DISSERTATION

TAXOL PRODUCTIVITY AND PHYSIOLOGICAL RELATIONSHIPS IN SUSPENSION CULTURES OF *TAXUS CUSPIDATA*

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY NOUSHIN MIRJALILI ENTITLED TAXOL PRODUCTIVITY AND PHYSIOLOGICAL RELATIONSHIPS IN SUSPENSION CULTURES OF *TAXUS CUSPIDATA* BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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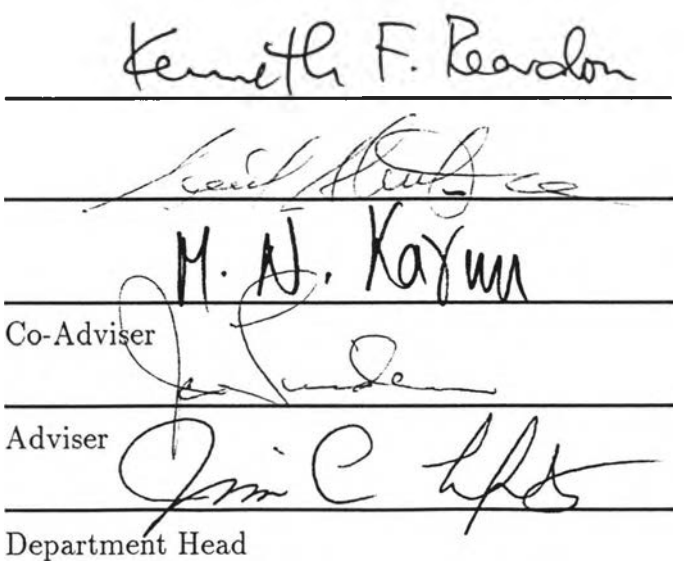

ABSTRACT OF DISSERTATION

TAXOL PRODUCTIVITY AND PHYSIOLOGICAL
RELATIONSHIPS IN SUSPENSION CULTURES OF *TAXUS CUSPIDATA*

Taxol, an extractive of the Pacific yew, is a plant secondary metabolite that has demonstrated anticancer activity. In an effort to prevent depletion of Pacific yew population and to obtain adequate supplies of taxol, alternative methods of production are being sought.

The goal of this research was to produce taxol in sufficient quantities for clinical use by manipulating engineering parameters that affect production of secondary metabolites in plant cell culture systems. Production of taxol in suspension cultures of *Taxus cuspidata* in shake flasks exposed to different concentrations and combinations of oxygen, carbon dioxide, and ethylene was investigated. The effect of each gas on cell growth and taxol production was studied using several sets of factorial design experiments. Low head space oxygen concentration (10% v/v) promoted production of taxol prior to day 14. High carbon dioxide concentration (10% v/v) inhibited taxol production. Taxol concentration increased as ethylene concentration was increased to 5 ppm; it leveled at 10 ppm ethylene.

The utilization patterns of sugars were dependent on headspace gas composition. Average calcium uptake rates into the cultured cells decreased and average phosphate uptake rates increased as the ethylene concentration was increased from 0 to 10 ppm. Ethylene concentration at 50 ppm had an inhibitory

effect on taxol production but not on phosphate uptake rate, suggesting independent regulation of taxol biosynthesis and physiological functions of the cell. The most effective gas mixture composition tested in terms of taxol production—10% (v/v) oxygen, 0.5% (v/v) carbon dioxide, and 5 ppm ethylene—is thought to be related to regulation of gene transcription.

To stimulate taxol production, suspension cultures of *Taxus cuspidata* were challenged with various concentrations and combinations of methyl jasmonate (an elicitor derived from jasmonic acid) and ethylene. Taxol productivity increased 19-fold compared to the basal level when *Taxus cuspidata* suspension cultures were exposed to 5 ppm ethylene and 10 μM methyl jasmonate. The induction of taxol biosynthesis occurred within 51 hours after elicitation. Simple induction models were proposed to explain the action and effects of both ethylene and methyl jasmonate with regard to receptor binding and transcription regulation in plants.

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I am very grateful to my parents for their faith in me and letting me do what I want.

دیدم بخواب خوش که بدستم پیاله بود — تعبیر رفت و کار بدولت حواله بود

چل سال رنج و غصه کشیدیم و دعا بگفت — تدبیر ما بدست شراب دوساله بود

آن نافه مراد که میخواستم ز بخت — در چین زلف آن بت مشکین کلاله بود

از دست برده بود خمار غم سحر — دولت مساعد آمد و می در پیاله بود

بر آستان میکده خون میخورم مدام — روزی ما ز خوان قدر این نوالہ بود

هر کو نکاشت مهر و ز خوبی گلی نچید — در رهگذار باد گمانبان لاله بود

بر طرف گلشنم گذر افتاد وقت صبح — آندم که کار مرغ سحر آه و ناله بود

دیدیم شعر دلکش حافظ به مدح شاه — یک بیت از این قصیده ز صد سال بود

آن شاه تند حمله که خورشید شیر گیر

پیشش بروز معرکه کمتر غزاله بود

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1

NATURE OF THE PROBLEM

1.1 Overview of this chapter

The problem addressed by this dissertation is presented in this introductory Chapter. After a brief review of plant cell culture systems, an attempt has been made to use this system to produce taxol. The first *in vitro* growth of plant cells and tissues was described approximately 50 years ago and the first commercial product from plant cell culture was introduced in the market 12 years ago (Verpoorte *et al.*, 1993). It is realized that while plant cell culture has seemed to be an adequate tool for lab scale studies, there are engineering aspects that need to be addressed. Hence in this dissertation, the emphasis is made on combination of biological science and engineering basics. Production of secondary metabolites from their respective plant cell culture system is an interdiciplinary study involving the plant sciences for growth and maintenance of the cultures and engineering basics for scale up considerations. The question of whether there is no obvious and fundamental limitation in the plant cell culture systems, which would prevent it from being an alternative route for producing secondary metabolites from plant cells, is raised. This question is placed in context by presenting the problem from an engineering point of view, as these properties have been less frequently addressed.

1.2 Goal and overview of this dissertation

In Chapter 1, the idea that plant cell culture system can be an alternative method of producing valuable chemicals is developed; this would fail if engineering basics are not considered.

The goal of this dissertation is to experimentally show that taxol can be produced in suspension cultures of various *Taxus spp.*; with the emphasis on engineering parameters, a number of variables have been used to enhance taxol production.

This dissertation is divided as follows.

In Chapter 2, the experimental approach is presented. An apparatus was constructed for implementing the goals of the study. The apparatus allows precise control of the desired variables in this study. The applicability of this apparatus to large scale reactors is also discussed.

In Chapter 3, the description and results of a number of experiments on taxol production are provided.

In Chapter 4, the physiological relationships between taxol production and culture activities are discussed.

In Chapter 5 the role of ethylene, its direct or indirect binding to a putative receptor and providing a function to the cell, is discussed.

In Chapter 6, the elicitation studies, their results and how an oligosaccharide can be used to stimulate taxol production are discussed.

In Chapter 7, the induction modelling as an analogy to the regulation of the *lac* operon in bacterial metabolism is presented. This is an attempt at a mathematical analysis of the various factors studied in this dissertation.

Chapter 8 concludes this dissertation.

Table 1.1: Commercially valuable plant products

1. Food colors, flavors and fragrances
Colors: anthocyanins, betacyanins, saffron
Flavors: strawberry, grape, vanilla, tomato, celery, asparagus
Oils: mint, rose, vetiver, jasmine, patchouly, sandalwood, lemon, onion, garlic
Sweeteners: stevioside, thaumatin, miraculin, monellin
2. Agricultural chemicals
Pyrethrins, retenone, azadirachtin, nerifolin, salannin, alleopathic chemicals
3. Pharmaceuticals
Codeine, morphine, scopolamine, atropine, vinblastine, L-Dopa, hyoscyamine, berberine, digoxin, quinine, vincristine, ajmalicine, serpentine, taxol

1.3 Plants as sources of valuable chemicals

Plants serve not only as food, but also as sources of chemicals. Just as mammalian cells can be manipulated to produce monoclonal antibodies, plant cells can be cultured to produce a variety of pharmaceuticals, dyes and flavors. About 25% of all U.S. prescriptions are for plant-derived compounds (Curtin, 1983); the most important are codeine and other morphine derivatives. A list of plant products that are of commercial interest is presented in Table 1.1 (Sahai and Knuth, 1985). Most of these chemicals are now produced by extraction from plant material that is cultivated or sometimes collected in the wild. There are several problems connected with this production method: variable quantities and qualities of the plant material, plants that need to grow several years before they are ready for harvesting, and over-collecting of endangered species. For the

high value products, alternative production methods are thus of great interest. Synthesis of natural products is an alternative to extraction. Although complete synthesis for most natural products has been shown to be possible, it is usually not economically feasible. Semi-synthesis, starting with more readily available precursors, has been successful in some cases.

Most plant products are a member of the class of chemicals known as secondary metabolites. Unlike primary metabolites (compounds like a protein, fat or carbohydrate, that are found in many organisms and are essential for cell function), a secondary metabolite occurs in only one or a few organisms and plays no obvious metabolic role. Secondary metabolites have not evolved to perform a necessary physiological function for the organism in which they are found, instead their role appears to be ecological. It is believed that many of the secondary metabolites produced by plants act in defense against the harmful effects of toxins, carcinogens, or mutagens found in the plant (Mitscher *et al.*, 1986; Williams *et al.*, 1989) or attack by external predators (Street, 1977). Due to their complex structures, plant secondary metabolites are still most efficiently produced by the plant (Verpoorte *et al.*, 1993). Therefore, in the past two decades, extensive research has been conducted in plant cell biotechnology using cell cultures rather than whole plants, as a possible alternative production method.

It may be possible to produce secondary metabolites from genetically engineered bacteria or yeast. The hurdle here is the lack of knowledge about plant gene expression and the fact that secondary metabolites are not products of single genes, each compound is the result of a multi-step, multi-enzyme process. Plant tissue culture offers the greatest potential for improving secondary metabolite production because tissue culture methods are more easily developed and more broadly applicable than genetic engineering. Secondary metabolites produced in culture have advantages over those obtained by conventional means. Supplies

would be reliable and regular, not dependent on weather, season or politics. Product quality would be uniform, and in those cases where the plant is rare or difficult to grow, the supply could be increased to meet demand. Analysts have determined that only products valued at more than $1000 per kilogram are worth producing by plant cell culture systems (Verpoorte *et al.*, 1993).

1.4 Plant cell culture systems

Plant cell culture encompasses of a number of techniques for growing plant cells. Cells from any plant species can be cultured aseptically on or in a nutrient medium. The cultures are started by planting a section of a sterile tissue on a nutrient medium solidified by agar. Within two or three weeks a callus (mass of unorganized cells) is produced. Callus can then be subcultured by transferring a small piece to fresh agar medium. Cell suspension culture is obtained when the callus is transferred to liquid medium and agitated when the vessel is placed on a shaker. Cell suspension cultures consist of mixtures of cell aggregates and single cells. The growth rates of such cultures are generally greater than on agar. The technique provides for better control of growth because most cells are surrounded by the medium (Gamborg, 1982). Usually two media are needed: the first is designed to maximize growth and the second medium is designed to maximize production of secondary metabolite.

The rapid measurement of cell mass in suspension cultures is complicated by the presence of macroscopic aggregates. For example, optical density measurements of plant cell suspensions are not successful. Generally, fresh weight (wet weight of filtered cells) or dry weight of the cells is used. The correlation between wet and dry weight, however, has been poor; the physiological state of the cells has been reported to affect this correlation (Shuler, 1981). If aggregation of the cells can be controlled, a Coulter Counter can be used to measure the

number and size of plant cells quantitatively. Another method of quantifying cell mass is to sonicate the cells to give a solution of cell debris and measure the optical density of this solution. Since most plant cells develop brown pigmentation due to phenolic accumulation as they enter the stationary phase, this method is limited to actively growing cells (Shuler, 1981).

Several strategies are being followed to improve yields of secondary metabolites in plant cell cultures. The screening and selection of high producing cell lines and the optimization of growth and production media are common approaches. Other approaches include culturing of differentiated cells (e.g., shoots, roots and hairy roots), induction by elicitors and metabolic engineering. The culture of differentiated tissues on a large scale in bioreactors is a major constraint (Verpoorte *et al.*, 1994); these systems are useful for biosynthesis studies. Induction and metabolic engineering are comparable with suspension cultures growing in fermenters. For the metabolic engineering approach to be successful, the biosynthetic pathway has to be known at the level of products, enzymes and genes, as well as the regulation at each level, including aspects of compartmentalization and transport (Verpoorte *et al.*, 1994).

