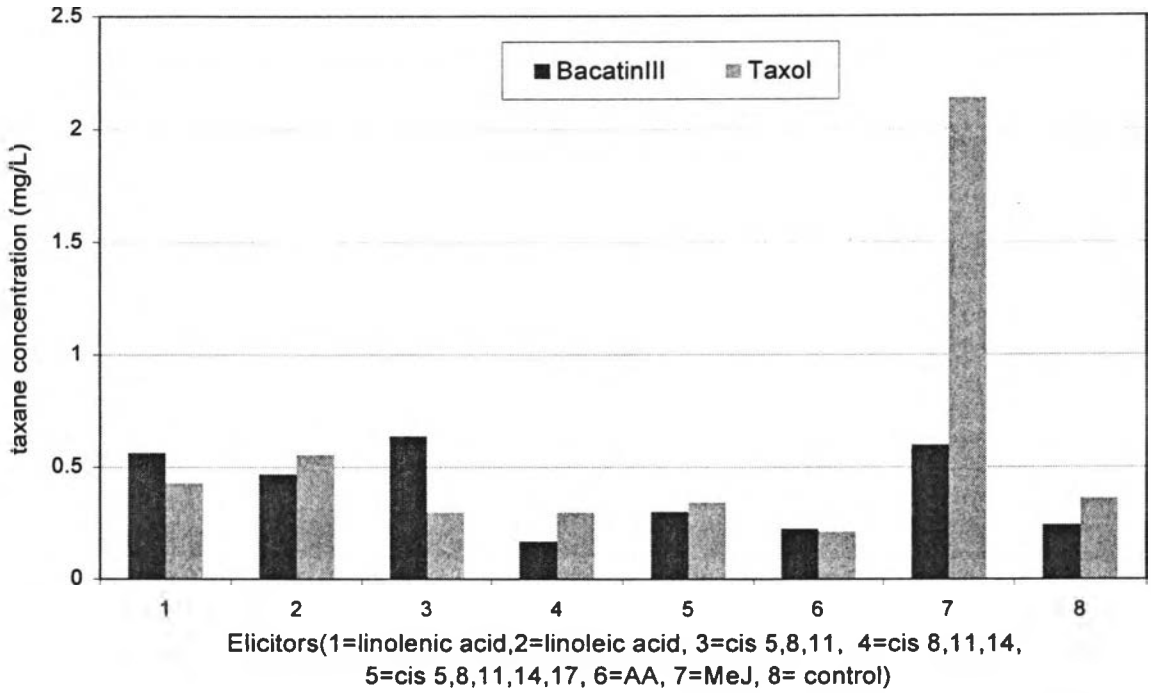

Figure D.1 Taxol and baccatin III at 21 days of incubation in the system provided 6 ppm C_2H_4, 15% O_2, and 0.5% CO_2 with 10 μM fatty acid.

The effects of elicitation of arachidonic acid, linolenic acid (water-soluble form), or linolenic acid (methanol-soluble form) together with MJ on accumulation of taxol (Figure D.2) and baccatin III (Figure D.3) were observed. Although some improvements of taxol production were observed with addition of 10 μM arachidonic acid or 5 μM linolinic acid (water-soluble form) compared to control without any elicitation, the addition of arachidonic acid or linolenic acid together with MJ did not improve taxol production relative to control elicitation with MJ alone. No significant increase of baccatin III was observed in the elicited system.