Once the cell line is selected and the optimum medium is developed, the cultures need to be transferred from laboratory scale to pilot plant. The major problem in growing larger volumes of plant cells is striking a balance between the need for mixing, in order to circulate nutrients and to prevent the cells from sticking together, and the shear sensitivity of plant cells.

1.5 Parameters affecting plant cell culture systems and secondary metabolite production

1.5.1 Cell line and nutrients

The techniques for increasing cell culture productivity often begin with the selection of high producing plants. Once the cell culture is established, further selection for higher productivity is an important parameter in increasing yields. The selection techniques take advantage of the fact that within a tissue or a new colony of cells, there is a wide distribution of cell types with differing capacities for product synthesis (Gamborg, 1982). By selecting for high yielding cells repeatedly, improvements in productivity and stability can be obtained.

Although the task of developing stable and productive cell lines is crucial to the success of any plant cell culture system, that of designing appropriate media is as important. The essential nutrients for plant cell systems are mineral salts, a carbon source, vitamins and growth regulators (Gamborg, 1982). Other compounds such as organic nitrogen compounds, organic acids, additional metabolites and extracts are optional. The optimization of medium is cell line dependent and involves trial and error experimentation. Extensive research is conducted on optimizing media for improving secondary metabolite production (see (Fosket, 1994) and references therein).

This dissertation does not involve cell line and media optimization for taxol production. That part of the work was conducted by the collaborators in the USDA Agricultural Research Service, laboratory of Dr. Donna Gibson in Ithaca, New York.

1.5.2 Elicitors

Application of elicitors has been used in plant cell and tissue culture to induce early production of desired secondary metabolites (Byun and Pedersen,

1994; Fluhr *et al.*, 1991; Malpathak and David, 1992; Watson *et al.*, 1986; Heinstein, 1985; Gundlach *et al.*, 1992). An elicitor acts as an external stress, which initiates a series of biochemical reactions leading to excretion of secondary metabolites. Elicitor action can also be evident by production of ethylene and changes in calcium and phosphate compartmentalization in the cell (Anderson *et al.*, 1993; Raz and Fluhr, 1992; Raz and Fluhr, 1993).

When the plant is stressed by fungal pathogens (Anderson *et al.*, 1993), osmotic stress (Handa *et al.*, 1983; Rudgek and Morris, 1986), or oxygen deficient conditions (Brailsford *et al.*, 1993; Jackson *et al.*, 1991) a series of signal transduction events begins. Darvill and Albersheim (1984), Eilert *et al.* (1987), and DiCosmo and Towers (1984) have worked toward elucidating the response mechanism of cells to elicitation. Exposure of poppy cells to elicitor preparations of arachidonic acid, chitosan, nigeran and homogenates of cultures of phytopathogenic fungi resulted in browning of the cultures within a few hours (Eilert *et al.*, 1987). This browning, which deepened over time, was thought to be the result of oxidation and polymerization of phenolic compounds. However, a direct correlation between intensity of the pigmentation and amount of alkaloids accumulated could not be seen. The occurence of phytoalexins (plant antibiotics) as a result of elicitation could be interpreted as excretion or as leakage due to cell breakdown. In *Papaver somniferum* cell cultures treated by fungal elicitors, however, tonoplast and plasmalemma membranes did not show any ruptures, and the cytoplasm contained the regular complement of organells (Eilert and Constabel, 1985). Therefore, the authors associated the occurence of the secondary metabolite sanguinarine to an excretion process; elicitation activated the pathway leading to benzophenanthridine synthesis by induction (Eilert and Constabel, 1985).

The nature of the plant defense response induced by elicitors depends on the type of elicitor and the plant being studied. Also, oligogalacturonides (cell wall fragments derived from the pectic fraction of plant cell walls) have been shown to induce the accumulation of phytoalexins in soybean, castor bean, pea and parsley (see (Hahn, 1995) and references therein). Biosynthesis of shikonin was also induced by an oligogalacturonoide (Tani *et al.*, 1992). It is thought that the oligogalacturonide elicitors are released during plant-pathogen interactions by the action of microbial pectic-degrading enzymes (endopolygalacturonase and endopectate lyase) (Hahn, 1995). Hahn (1995) suggested that oligogalacturonide perception occurs at the plasma membrane of responsive cells. For example, oligogalacturonide mixtures depolarized the membranes of tomato leaf mesophyll cells within 5 minutes (Thain *et al.*, 1990). Oligogalacturonide elicitation induced a transient stimulation of K^+ efflux, alkalinization of the extracellular medium, depolarization of the plasma membrane, and a decrease in the external calcium concentration in suspension cultures of tobacco (Mathieu *et al.*, 1991).

On the other hand, a partially purified extract from yeast was shown to induce ethylene biosynthesis and phenylalanine-ammonia-lyase (PAL) activity in suspension cultures of tomato (Grosskope *et al.*, 1991). The molecules in the yeast extract that are responsible for these activities were shown to be glycopeptides (Basse and Boller, 1992). Chitin and oligosaccharides derived from chitin have also been shown to induce several plant defense responses (Mauch *et al.*, 1984; Pearce and Ride, 1982; Broglie *et al.*, 1986; Roby *et al.*, 1991; Seifert *et al.*, 1994).

In this dissertation, the elicitor methyl jasmonate, was used to induce early taxol production. The effect of methyl jasmonate and its interaction with ethylene on production of taxol is described in Chapter 6.

1.6 Regulatory pathways in plant cells

Most plants have a series of alternative pathways for development. According to Fosket (1994) the special regulatory molecules that stimulate or suppress growth and can initiate or change developmental pathways are called hormones. There are five classes of plant hormones: auxins, cytokinins, gibberellins, ethylene and abscisic acid (Salisbury and Ross, 1992). Systemin has been recently added to this list of plant hormones (Fosket, 1994). Hormones regulate many aspects of plant growth and development. Most hormones have a receptor or receptors to which they bind; the binding of a hormone to its receptor initiates a cascade of cellular changes that includes a signal transduction pathway (Fosket, 1994). In some cases, the binding of a hormone to its receptor results in the accumulation of a second messenger (such as calcium, inositol phosphate, and jasmonic acid) and the second messenger then initiates the regulatory process (Raz and Fluhr, 1992). Receptors for auxin, gibberellin and abscisic acid have been identified. The gibberellin and abscisic acid receptors are on the cell surface and the auxin receptors are found both on the cell surface and in the cytoplasm (Fosket, 1994).

Since taxol is believed to be produced by the native plant as a response to pathogen attack, a brief summary of the signal transduction in response to pathogen attack follows in the next section.

1.6.1 Signal transduction in response to pathogen attack

Many plants synthesize proteinase inhibitors I and II in response to insect attack. Proteinase inhibitor I is an inhibitor of the digestive enzyme chymotrypsin and proteinase inhibitor II blocks the degradation of proteins by trypsin and chymotrypsin (Fosket, 1994). Mechanical wounding initiates the

production of signalling molecules that trigger the transcription of the genes encoding the proteinase inhibitors. Several different signalling molecules will induce this response, including abscisic acid and variety of oligogalacturonides. Since oligogalacturonides are not readily transported in plant tissue, a local response initiates the binding of the oligogalacturonide to its receptor; this receptor has been identified and is on the plasma membrane (Fosket, 1994). On the other hand, systemin, which is an 18-amino acid peptide and is produced as a response to wounding, can be transported easily through the plant, inducing proteinase inhibitors; systemin's receptor resides on the cell surface (Fosket, 1994). So both systemin and oligogalacturonides, having different receptors, induce proteinase inhibitors through the same mechanism. Jasmonic acid, in some instances, can be formed as the result of the binding of an oligonuronide or systemin to their respective receptor (through hydrolysis of the ester bond in linolenic acid). Both jasmonic acid and methyl jasmonate (a derivative of jasmonic acid) induce proteinase inhibitor synthesis (Tamari *et al.*, 1995; Creelman *et al.*, 1992; Dittrich *et al.*, 1992; Farmer *et al.*, 1992). It is believed that methyl jasmonate is converted to jasmonic acid after it enters the plant cell and this conversion is responsible for its activity (Fosket, 1994). Methyl jasmonate can transmit a signal from a plant under attack to neighboring plants, inducing proteinase inhibitor synthesis (Farmer and Ryan, 1990). The genes encoding proteinase inhibitors I and II contain jasmonate response elements in their promoters; these sequences when introduced into the promoter of other genes, cause them to be methyl jasmonate or wound inducible (Fosket, 1994). A model for the signalling pathway leading to proteinase inhibitor synthesis can be found in Farmer and Ryan (1992).

Many plant hormones stimulate an increase in cytoplasmic calcium levels, triggering a variety of cellular responses (Raz and Fluhr, 1992). Calcium

ion levels are very low in the cytosol but high in some membrane-bound compartments and in the extracellular environment. Some hormones cause cytosolic calcium levels to increase transiently (Fosket, 1994). Calcium-dependent protein kinases can cause changes in gene expression since transcription factors must be phosphorylated to bind to regulatory elements in genes (Raz and Fluhr, 1992).

In this dissertation, Chapters 5 through 7 are aimed toward an understanding of the mechanism of action of the hormone ethylene and the signal transduction cascade involving methyl jasmonate and ethylene.

1.7 Engineering aspects of plant cell biotechnology

In order for the secondary metabolites to be produced commercially, plant cells have to be grown in large fermenters and the price of a product from large scale plant cell system must be competitive with the existing production methods. Plant cells are characterized by a number of features which distinguish them from microbial cells and result in problems that might not be encountered when working with microbial systems. For example, plant cells are larger than microbial cells; they have diameters ranging from 100–200 μm, about 10–1000 times bigger than microbial cells (Taticek *et al.*, 1991). Plant cells have spherical to cylindrical shapes, and their cell size changes during the growth period. The majority of the cell volume, particularly that of older cells, is taken up by the vacuoles; this makes the plant cells sensitive to changes in osmotic potential and physical stress (Street, 1977). Also their cellulose-based cell wall and the greater size of the plant cells make them more sensitive to shear compared to microbial cells. Because of this sensitivity of plant cells to physical stress, various types of bioreactors have been the subject of much research. One of the main concerns in the bioreactor is the availability of necessary gases for the growth of plant cells. In microbial systems, oxygen is considered to be the main gas of

interest. Sufficient supply of oxygen is provided by controlling the agitation and aeration rates in microbial fermentations. In plant cell culture systems, on the other hand, high agitation and aeration rates can be detrimental to growth and secondary metabolite production. Tanaka (1981) found that the stress on plant cells generated as a result of agitation and aeration decreased as the size of the plant cell aggregates decreased. However, cultures containing small aggregates were found to have higher viscosities and hence lower oxygen transfer rates. In addition, the level and degree of aggregation, which affect cellular biochemistry, is related to vessel geometry and design.

Because of the problems associated with working with cell aggregates, a number of methods (enzymatic, chemical and physical) have been used to eliminate aggregation (Shuler, 1981); none of these methods has given a single-cell culture unchanged in biochemical properties and capable of sustaining the single-cell condition for long periods. Therefore, conditions optimal for growth of plant cells involve a trade-off between shear damage and effective gas and nutrient supply. Additionally, oxygen is not the only important gas in plant cell culture systems. The importance of gas composition on plant cell culture systems is discussed in the next section.

1.7.1 Headspace gas composition

Tissue-cultured plants are different from normal plants in part because of the environmental conditions in the container flask or bioreactor (Debergh *et al.*, 1992). The container and closure device of a tissue culture system is of significant importance in the determination of the headspace composition, and influences plant growth and secondary metabolite production (Lee and Shuler, 1991; Debergh *et al.*, 1992). Also, the headspace composition requirement seems to be species dependent (Righetti *et al.*, 1990). Correlations between gas transfer

rate and culture performance have been reported by many investigators (Smith *et al.*, 1990; Tate and Payne, 1991; Taticek *et al.*, 1990; van Gulik *et al.*, 1994).

The three gases, oxygen, carbon dioxide, and ethylene, are important in plant cell systems. Oxygen has an important role in respiration and other aspects of cell metabolism, such as stoichiometry of nutrient conversion (Payne *et al.*, 1991). The effect of oxygen on plant cell growth has been the topic of several studies (Tate and Payne, 1991; Kvaalen and von Arnold, 1991; Gao and Lee, 1992). High oxygen levels stimulated cell growth and increased oxygen consumption rate and the level of phenolics in suspension cultures of genetically modified tobacco cells (Gao and Lee, 1992). Various methods of oxygen supply for cultures of shear-sensitive organisms were reviewed by Mano *et al.* (1990). When oxygen was supplied with perfluorocarbons saturated with air, the overall mass transfer coefficient was five-fold greater than that of the surface aeration (Mano *et al.*, 1990).

Various investigators have reported the influence of carbon dioxide on heterotrophically grown cell suspension cultures (Ducos and Pareilleux, 1986; Hegarty *et al.*, 1986; Smart and Fowler, 1981; Maurel and Pareilleux, 1985; Tate and Payne, 1991; van Gulik *et al.*, 1994). Increasing carbon dioxide levels in the incubator resulted in enhanced biomass formation, faster carbon source utilization and higher specific growth rates in suspension cultures of *Catharanthus roseus* (Maurel and Pareilleux, 1985). Payne *et al.* (1990) reviewed the experimental constraints for studying the effects of oxygen and carbon dioxide on plant cell growth. Smith *et al.* (1990) have developed a strategy to control the concentrations of oxygen and carbon dioxide at constant shear in a plant cell bioreactor.

The effect of ethylene on cell cultures is reviewed by Biddington (1992). Ethylene has many physiological effects, including inhibition of growth and stimulation of production of secondary metabolites from various plant tissue and cell

cultures (Chi and Pua, 1989; Cho *et al.*, 1988; Songstad *et al.*, 1989). Carbon dioxide can inhibit or delay many ethylene responses (Biddington, 1992; Sisler and Wood, 1988; Yang, 1985). For instance, 2% carbon dioxide and 21 ppm ethylene each increased volumetric production of berberine two-fold; whereas enriching the inlet gas with both carbon dioxide and ethylene at those concentrations increased the productivity by three-fold (Kim *et al.*, 1991). Kobayashi *et al.* (1991) reported the reduced production of berberine and browning of the *Thalictrum minus* cell cultures by removal of carbon dioxide from the culture medium by air bubbling; the physiological damage caused by aeration was prevented by adding 2% (v/v) CO_2. The induction of cell browning by exogenously applied ethylene suggested that carbon dioxide might act antagonistically against endogenously generated ethylene (Kobayashi *et al.*, 1991).

These results clearly indicate the important roles of carbon dioxide and ethylene in secondary metabolism. Understanding the effects of volatile gases on plant cells is necessary for scale-up of plant cell culture systems. Production of secondary metabolites is usually reduced as the system is scaled up from shake flasks to bioreactors. One reason for this is that gas transfer requirements of the culture are not well known. It is important to study the effect of various concentrations and combinations of gases without affecting shear energy on the cells. Changing aeration or agitation rates to bring about given dissolved gas concentrations affects, among other things, the shear stress on plant cells. In air sparged bioreactors, ethylene and carbon dioxide, which are important parameters in production of secondary metabolites from plant cell cultures, can be swept out of the solution, even if they are produced by the cells. This sweeping is dependent on the aeration and agitation rates, mass transfer coefficients and reactor configuration (Ducos and Pareilleux, 1986; Smart and Fowler, 1981). The narrow range of mass transfer coefficient values ($k_l a$ = 5–10 hr^{-1}) over which

Table 1.2: Large scale secondary metabolites from plant cell culture systems

Compound	Cell culture system	Bioreactor/Volume	Producer(s)
Berberine	*Thalictrum mimus*, *Coptis japonica*	batch & continuous impeller driven 4000 L	Mitsui Petrochemical Industries, Japan
Shikonin	*Lithospermum erythrorhizon*	batch 750 L	Mitsui Petrochemical Industries, Japan
Saponins	*Pamax ginseng*	cell & root cultures 20,000 L	Nitto Denko Japan
Vanillin	*Vanilla planifolia*	impeller driven reactor, 72 L	ESCAgenetics, USA
Taxol	*Taxus spp.*	2-stage system impeller driven airlift reactor 75,000 L	ESCAgenetics, USA Phyton Catalytic, USA Nippon Oil Company, Japan
Anthocyanins	*Euphorbia millii*	rotary culture system	Nippon Paint, Company, Japan
Sanguinarine	*Papaver somniferum*	airlift reactor 300 L	Vipont Research Labs, USA

plant cell bioreactors may be operated without causing either oxygen limitation or carbon dioxide stripping is an important constraint in large scale production of plant metabolites (Smart and Fowler, 1981).

1.7.2 Large scale production of plant metabolites

The large scale, commercial production of secondary compounds from cell culture systems has been reported by Smith (1995). Because of proprietary restrictions, detailed information of bioreactor configurations are not published. These and other semi-commercial large scale secondary metabolites are shown in Table 1.2 (Smith, 1995).

Because plant cells are considered to be very sensitive to shear forces, various types of low-shear bioreactors have been used for large scale production

of secondary metabolites. The appropriate selection of a bioreactor for mass cultivation of a specific cell line depends on the shear tolerance of the cells. Pneumatically agitated fermenters, providing uniform low shear hydrodynamics, have been used by several researchers (Hegarty *et al.*, 1986; Smart and Fowler, 1981; Byun and Pedersen, 1994; Kim *et al.*, 1991). A number of research groups have studied the shear tolerance of various plant cell suspensions. Scragg *et al.* (1988), using a stirred tank bioreactor, subjected *C. roseus, H. annuus*, and *P. quassioides* to shear rates between 73 and 167 s^{-1} for up to 5 hours (impeller speeds of 440–1000 rpm). Between 20 and 30% of the original cells present ruptured by the end of 5 hours. Hooker *et al.* (1989) used a Couette type viscometer to study the effect of shear rate on the viability of *N. tabacum*. Shear rates under 298 s^{-1} (corresponding to 200 rpm) had no detrimental effect on culture viability. Higher shear rates (596–1193 s^{-1}) resulted in significant decreases in viability that increased with increasing shear. After 12 hours at 800 rpm, the viability of the cells had dropped to less than 10%; cells in the stationary phase were more susceptible to shear than cells from earlier in the growth cycle (Hooker *et al.*, 1989). This fact may be due to the increase in the cell size associated with growth and expansion resulting in increased shear sensitivity (Taticek *et al.*, 1991). The type of impeller used affects the applicability of stirred tank reactors. Turbine impellers operating at 28 rpm have produced cell lysis of *C. roseus*, while flat blade impellers operating at 200 rpm have not (Fowler, 1981). Spiral type impellers have also been used to mix plant cultures without cell damage (Spieler *et al.*, 1985; Ulbrich *et al.*, 1985).

Large scale cultivation of plant cells at high density is reviewed by Tanaka (1981). The effects of bioreactor configuration on growth and secondary metabolite production in suspension cultures of *Eschscholtzia californica* and *Catharanthus roseus*, is described by Taticek *et al.* (1990) and ten Hoopen *et al.*

Table 1.3: Various bioreactors used in plant cell culture systems

Bioreactor type	Volume (L)	Species	Reference
Jar fermenter	14 & 65	*N. tabacum*	(Kato *et al.*, 1975)
Airlift	30	*C. roseus*	(Bond *et al.*, 1988)
STR	2	*C. roseus*	(Smith *et al.*, 1990)
STR	3	*C. roseus*	(Schlatmann *et al.*, 1994)
Airlift	1	*E. californica*	(Taticek *et al.*, 1990)
STR	1	*E. californica*	(Taticek *et al.*, 1990)
STR	12	*C. roseus*	(Leckie *et al.*, 1991)
Helical ribbon	1	*C. roseus*	(Jolicoeur *et al.*, 1992)
STR	3	*C. roseus*	(Schlatmann *et al.*, 1993)
STR	3	*C. roseus*	(ten Hoopen *et al.*, 1994)
STR	2	*C. roseus*	(van Gulik *et al.*, 1994)
Airlift	3	*E. californica*	(Byun and Pedersen, 1994)
Airlift	20	*C. rubrum*	(Fischer *et al.*, 1994)
STR	3	*N. tabacum*	(Ho *et al.*, 1995)
Jar fermenter	2	*N. tabacum*	(Nagai *et al.*, 1994)
STR	15	*C. roseus*	(Schlatmann *et al.*, 1995)

(1994), respectively. ten Hoopen *et al.* (1994) reported that growth of biomass was not affected by the type of culture vessel and the production of ajmalicine could be restored by creating a gas regime in the bioreactor comparable to that in a shake flask. The types of bioreactor used in plant cell culture systems are listed in Table 1.3. As can be seen, the stirred tank reactor (STR) is the most commonly used reactor.

Many of the considerations applicable to scale up of microbial systems are also applicable to plant cell cultures. These include nutrient supply, uniformity of mixing, and adequate gas transfer. Added to these considerations must be the effect of shear intensity and air bubble dispersion. Growth of cultures can be limited by the availability of oxygen in the liquid phase. One of the objectives of the bioreactor design is to optimize oxygen transfer while operating within the shear tolerance limits of the plant cells. This requires a clear understanding of oxygen transfer and utilization by cell systems to design reactors. The overall

process of oxygen transfer, as well as that of other nutrients, is described in detail by Moo-Young and Blanch (1981) and Arrua *et al.* (1990).

Large scale production of taxol is not studied in this dissertation. Problems in the shake flasks have to be solved before attempts toward scale up parameters are considered.

1.8 Shikonin, a case study

In spite of the high interest in plant cell culture systems, there has been little industrial scale production of pharmaceutical compounds using this method. The first chemical compound produced by plant tissue culture was shikonin, a dye and pharmaceutical that Mitsui Petrochemical Industries Ltd. (Tokyo) is selling for $4000 per kilogram (Curtin, 1983). The production of shikonin from plant cell culture provides a case study for producing secondary metabolites from plant culture systems.

Shikonin is found in the root of the plant *Lithospermum erythrorhizon*, or shikon. Shikon is a traditional medicine in Japan, where it is used for its anti-flammatory effects, principally for treatment of burns and hemorrhoids (Becker and Sauerwein, 1990). Shikonin is bright red, and it is used in dyes as well as pharmaceuticals. Problems with conventional methods of shikonin production made the substance attractive as a candidate for plant cell culture systems. *Lithospermum* plants must grow for 5–7 years before the shikonin concentration in their roots reaches 1–2 percent (Curtin, 1983). Since pure shikonin costs about $4500/Kg, it was worth while considering plant cell culture system as a way of producing shikonin. For this production, Mitsui Petrochemical Industries had to overcome a number of problems before it could commercialize plant cell culture produced shikonin. They had to develop productive cell lines and media, and solve the problems of fermenter design and scale up. The goal of the company's

strain development was to select a cell line whose yield of shikonin was greater than the plant yield (Fujita and Tabata, 1987). After examining various methods of screening, they obtained a high producing cell line for shikonin derivatives from protoplast cultures of *L. erythrorhizon* (Fujita and Tabata, 1987). The productivities of shikonin in the protoplast derived cell lines were distributed widely around the corresponding value for the parent line, and the average productivity was similar to that of the parent. For example, when a high producing cell line (4.2 g shikonin/g of inoculum/23 days) was the parent, a daughterline with 1.5 times higher productivity was obtained (Fujita and Tabata, 1987). They obtained a more stable productivity using protoplast selection than the parent line which had been obtained by the conventional selection method. This selection process was very labor intensive; the cell lines derived from an aggregate or from a protoplast had to be each examined for productivity of shikonin. This procedure was later shortened by use of a cell-sorting machine to select only superior cells in the single cell stage (Fujita and Tabata, 1987). Shikonin is a dye, so productive *L. erythrorhizon* cells are bright red and could be selected visually in the early stages of selection.

Another significant effort in this process was to develop productive cell lines that were stable in culture. The nutritional environment of cells had also an influence on the productivity of cell cultures. A detailed study of the influence of the nutrient medium on the production of shikonin by cell suspension cultures of *L. erythrorhizon* was published by Fujita *et al.* (1981a) and Fujita *et al.* (1981b). When *L. erythrorhizon* cells were cultured in liquid Linsmaier-Skoog (LS) (Linsmaier and Skoog, 1965) medium suitable for growth, they produced no shikonin; but shikonin was produced in White's medium (White, 1954). By optimizing each factor of White's medium, Fujita and Tabata (1987) were able to increase the shikonin production from 2% of dry weight to 12.4%. In addition to

nutrients, phytohormones played an important role in optimization of medium; a negative effect of gibberellin on production of shikonin was reported (Fujita and Tabata, 1987).