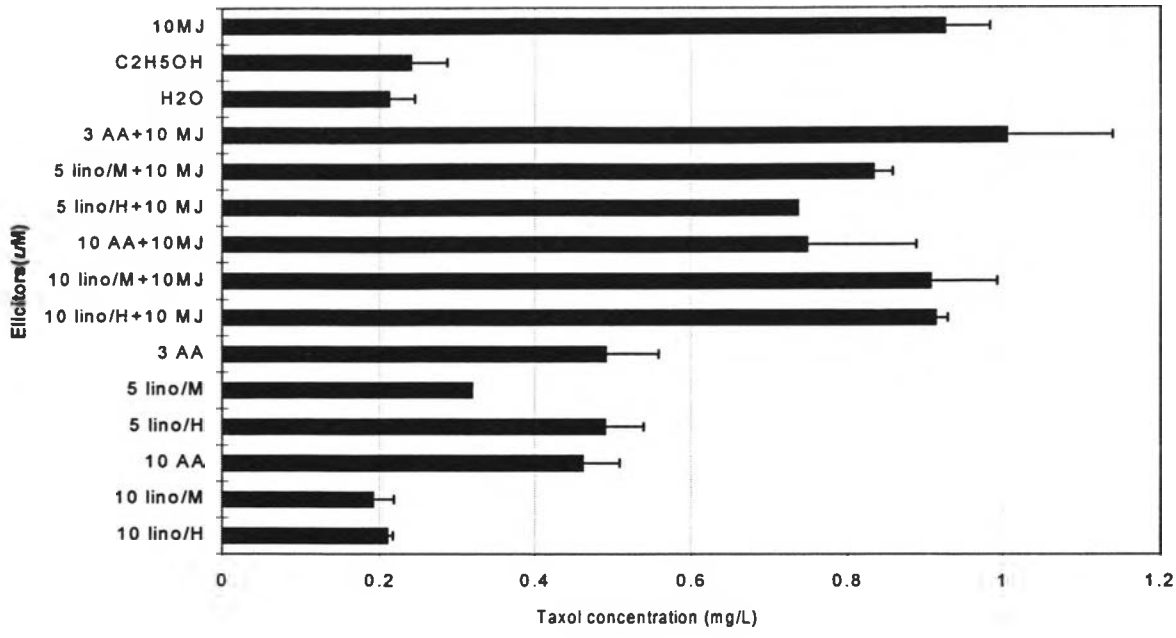

Figure D.2 Effects of supplementation of fatty acid with and without addition of MJ on accumulation of taxol.

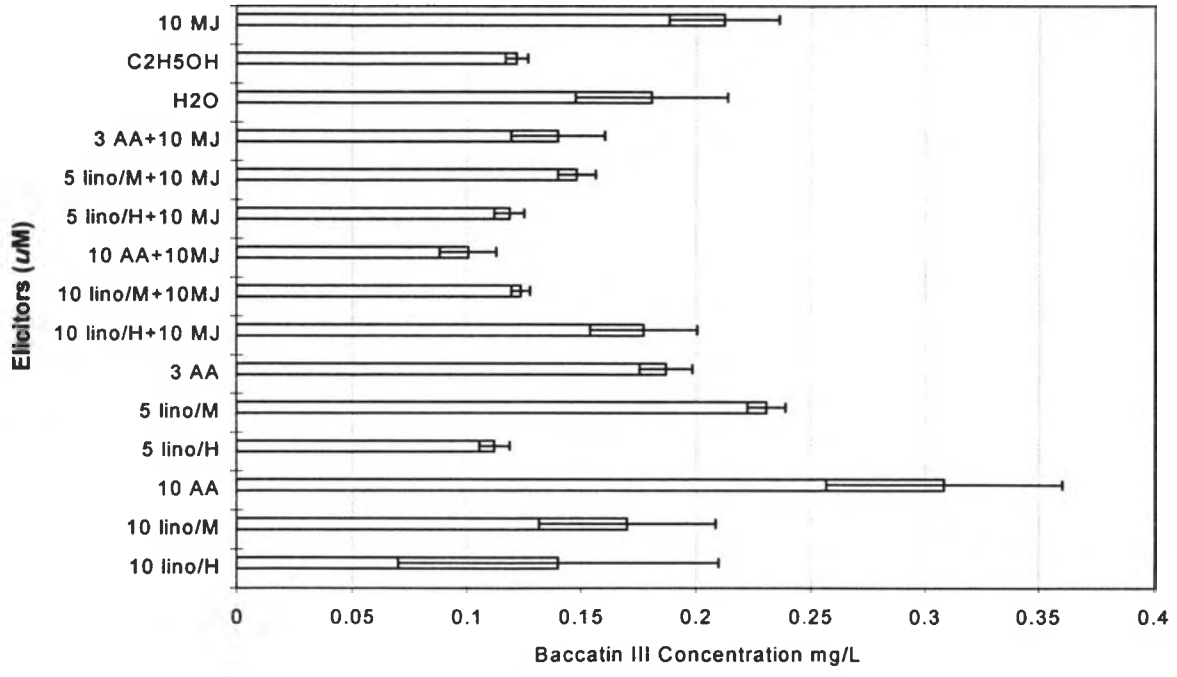

Figure D.3 Effects of supplementation of fatty acid with and without addition of MJ on accumulation of baccatin III.