Once the cell line and the media were optimized, the step from laboratory to pilot plant was conducted. Fujita and Tabata (1987) observed sufficient supply of oxygen with minimum impact on the cells, as the most important factor in the scale up process of shikonin. Both an aeration-agitation type vessel with a modified impeller and an airlift type vessel were used for production of shikonin. In the former, the cell grew well but the yield of shikonin was lower than in the usual flask culture; the greater the rotation speed of the impeller, the more shikonin yield decreased because of injuries to the cells (Fujita and Tabata, 1987). In the airlift reactor, the cells were suspended, even at the cell density of 15 g/L (dry weight basis), but as the cells adhered to the tank wall above the liquid surface level because of bubbling up, the yield of shikonin production was reduced (Fujita and Tabata, 1987). Therefore, a rotary drum type vessel was designed and constructed for the culture of *L. erythrorhizon* cells, where little injury was caused because the culture is moved by the revolution of the tank itself. They were able to scale up the volume to 1 m^3 with no decrease in the yield of shikonin. Approximate shikonin yields at various volumes of the rotary drum type tank and aeration type tank is listed in Table 1.4. This data was taken from a graph by Fujita and Tabata (1987).

The Mitsui process of shikonin production involves culturing the cells in a 250 liter fermenter for the growth phase; subsequently the cells are inoculated into a 750 liter fermenter for the production phase (Curtin, 1983). The cells are cultured for a total of three weeks and shikonin is then extracted from the cells by conventional methods. According to Curtin (1983), Mitsui Petrochemical produces 5 Kg of pure shikonin from each batch fermentation.

Table 1.4: Shikonin yields from suspension cultures of *L. erythrorhizon* in various bioreactor scales and configurations

Reactor Type	Volume (L)	Shikonin Yield mg/L
Rotary drum	1	3800
"	10	3800
"	100	3800
"	1000	3800
Aeration-agitation	1	3700
"	10	3300
"	100	3200
"	1000	3000

Although there are problems associated with plant cell culture, there are many benefits to this alternative production strategy. These benefits result from the difficulties associated with the present methods used to obtain plant products. Many of the plant products are derived from plants grown in tropics, the levels of supply of raw material may be erratic due to natural disasters or due to economic and political instabilities. The natural variation in plant material and variations in product content can complicate the product recovery systems and reduce their efficiency. Production of secondary metabolites from plant cell culture system can provide a reliable and homogenous supply of plant material. Cultured cells can be more easily manipulated than whole plants to increase production of secondary compounds. Products obtained from cultured cells have simpler down stream processing and product recovery costs compared to conventional technology (Taticek *et al.*, 1991).

1.9 Taxol, an anticancer agent

Taxol (Figure 1.1) was first isolated in June 1967 by Wall and Wani (1995). Using a countercurrent distribution method, 0.5 g of taxol was isolated

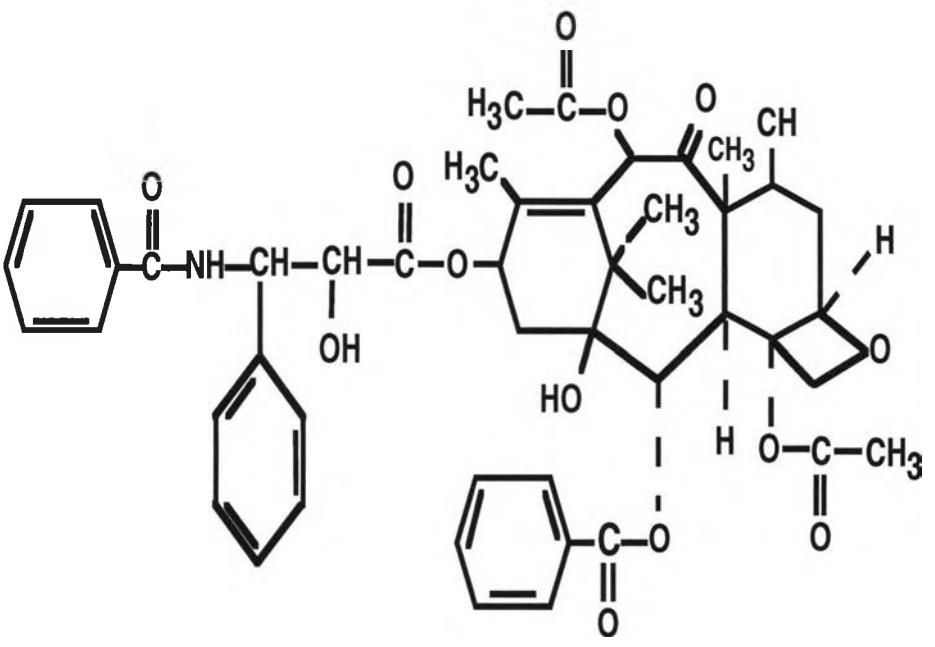

Figure 1.1: Chemical structure of taxol: $C_{47}H_{51}NO_{14}$; MW = 853.

starting with 12 kg of air dried stem and bark from *Taxus brevifolia*. *T. brevifolia* is a slow growing tree, native to western North America (Campbell and Whitney, 1995). Most parts of the *T. brevifolia* plant, the bark, wood, and needles, contain taxol. Taxol is now a registered trademark name; its generic name is paclitaxel (Campbell and Whitney, 1995). In this dissertation, the word taxol is used instead of paclitaxel.

Taxol was discovered as a result of a screening program initiated in 1960 by the National Cancer Institute. The ecological role of *T. brevifolia* in the forest is not well understood. The tree is utilized as food and habitat by many forest species (Campbell and Whitney, 1995).

Wall and Wani (1995) reported activity of taxol against various cancer cells in 1967; animal toxicology of taxol started in 1982 followed by clinical trials in 1983. Great interest was generated in taxol by the announcement of activity of taxol

against ovarian cancer (McGuirem *et al.*, 1989). Taxol has also shown antitumor activity in multiple clinical trials in cancers of the ovary, breast, head and neck, lung and gastrointestinal tract (Holmes *et al.*, 1995).

The mechanism of action of taxol was elucidated by Horwitz *et al.* (1981). Taxol is a mitotic inhibitor; the mechanism of its action is unique in that it stabilizes microtubules and inhibits depolymerization of tubulin to form microtubules (Horwitz *et al.*, 1981; Horwitz, 1992).

In 1989, approximately 60,000 pounds of bark of *T. brevifolia* were collected from federal lands and sent to the National Cancer Institute (Campbell and Whitney, 1995). In 1990, this number increased to 74,000 pounds and in 1991 to 850,000 pounds. Due to this extensive harvest, many concerns over the environmental impact surfaced, which resulted in use of Pacific yew bark as a transition source, and development of alternative programs for obtaining taxol.

1.9.1 Strategies in producing taxol

Owing to concern over depletion of *Taxus brevifolia* populations and obtaining adequate supplies of taxol, alternative methods of production are being studied. A brief list of alternate methods follows.

1. Complete chemical synthesis; the process has been achieved, is very involved and requires many steps (Nicolaou *et al.*, 1994; Holton *et al.*, 1994).

2. Semi-synthesis of taxol starting with baccatin III or 10-deacetylbaccatin III; these compounds can be obtained from needles of different *Taxus* spp. in greater quantity than taxol (Denis *et al.*, 1988; Wall and Wani, 1995; Kingston *et al.*, 1982; Borman, 1991; Kingston *et al.*, 1992). Baccatin III and 10-deacetylbaccatin III do not have the side chain necessary for the cytotoxicity of taxol (Kingston *et al.*, 1990). The chemical structure of

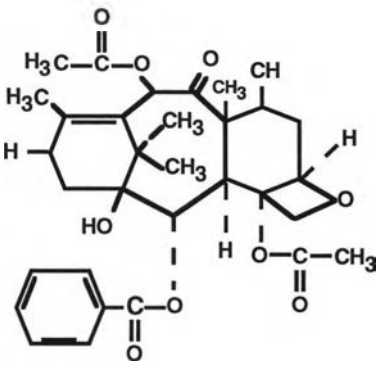

Figure 1.2: Chemical structure of 10-deacetylbaccatin III.

10-deacetylbaccatin III is shown in Figure 1.2. This is the current method of supplying taxol for the clinical trials.

3. Extraction of taxol from needles of Pacific yew; this is a renewable source, but taxol concentration is very low (0.0013%, on a dry weight basis) (Witherup *et al.*, 1990). Direct extraction from needles presents significant problems in purification (more than from other source materials) (Wall and Wani, 1994). Also environmental concerns such as over harvesting and collection costs for needles can increase as the market size expands.

4. Production of taxol from *Taxomyces andreanae*, a fungus that was isolated from the phloem of Pacific yew; again taxol production using this method is extremely low (24 to 50 ng/L) (Stierle *et al.*, 1993).

5. Plant tissue and cell culture of different *Taxus spp.* (Srinivasan *et al.*, 1995; Hirasuna *et al.*, 1994; Fett-Neto *et al.*, 1994b; Gibson *et al.*, 1993; Mirjalili and Linden, 1995a; Mirjalili and Linden, 1995b); this is the subject of this dissertation.

Matthew Suffness of the National Cancer Institute has reportedly declared: "There is no doubt that taxol can be produced by cell culture; the only question is whether the cost will be competitive with semisynthesis" (Holmes *et al.*, 1995).

1.9.2 Taxol production using plant cell culture

Plant cell culture production of taxol started in the late 1970s (Suffness, 1995). Screening of cell cultures (Christen *et al.*, 1989) gave promising results toward this alternative method of producing taxol. Several reports (Fett-Neto *et al.*, 1994b; Mirjalili and Linden, 1995a; Seki *et al.*, 1995; Fett-Neto *et al.*, 1994a; Gibson *et al.*, 1993; Hirasuna *et al.*, 1994; Ketchum and Gibson, 1994; Shuler *et al.*, 1992; Wickremesinhe and Arteca, 1994; Srinivasan *et al.*, 1995) have established the potential of producing taxol using plant cell culture systems. Fett-Neto *et al.* (1994b) have obtained 0.15 mg/L taxol during stationary phase (0.004 mg/L·day) and a specific productivity of 0.075 μg/g·day (Fett-Neto *et al.*, 1994a) using *T. cuspidata* cell cultures. Today taxol is being produced in "reasonable" quantities by Phyton Catalytic (Ithaca, NY) and ESCAgenetics (San Carlos, CA) in the United States; no actual data has been published by either company. Phyton Catalytic is utilizing 75,000 L stirred tank reactors in Germany to produce taxol (Bringi, V., personal communication).

Although the mechanism of action of taxol is known, there is a lack of understanding about taxol biosynthesis and plant physiology affecting taxol production. Plant cell culture can be used as a tool to exploit biosynthetic and genetic manipulation of taxol. Another advantage with plant cell culture is the production of taxol analogues. Ma *et al.* (1994) have reported that *Taxus* cell cultures are capable of producing taxanes which have not previously been identified from plant extracts. Commercialization of taxol requires production of

taxol in much higher concentrations than obtained from the bark. Productivity values in the range of 1–2 mg/L·day are needed for economic viability of taxol production using plant cell culture system (Shuler *et al.*, private communication). This subject will be discussed in more detail in later chapters.

Chapter 2

APPROACH

As discussed in Chapter 1, the problem in hand was to increase taxol production in suspension cultures of *Taxus cuspidata*. Our project was part of a five member consortium that was supported by the National Cancer Institute between 1990 and 1995 under RO1 CA55138–04. The other consortium members were Cornell University, USDA Agricultural Research Service, Phyton Catalytic Inc. and Hauser Chemical Inc. Each consortium member investigated a certain set of parameters. The main parameter of interest for our part of the study was the role of volatile gases on taxol production and physiology of *Taxus cuspidata* suspension cultures. Another approach was to stimulate taxol production by elicitation. These two broad approaches will be briefly discussed in this Chapter.

2.1 Volatile gas studies

We needed to study the effect(s) of oxygen, carbon dioxide, and ethylene without affecting other variables such as shear stress, which can indirectly change taxol production and physiology of the cells. We also needed to identify the effect of each gas and its possible interaction with other gases. Hence, an apparatus was required that provided independent control of each gas. An existing apparatus was modified to achieve this goal; several sets of experiments were designed and performed. The results of the controlled headspace gas studies are discussed in Chapters 3 and 4. The materials and methods for these experiments and the gas apparatus are explained in detail in the following sections.

2.1.1 Cell line and growth conditions

The cell lines used in this dissertation were kindly provided by Dr. Donna Gibson and Dr. Ray Ketchum (Plant, Soil and Nutrition Laboratory of the USDA Agricultural Research Service, Ithaca, New York). Originally, the cells were received on solid medium as callus. Cell suspension cultures were obtained by transferring small pieces of callus into liquid. This method resulted in suspension cultures with aggregates bigger than 2 mm in diameter. Subsequent subculturing and screening was required to reduce the aggregate size to less than 1 mm. This process was very time consuming with low rates of success. Consequently, 10 ml aliquots of suspension cultures, developed in the USDA laboratory, was sent to us via over night delivery carriers. The 10 ml aliquots were placed in sterile culture flasks; kept cool by ice packages in styrofoam containers. Generally 5–10 culture flasks were shipped to us in one styrofoam container. Upon arrival to our laboratory, each 10 ml aliquot was transferred into 20 ml of fresh medium and placed on a shaker rotating at 125 rpm at 25^{o}C.

The first cell line used in the studies described in this dissertation was *Taxus cuspidata* P991A2 (the name adopted by the USDA researchers). In this dissertation, this cell line is referred to as TC1 and was used in the preliminary experiments and experiments A-C. Due to loss of the air-conditioning in our laboratory on June 5, 1994, this cell line was lost. A subclone of TC1 was received from the laboratory of Dr. Donna Gibson as described above on July 20, 1994. This cell line was subsequently referred to as TC2 and was used in experiments D through F and the elicitation studies.

The growth medium used was according to Gibson *et al.* (1993). Gamborg's B5 basal salts medium supplemented with 2% sucrose, without auxins and cytokinins was purchased from Gibco BRL, Gaithersburg, MD. Naphthaleneacetic acid (5μM) and 6-benzylaminopurine (0.01μM) were added to the liquid

medium prior to sterilization. Glutamine (2mM) and ascorbic acid (50 mg/L) were filter sterilized and added to the cultures after sterilization and immediately prior to subculturing. All the reagents were plant cell culture tested and were purchased from Sigma Chemical Company, St. Louis, MO. The cultures were subcultured every two weeks in 125 ml Erlenmeyer flasks; 10 ml inoculum was added to 40 ml of fresh medium. For maintenance, the culture flasks were capped with 28 mm I.D. Bellco silicone closures (Vineland, NJ), and agitated at 125 rpm in a New Brunswick incubator shaker at 25°C in the dark.

2.1.2 The gas mixing apparatus

The apparatus used for controlling the gas flow into each flask (Figure 2.1) was developed from that described by Barmore and Wheaton (1978) and Haigh (1993). Oxygen, carbon dioxide, and nitrogen flowed from their respective cylinders through separate flowmeters into corresponding manifolds. Typically the flow rates of the three gases, O_2, CO_2, and N_2, were 80, 80, and 65 ml/min respectively. The oxygen, carbon dioxide, and nitrogen flows were regulated at 20 psi. Ethylene from the cylinder (20 psi) was diluted twice with compressed air and flowed into the ethylene manifold. The outlet pressure of the first ethylene dilution device was 14, and of the second 4 psi. The oxygen and carbon dioxide manifolds each had 15 metering valves; the ethylene manifold had 12 metering valves. The nitrogen was humidified and distributed from a manifold with 15 flow restrictors, rather than metering valves, to each flask. The flows from the oxygen, carbon dioxide, and ethylene manifolds were controlled by using the corresponding metering valves. The total flow to each flask was 40 ml/min, nitrogen was used as the balance gas. This arrangement allowed independent control of the compositions of oxygen, carbon dioxide, and ethylene, enabling full factorial experimental design. The gas apparatus was adjusted to the desired

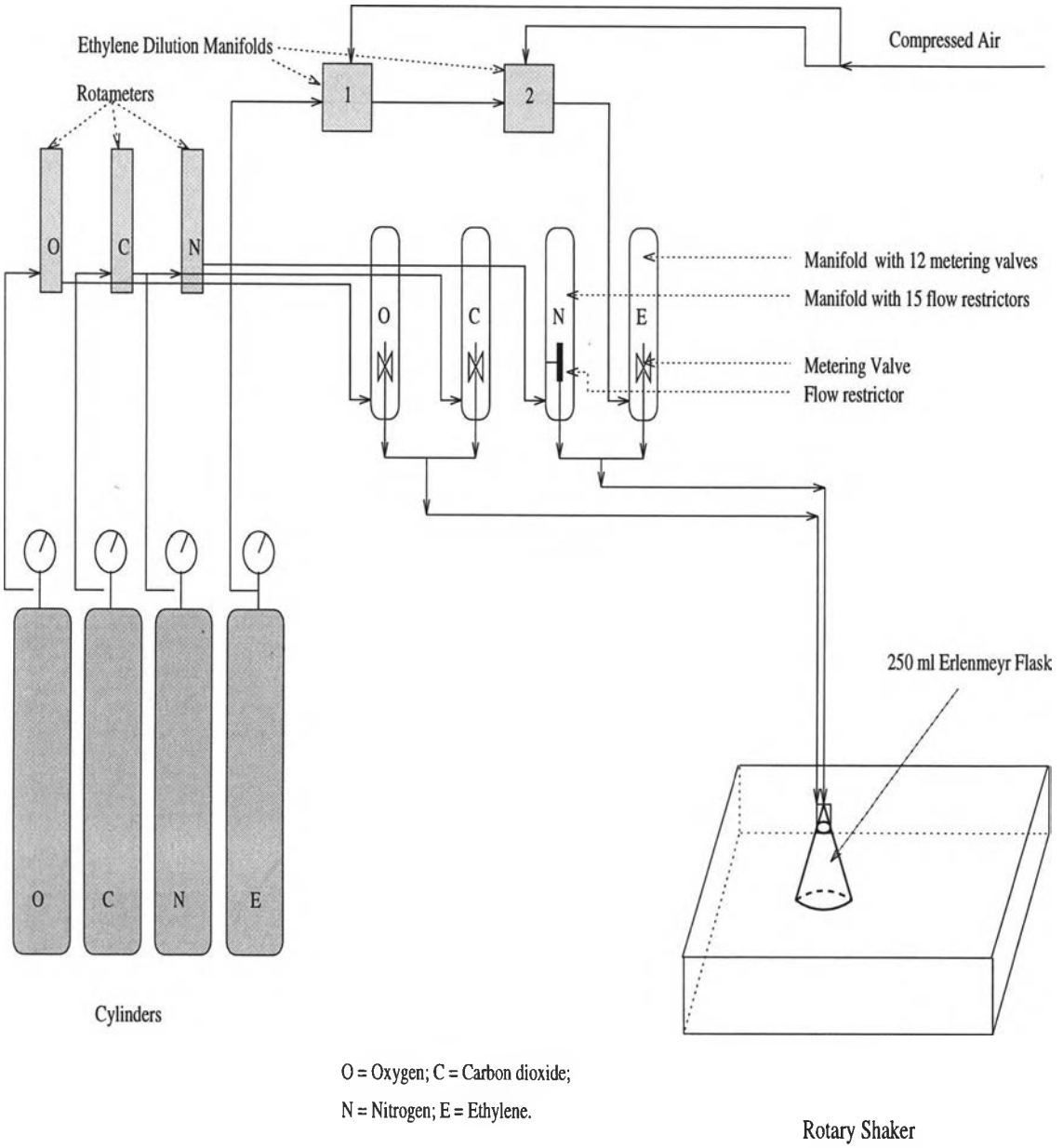

Figure 2.1: Schematic diagram of the gas mixing apparatus.

settings before the start of each experiment. The gas composition into each flask was measured by collecting gas samples in saran bags (Anspec Inc., Ann Arbor, MI) from the out-flow of each flask biweekly throughout the course of the experiments and analyzed using gas chromatography. Figure 2.2 shows typical concentrations of oxygen, carbon dioxide and ethylene over the 28 days of the experiments when the gas mixing apparatus was set to 10% (v/v) oxygen, 0.5% (v/v) carbon dioxide and 5 ppm ethylene.

2.1.3 Experimental conditions and procedures

Cultures were subcultured into 250 ml Erlenmeyer flasks for the experiments (10 ml inoculum into 40 ml fresh medium). A rubber stopper equipped with two 0.2 μm membrane filters was used as the closure for test flasks. The desired concentrations of oxygen, carbon dioxide, ethylene and nitrogen were fed into each flask through one of the filters at a total gas flow rate of 40 ml/min. Experiments were run for four weeks. The flasks were weighed after inoculation as well as before and after each sampling; the volume of water that had evaporated was added through a 0.45 micron cellulose acetate filter (Corning, NY) before each sampling. The weekly or biweekly sample volumes had to be limited to 1.0–1.5 ml to minimize the percent volume change during the course of the experiment. Following centrifugation at 6000 g for 15 minutes, supernatants were analyzed for sucrose, glucose, fructose, calcium, phosphate and taxol. The fresh weight data that were obtained from such small samples were not reliable enough for quantifying growth. Therefore, the DNA content was used to quantify growth of the cultures.

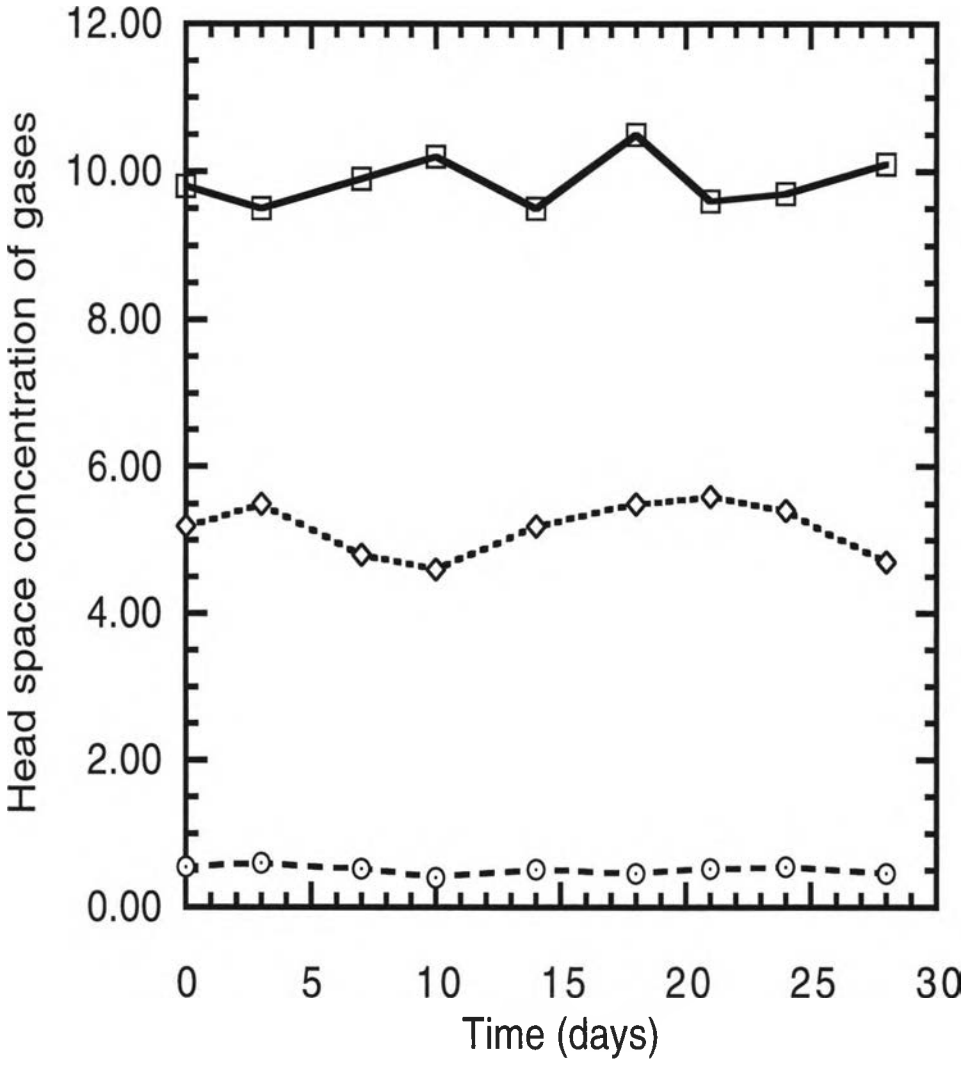

Figure 2.2: Typical concentrations of oxygen, carbon dioxide and ethylene as measured through the out-flow filter unit; the gas apparatus was pre-adjusted to 10% (v/v) O_2, 0.5% (v/v) CO_2 and 5 ppm ethylene. $\square$ = oxygen; $\diamond$ = carbon dioxide; $\odot$ = ethylene.

Reference cultures

In order to be able to compare our experimental results with those of other consortium members, various types of reference (control) cultures were used in each experiment. These reference cultures were as following.

1. 125 ml flasks capped with Bellco silicone closures.

2. 250 ml flasks capped with Bellco silicone closures.

3. 250 ml flasks with 40 ml/min of humidified air flowing into them.

2.1.4 Oxygen and carbon dioxide measurements

Oxygen was measured on a Gow Mac model 550 gas chromatograph equipped with a thermal conductivity detector and a molecular sieve column (Alltech 5A, 0.085 in I.D., 4 ft, SS). The mobile phase was helium at 25 ml/min. The column and the detector were kept at 50°C. Gas samples of 1.0 ml were injected and quantified against gas mixtures of known concentration. Carbon dioxide was measured on the same gas chromatography system under the same conditions using an Alltech Porapak N (0.085 in I.D. 6 ft, SS) column. Air (21% oxygen) was used as the O_2 standard. Samples from a gas cylinder containing 5% carbon dioxide in air were used as the CO_2 standard.

2.1.5 Ethylene measurement

Ethylene was measured on a Hewlett Packard model 5840A gas chromatography system equipped with a flame ionization detector (FID). An Alltech Porapak N (6 ft, 0.2 mm I.D., SS) column was used at 75°C. The injection port and detector temperatures were 90°C and 180°C, respectively; the mobile gas was helium at 20 ml/min; sample size was 0.2 ml. Pure ethylene was diluted to 100, 50, 25, 10 and 5 ppm concentrations by injecting a known volume into

empty 500 ml flasks. These concentrations of ethylene were used to construct an ethylene calibration curve.

2.2 Elicitation studies

Elicitation is an example of secondary metabolite production as a result of induced enzyme synthesis involving transcription and translation (Croes *et al.*, 1994). Several reports (DiCosmo and Misawa, 1985; Kutchan, 1991; Gundlach *et al.*, 1992; Xu *et al.*, 1994; Enyedi *et al.*, 1992; Dixon *et al.*, 1990) have demonstrated the induction of secondary metabolites in plant cell culture systems. It is reported that both ethylene and methyl jasmonate activate plant defense genes, are both involved in the signal transduction of plants and are both produced by plants as a response to various forms of stress (e.g., pathogenic attack, wounding, etc.). The effect(s) of ethylene/methyl jasmonate on production of taxol was investigated using the following sets of methods.

2.2.1 Materials and methods

As mentioned in Section 2.1.1, the cell line TC2 was used in the elicitation experiments. The growth medium and subculturing procedures were the same as described in Section 2.1.1. As will be discussed in later Chapters, the growth rate of this cell line was 67% that of the previous one. In order to be able to compare the gas studies with elicitation studies, taxol production and phosphate uptake rates per gram glucose consumed was calculated for both studies. The result of the elicitation experiments are discussed in Chapter 6.

2.2.2 Experimental conditions

The gas mixing apparatus explained in Section 2.1.2 was used to study the various concentrations and combinations of ethylene and methyl jasmonate. Elicitation was started seven days after growth was initiated under 10% (v/v)

oxygen and 0.5% (v/v) carbon dioxide and variable headspace ethylene concentrations. Methyl jasmonate (Bedoukian Research Inc., Danbury, CT) at concentrations of 0, 10 and 100 μM was added to the cultures in 5 μl of ethanol per ml of culture (Gundlach *et al.*, 1992). Equal volumes of ethanol (250 μl) was added to all cultures. After elicitation, samples were taken every 8 hours for the first day and then every 24 hours until day 14. Samples were analyzed for extracellular sucrose, glucose, fructose, phosphate, calcium and taxol.

2.3 Sample assays

2.3.1 DNA analysis

Weighed fresh cells (0.1–0.5 g) were washed with 2 ml of washing solution (150 mM NaCl, 15 mM citrate, 3 mM EDTA, pH 7) and centrifuged at 700 g for 10 minutes. The pellets were extracted with 0.05 ml of extraction solution (1 N NH_4OH, 0.2% Triton x-100) by mixing and incubating at 37°C for 10 minutes. Cell lysates were diluted to 2.0 ml with assay buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris, pH 7), centrifuged at 2500 g at 4°C for 30 minutes and kept on ice until assayed. Different concentrations of calf thymus DNA standard were prepared containing the same concentration of extraction solution and assay buffer. Fluorescence was measured on a Perkin Elmer model MPF–2A spectrofluorometer at an excitation wavelength of 350 nm and emission wavelength of 455 nm. The procedure was according to that provided with Pierce DNA kit (cat.# 23225). DNA concentrations were expressed as μg DNA per gram fresh weight.

2.3.2 Sugar assay

Sucrose, glucose, and fructose were measured using a Waters model 6000 High Pressure Liquid Chromatography (HPLC) system equipped with a

refractive index detector. The column was a Biorad HPX-87P monosaccharide (300×7.8 mm) column. The mobile phase was distilled water; the temperature of the mobile phase and column was 80°C. Standards at concentrations of 10, 5, 2.5, 1.25, and 0.625 g/L sucrose, glucose, and fructose were prepared and used to construct a calibration curve for each sugar.

2.3.3 Taxol analysis

Taxol concentration was measured on a Waters model 501 HPLC system equipped with a Waters model 486 UV detector at 228 nm. The column used was Phenomenex Curosil G (250×4.6 mm). Acetonitrile : water (45:55) at 1.0 ml/min was used as the mobile phase. Extracellular taxol was measured by mixing 250 μl of supernatant with 750 μl methanol, filtering through a 0.45 μm nylon filter into 1.5 ml vials. To measure intracellular taxol, weighed cells (0.1–0.4 g) were dried; 1.0 ml methanol was added and the mixture was sonicated in a bath sonicator for 1.0 hour; then centrifuged into pellets. Following centrifugation, supernatants were collected and dried; 100 μl methanol was added. The sample was then filtered through a 0.2 μm nylon membrane into 0.2 ml vials. Taxane standards were kindly provided by Hauser Chemical Inc., Boulder Colorado. Taxol standards at concentrations of 0.05, 0.1, 0.5, 1, 2, 4, and 10 mg/L were prepared and used to construct a calibration curve.

2.3.4 Calcium assay

Extracellular calcium concentration was measured colorimetrically by using Arsenazo 3 solution (Sigma cat. # 588). The procedure involved incubation of 10 μl of sample with 1.0 ml of the reagent for one hour at room temperature; absorbance at 600 nm was then read on a Bausch and Lomb Spectronic 21 and compared to a standard curve. The standard curve was constructed by using calcium chloride at concentrations of 200, 100, 50, and 25 mg/L.

2.3.5 Phosphate assay

Extracellular phosphate concentration in the sample was measured colorimetrically (Kagawa and Sone, 1979). The sample (100 μl) was incubated with 350 μl of an ammonium molybdate solution for 10 minutes at 37^{o}C. Absorbance of the mixture was read at 700 nm after cooling for approximately 10 minutes. Sodium phosphate ($NaH_2PO_4.H_2O$) at concentrations of 200, 100, 50, and 25 mg/L was used to construct a calibration curve.

2.4 Statistical analysis

Statistical experimental design was used in all the experiments in this dissertation to determine variables that significantly affect process performance (i.e. taxol production, nutrient uptake). The results of the experiments were interpreted based on the estimates of how each of the experimental factors affected taxol production. The design of experiments enabled us to know which variables should be changed, and in which direction, to improve the taxol productivity.

The experiments described in Chapters 3 and 6 were full factorial design experiments (with either 2 or 3 factors). A full factorial design experiment investigates all of the possible combinations of values of each experimental factor. Full factorial designs can also provide good estimates of experimental error or noise (Haaland, 1989). Our experiments were conducted using a two-level factorial design, providing information about all main effects and two-factor interactions. The data from each gas experiment was analyzed with the aid of a statistical program (Minitab). A linear regression model was constructed with statistically valid ($p \leq 0.05$) factors at each time point (i.e. time was not a variable). The sign and magnitude of the coefficients of each factor allowed comparison of the relative importance of the estimated effects. The factors had dimensional units

of the respective process performance they were defining. A sample of a linear regression model is explained below.

$$Taxol(day3) = 0.028 - 0.029[CO_2] + 0.031[C_2H_4] - 0.055[CO_2 \times C_2H_4] \quad (2.1)$$

Equation 2.1 shows that carbon dioxide, ethylene and the interaction of carbon dioxide and ethylene have effects at the 95% significance level on taxol concentration on the 3rd day of the experiment. The effect of carbon dioxide is negative while ethylene has a positive effect on the taxol production. This means that taxol concentration decreased going from low to high concentration of carbon dioxide; whereas taxol concentration increased going from low to high concentration of ethylene. The interaction of carbon dioxide and ethylene had the highest effect on production of taxol on Day 3 of the experiment.

Chapter 3

CONTROLLED HEADSPACE GAS EXPERIMENTS

The use of plant cell systems as a potential method of producing taxol was discussed in Chapter 1. However, the concentration of taxol using this method is still low. Commercialization of taxol using plant cell culture requires relatively high productivity. Productivity can be thought of in two ways : 1) product concentration per total dry mass synthesized per unit time and 2) product concentration per liter of cultured medium per unit time. In either case, reducing the time involved is important. The purpose of this study was to find conditions under which taxol is produced early and in high concentrations in suspension cultures of *Taxus cuspidata*.

The roles of oxygen and carbon dioxide and ethylene on growth and secondary metabolite production were reviewed in Chapter 1 (Section 1.7.1). Application of ethylene, which frequently is a product of stress imposed on plant cells, might induce excretion of secondary metabolites from cells. Taxol may be produced by plants as a response to pathogen attack, which is one form of stress. This is the rationale for studying the effect of ethylene on inducing early taxol formation and increasing productivity. The gas mixing apparatus (described in Chapter 2) enabled us to study the roles of oxygen, carbon dioxide, ethylene and their possible interactions on taxol production.

The preliminary experiments were treated as screening exercises. Full factorial design experiments with three factors, each at two levels, require eight

treatments. The gas mixing apparatus had the capability of accommodating 15 treatments; we could duplicate all but one of the eight treatments. Therefore, we needed to perform screening experiments to determine whether any factor is small and can be discarded. The factor(s) whose effects are small can be held constant and not varied, while other factors can be studied in more detail with additional designed experiments (Deming and Morgan, 1993). The purpose of the screening experiments was to choose from the three factors those that are most influential to make initial improvements in the system. Following the preliminary experiments, the definitive experiments were conducted, exploring the roles of carbon dioxide and ethylene in more detail. This Chapter provides the results of preliminary and definitive experiments on taxol production, while Chapter 4 discusses the effect of the above mentioned gases on growth and nutrient patterns in suspension cultures of *Taxus cuspidata.*

3.1 Preliminary experiments

Two sets of preliminary experiments were conducted to determine the correlations between gas composition and taxol production. The experiments were full factorial design with three factors, each at two levels. The factors and their levels (head space concentrations) shown in Table 3.1 were selected on the basis of results from a similar study on an *Artemisia annua* cell culture (Haigh, 1993).

Table 3.1: The factors and the levels studied in the preliminary experiments.

Factor	High	Low
O_2	25%	10%
CO_2	10%	0.5%
C_2H_4	2ppm	0ppm

Table 3.2: Taxol production in the first preliminary experiment.

Treatment ($O_2/CO_2/C_2H_4$)	Taxol(wk1) mg/L±SD	Taxol(wk2) mg/L±SD	Taxol(wk3) mg/L±SD	Taxol(wk4) mg/L±SD
25/10/2	0	0.072±0.002	0.047±0.002	0.055±0.001
25/10/0	0	0	0	0.034±0.001
25/0.5/2	0	0.16±0.02	0.039±0.001	0.034±0.001
25/0.5/0	0	0	0.13±0.002	0.062±0.002
10/10/2	0	0.27±0.08	0.21±0.02	0.13±0.06
10/10/0	0	0	0	0.022±0.007
10/0.5/2	0.29±0.07	0.15±0.05	0.16±0.06	0.05±0.03
10/0.5/0	0.27±0.04	0.24±0.07	0.26±0.09	0.11±0.06
21/0.03/0	0	0	0.034±0.009	0.16±0.06

The three types of reference cultures (see Chapter 2), were used throughout the experimentation as controls. The head space gas concentrations in the flasks under reference operating conditions were measured, which showed these cultures were exposed to 21% (v/v) oxygen, 0.03% (v/v) carbon dioxide and 0 ppm ethylene during the four weeks of the experiments.

The most important result obtained from the preliminary experiments was that there was no taxol production during the first week using oxygen concentrations of ambient or above. Taxol production in the first preliminary experiment is presented in Table 3.2. Under ambient conditions, taxol was not detected until Day 21. The negative effect of oxygen and positive effect of ethylene are depicted in Equation 3.1.

$$Taxol(wk1) = 0.028 - 0.029[O_2] + 0.031[C_2H_4] - 0.055[CO_2 \times C_2H_4] \quad (3.1)$$

The second preliminary experiment was run for two weeks only. In order to obtain good biomass data, whole contents of the flasks were harvested on the 14th day; no samples were taken during the two weeks. The growth data will be discussed in Chapter 3. Table 3.3 shows extracellular taxol concentration

Table 3.3: Taxol production in the second preliminary experiment.

O_2% (v/v)	CO_2% (v/v)	C_2H_4 ppm	Comments	14 day Taxol mg/L±SD
25	10	2	–	2.10±0.11
25	10	0	–	1.09±0.01
25	0.5	2	–	1.03±0.05
25	0.5	0	–	1.98±0.13
10	10	2	–	1.68±0.15
10	10	0	–	1.71±0.11
10	0.5	2	–	1.37±0.11
10	0.5	0	–	1.53±0.16
21	0.03	0	40 ml/min air	0
21	0.03	0	250 ml, Bellco cap	0
21	0.03	0	125 ml, Bellco cap	1.8±0.05

after 14 days of growth under the full factorial design experiment. The reason taxol levels were higher in the second preliminary experiment was due to the replacement of the hormone picloram by naphthaleneacetic acid (NAA) in the growth medium, which was recommended by our collaborators at the USDA laboratory. The statistical regression analysis for this experiment (Equation 3.2) also showed a negative effect of oxygen and a positive effect of ethylene on taxol production.

$$Taxol(day14) = 1.4 - 0.15[O_2] + 0.14[C_2H_4] - 0.13[CO_2 \times C_2H_4] - 0.36[O_2 \times CO_2 \times C_2H_4] \quad (3.2)$$

Both Equations 3.1 and 3.2 showed the interaction of carbon dioxide and ethylene on production of taxol, as an important factor. The oxygen concentration was set at 10% (v/v) for subsequent experiments; the two-factorial effects of carbon dioxide and ethylene were subsequently studied in greater detail in the definitive experiments described below.

3.2 Definitive experiments

The results of the definitive experiments, A through F, are discussed in detail here. The most effective treatment from one experiment was repeated in the subsequent experiment.

3.2.1 Experiment A

The experimental design for Experiment A is shown in Table 3.4. The carbon dioxide and ethylene concentrations were each varied at two levels in quadruplicate to obtain a better estimate of the experimental error. Samples of 1.5 ml (cells plus liquid) were taken from each flask every week for four weeks. Figure 3.1 shows taxol production for experiment A under the four treatments.

Table 3.4: Experimental design for Experiment A.

Treatments	O_2%(v/v)	CO_2%(v/v)	C_2H_4(ppm)	Comments
1–4	10	10	2	–
5–8	10	10	0	–
9–12	10	0.5	2	–
13–16	10	0.5	0	–
17–20	21	0.03	0	humidified air

The greatest taxol concentration was observed on day 6 (3.6 mg/L) with 10% oxygen, 0.5% carbon dioxide and 2 ppm ethylene. The standard deviation of replicates was less than 15 percent of the mean values for taxol concentration in the medium. As shown in Table 3.5, taxol was not observed until day 21 in the reference cultures exposed to ambient concentration of oxygen, carbon dioxide and ethylene.

The higher ethylene concentration (2 ppm) had a positive effect on taxol concentration. The coefficients of the terms in the statistical regression equation (Equation 3.3), reveal the sign and relative size of effects on taxol production

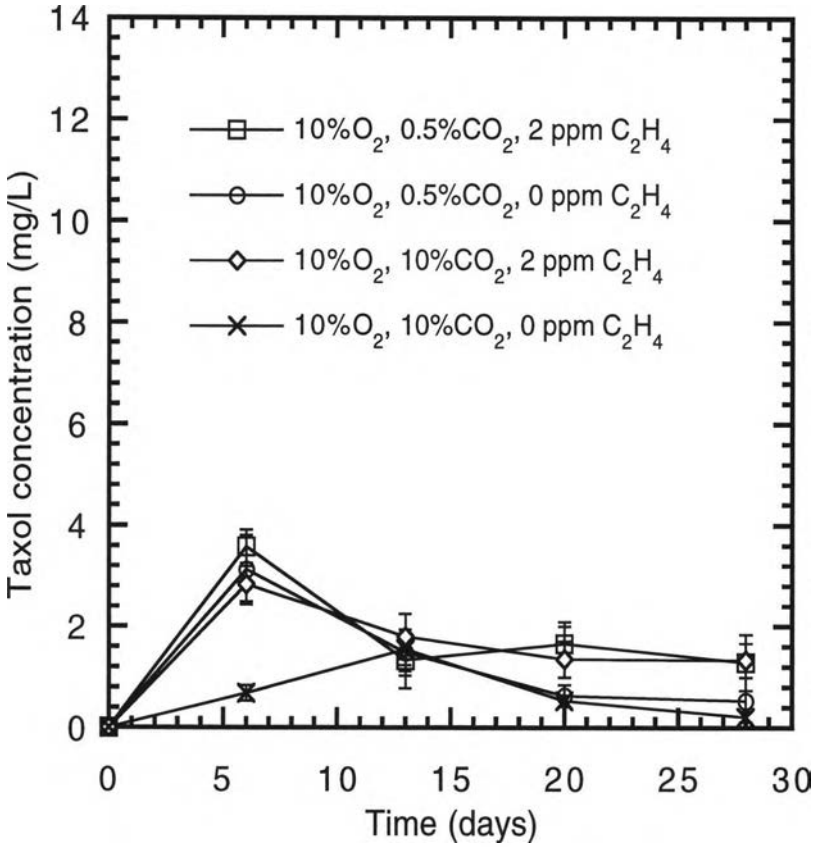

Figure 3.1: Kinetics of taxol production when cultures were grown under the experimental design for Experiment A.

Table 3.5: Taxol data for Experiment A.

Treatment ($O_2/CO_2/C_2H_4$)	Taxol(wk1) mg/L±SD	Taxol(wk2) mg/L±SD	Taxol(wk3) mg/L±SD	Taxol(wk4) mg/L±SD
10/10/2	2.84±0.27	1.80±0.13	1.35±0.16	1.33±0.15
10/10/0	0.68±0.05	1.55±0.15	0.53±0.05	0.21±0.05
10/0.5/2	3.58±0.21	1.33±0.16	1.65±0.21	1.29±0.21
10/0.5/0	3.12±0.25	1.48±0.15	0.63±0.05	0.53±0.09
21/0.03/0	0	0	1.11±0.20	1.74±0.35

accordingly. The greater carbon dioxide concentration (10% v/v) had a negative effect on taxol production. Ethylene had a positive effect and the interaction between carbon dioxide and ethylene had the greatest effect on taxol production (Equation 3.3).

$$Taxol(6days) = 2.56 - 0.42[CO_2] + 0.55[C_2H_4] - 0.79[CO_2 * C_2H_4] \quad (3.3)$$

Early production of taxol under 10% (v/v) oxygen (day 6) was noted in all cultures except for cultures exposed to 10% (v/v) CO_2 and 0 ppm C_2H_4 and the reference cultures. Hence, carbon dioxide concentrations were reduced and ethylene concentrations were increased for the next set of experiments.

3.2.2 Experiment B

Based on the results of Experiment A, the higher carbon dioxide concentration was reduced to 0.1% (v/v) and the higher ethylene concentration was increased to 5 ppm; oxygen was maintained at 10% (v/v) in all flasks. This two factor, two level experimental design was conducted in triplicate (Table 3.6). In order to get more information on kinetics of taxol production, samples of 1.0 ml were taken biweekly from each flask. All three types of reference flasks explained in Chapter 2 were used in this experiment. The triplicate treatments of 4–6 repeated the best result of experiment A.

In experiment B (Figure 3.2), as well as in experiment A (Figure 3.1), taxol levels declined after the initial peak (day 7) in all cultures except for those exposed to 10% (v/v) oxygen, 0.5% (v/v) carbon dioxide and 5 ppm ethylene. Using this combination of applied gases, taxol was observed first on day 7 (3.5 mg/L) and continuously increased until day 21 (6.5 mg/L) before the decline was noted on day 25. The cause and result of the decline is not known; a coincident increase in other taxanes has not been observed in chromatograms. The coefficient of variance for this experiment was less than 10 percent.

Table 3.6: Experimental design for Experiment B.

Treatments	O_2%(v/v)	CO_2%(v/v)	C_2H_4(ppm)	Comments
1–3	10	0.5	5	–
4–6	10	0.5	2	–
7–9	10	0.1	5	–
10–12	10	0.1	2	–
13–15	21	0.03	0	humidified air
16–18	21	0.03	0	250 ml Bellco capped
19–21	21	0.03	0	125 ml Bellco capped

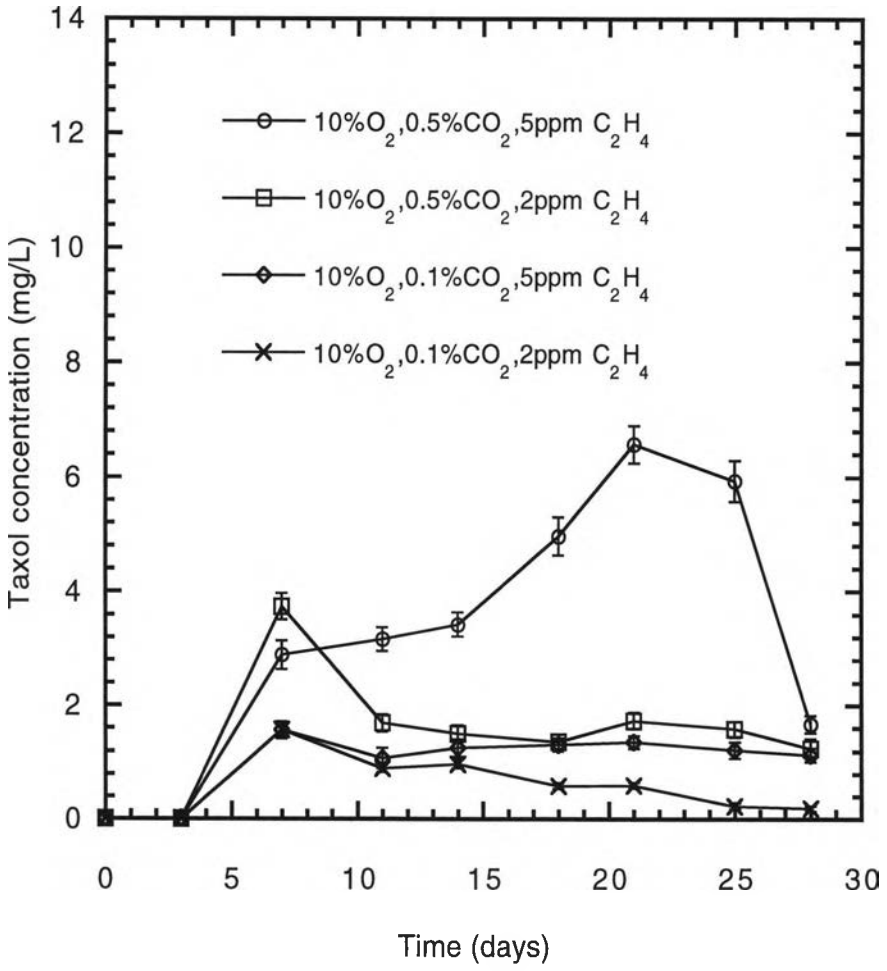

Figure 3.2: Kinetics of taxol production when cultures were grown under the experimental design for Experiment B.

Table 3.7: Taxol data for experiment B

Treatment ($O_2/CO_2/C_2H_4$)	Comments	Taxol(day3) mg/L±SD	Taxol(day7) mg/L±SD	Taxol(day11) mg/L±SD
10/0.5/5	–	0	2.88±0.25	3.16±0.21
10/0.5/2	–	0	3.73±0.23	1.69±0.15
10/0.1/5	–	0	1.57±0.11	1.07±0.18
10/0.1/2	–	0	1.56±0.15	0.89±0.07
21/0.3/0	40 ml/min air	0	0.56±0.05	1.07±0.11
21/0.3/0	125 ml Bellco	0	0.82	1.54
21/0.3/0	250 ml Bellco	0	0.55	1.50

Taxol(day14) mg/L±SD	Taxol(day18) mg/L±SD	Taxol(day21) mg/L±SD	Taxol(day25) mg/L±SD	Taxol(day28)
3.42±0.21	4.96±0.33	6.57±0.33	5.93±0.36	1.67±0.15
1.50±0.15	1.36±0.13	1.73±0.15	1.58±0.14	1.24±0.17
1.25±0.15	1.31±0.11	1.35±0.11	1.21±0.14	1.13±0.12
0.97±0.08	0.58±0.03	0.59±0.05	0.22±0.01	0.18±0.05
1.42±0.16	1.94±0.11	1.74±0.13	1.57±0.12	NA
2.23±0.21	2.20±0.16	2.55±0.19	3.25±0.16	2.01
2.18±0.22	2.29±0.29	2.59±0.35	NA	NA

Carbon dioxide had a positive effect on taxol concentration; i.e. 0.5% (v/v) was better than 0.1% (v/v) as seen in Equations 3.4 and 3.5. Ethylene had a negative effect on taxol concentration as measured on day 7, but a positive effect as measured throughout the rest of the experiment; i.e. 2 ppm was better during the first week, but 5 ppm was better during the last 3 weeks of the experiment (Equations 3.4 and 3.5). The reference cultures produced 3.25 mg/L taxol on day 25 (Figure 3.4).

$$Taxol(day7) = 2.43 + 0.87[CO_2] - 0.21[C_2H_4] + 0.21[CO_2 * C_2H_4] \quad (3.4)$$

$$Taxol(day11+) = 1.70 + 0.72[CO_2] + 0.41[C_2H_4] + 0.32[CO_2 * C_2H_4] \quad (3.5)$$

The taxol data for this experiment are shown in Table 3.7.

Table 3.8: Experimental design for Experiment C.

Treatments	O_2%(v/v)	CO_2%(v/v)	C_2H_4(ppm)	Comments
1–3	10	0.5	5	–
4–6	10	0.5	10	–
7–9	10	0.5	5	1% fructose on day 11
10–12	21	0.03	0	250 ml Bellco capped
13–15	21	0.03	0	125 ml Bellco capped

3.2.3 Experiment C

In Experiment C, the oxygen and carbon dioxide concentrations were held at 10% (v/v) and 0.5% (v/v), respectively; the ethylene concentration was increased to 10 ppm. Table 3.8 shows the experimental design for this experiment. The triplicate treatments 1–3 repeated the best results from Experiment B. The results of the sugar analysis of the cultures in Experiment B showed that the cultures were depleted of glucose during the second week and of fructose during the third week of the experiment (see Chapter 4). Therefore, 10 g/L fructose was added to one set of the treatments on day 11 of the growth.

The higher ethylene concentration (10 ppm) used in Experiment C was not inhibitory, but taxol levels did not significantly increase over that of 5 ppm (Figure 3.3). Cultures exposed to 5 ppm ethylene produced similar taxol levels in this experiment as well as in experiment B. The greatest taxol concentration was observed in the cultures to which fructose was added on day 11. These cultures continued production of taxol until day 25 (12.2 mg/L). Effect of fructose on taxol production was also reported by our collaborators at Cornell University. Hirasuna *et al.* (1994) reported that fructose at concentration of 10 g/L increased taxol production 2–7 fold when added to low and moderate producing cell lines; the effect of fructose on the high producing cell line was negative. The stimulatory and inhibitory effects of fructose was attributed to stimulation or inhibition

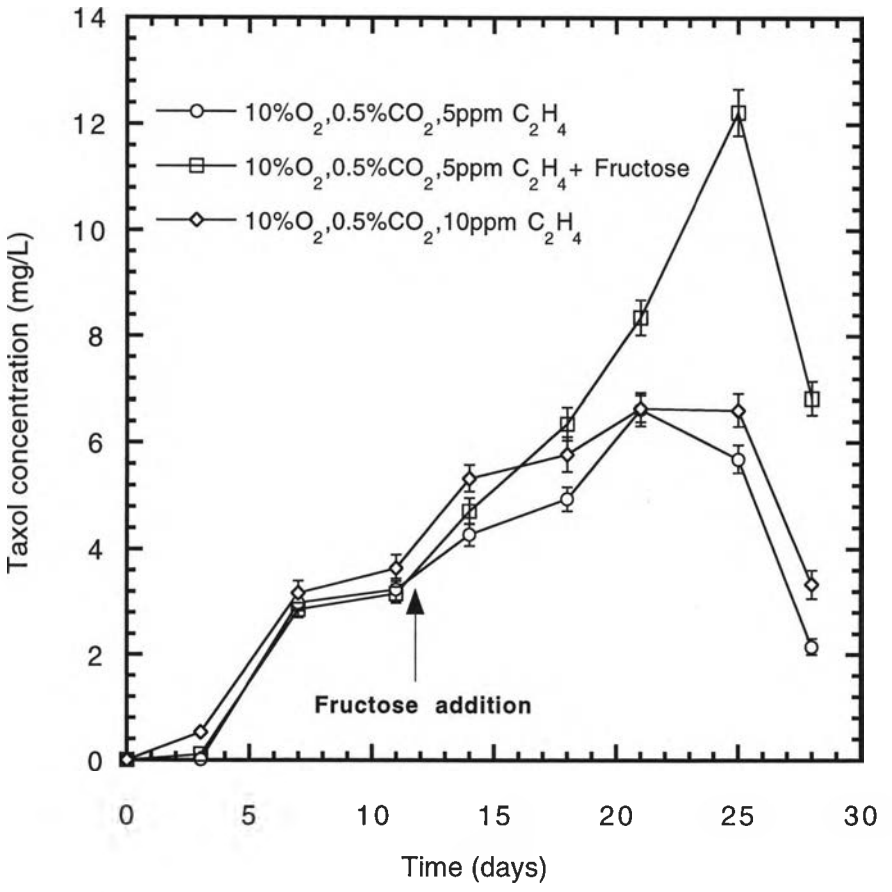

Figure 3.3: Kinetics of taxol production when cultures were grown under the experimental design for Experiment C.

of a limiting step in taxol synthesis (Hirasuna *et al.*, 1994); 10 g/L glucose was assumed to have inhibitory effect on this limiting step. In experiment C, the intracellular concentration of taxol was also measured. No intracellular taxol was detected. Srinivasan *et al.* (1995) have reported that the intracellular taxol concentration was always less than 10% of total (intracellular and extracellular) taxol. Taxol data for Experiment C are shown in Table 3.9.

Table 3.9: Taxol data for experiment C

Treatment ($O_2/CO_2/C_2H_4$)	Comments	Taxol(day3) mg/L±SD	Taxol(day7) mg/L±SD	Taxol(day11) mg/L±SD
10/0.5/5	–	0	2.98±0.15	3.24±0.21
10/0.5/10	–	0	3.17±0.23	3.64±0.25
10/0.5/5	1% fructose on day 11	0	2.86±0.15	3.16±0.18
21/0.03/0	125 ml,Bellco	0	0.88	1.7
21/0.03/0	250 ml,Bellco	0	0.53	1.89

Taxol(day14) mg/L±SD	Taxol(day18) mg/L±SD	Taxol(day21) mg/L±SD	Taxol(day25) mg/L±SD	Taxol(day28) mg/L±SD
4.27±0.21	4.94±0.23	6.63±0.31	5.69±0.26	2.12±0.15
5.33±0.25	5.78±0.33	6.65±0.25	6.61±0.31	3.33±0.27
4.71±0.25	6.35±0.31	8.35±0.33	12.20±0.44	12.08±0.32
2.33	2.75	3.28	3.15	4.04
2.18	2.53	NA	NA	NA

3.2.4 Taxol in reference cultures

The data shown in Figure 3.4 are representative of reference cultures in experiments A, B, and C. The reference cultures produced less taxol on day 7 than the treated cultures (0.5 vs. 3.5 mg/L). In the 125 ml Bellco capped shake flasks, taxol production continued until day 25 (3.1 mg/L). Reference cultures in 250 ml Bellco capped flasks produced 2.4 mg/L taxol on day 21, but turned red on day 22. Reference cultures flushed with 40 ml/min humidified air produced 1.9 mg/L taxol on day 18 and taxol levels declined thereafter; these cultures turned red on day 25. The red coloration is believed to be associated with an as yet undefined stress response and always leads to cell death.

3.2.5 Experiments D, E, and F

As mentioned in Chapter 2 Section 2.1.1, the cell line TC2 was used in Experiments D, E, and F. In experiment D, the oxygen and carbon dioxide concentrations were 10% (v/v) and 0.5%(v/v), respectively; ethylene concentrations of 5 and 50 ppm were studied in duplicate cultures. Table 3.10 shows the design for this experiment.

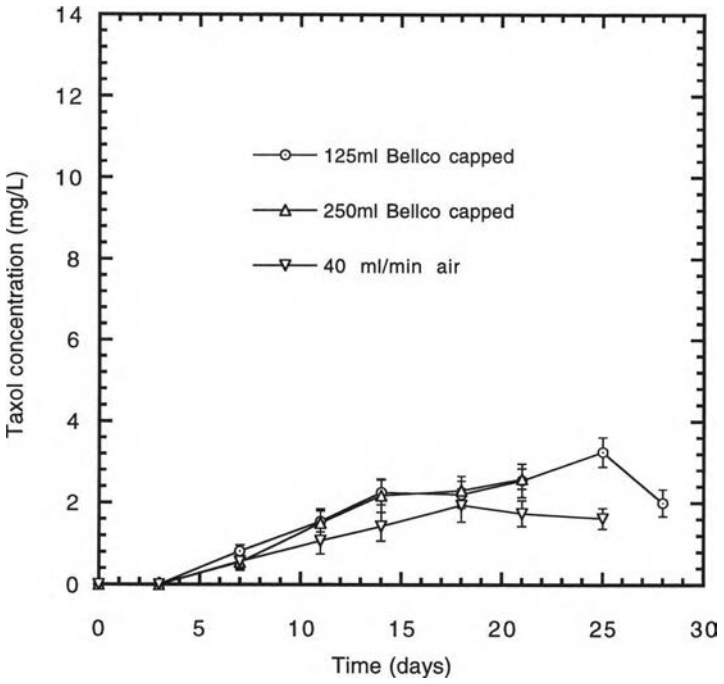

Figure 3.4: Average taxol production in reference cultures from Experiments A-C.

Table 3.10: Experimental design for Experiments D, E, and F for which the cell line TC2 was used.

Treatments	O_2%(v/v)	CO_2%(v/v)	C_2H_4(ppm)	Comments
1–2	10	0.5	5	–
3–4	10	0.5	50	–
5–6	21	0.03	0	125 ml Bellco capped

Table 3.11: Results of Experiment D.

Treatment ($O_2/CO_2/C_2H_4$)	Taxol(day3) mg/L±SD	Taxol(day7) mg/L±SD	Taxol(day10) mg/L±SD
10/0.5/5	0.61±0.08	0.43±0.05	0.50±0.02
10/0.5/50	0	0	0.50±0.01
21/0.03/0	0	0	0.85±0.11
Taxol(day14) mg/L±SD	**Taxol(day21) mg/L±SD**	**Taxol(day24) mg/L±SD**	**Taxol(day28) mg/L±SD**
0.47±0.05	0.48±0.01	0.07±0.02	0.06±0.01
0.39±0.08	0.43±0.12	0.43±0.11	0.53±0.15
NA	NA	NA	NA

Kinetics of taxol production in experiment D are shown in Table 3.11. The TC2 cell line grew more slowly and produced less taxol (0.5–0.8 mg/L vs. 3–4 mg/L) compared to TC1. The cultures exposed to 5 ppm ethylene produced the highest concentration of taxol on Day 3 (0.61 mg/L); taxol levels declined thereafter. The cultures exposed to 50 ppm ethylene did not produce taxol until day 10 (0.50 mg/L). The reference cultures in experiment D turned red after the 10th day of growth. Due to the surprising lower concentration of taxol in the 50 ppm treatments as compared to the 10 ppm ethylene treatments, this experiment was repeated twice more in experiments E and F, following the same experimental design as experiment D. Experiment E was only run for one week (cultures became contaminated on Day 7). Experiment F was run for 28 days as was done for the Experiments A-D.

Cultures exposed to 50 ppm ethylene always produced less taxol compared to the cultures exposed to 5 ppm ethylene (Tables 3.12 and 3.13). Even though saturation kinetics are expected for ethylene action (Sisler, 1991), 5 and 10 ppm ethylene resulted in the highest taxol levels in our studies. There are instances given in the literature where hormone action occurs at defined optimum

Table 3.12: Taxol data for Experiment E.

Treatment ($O_2/CO_2/C_2H_4$)	Taxol(day3) mg/L±SD	Taxol(day7) mg/L±SD
10/0.5/5	0.85±0.15	0.94±0.22
10/0.5/50	0.23±0.10	0.58±0.15
21/0.03/0	0	0

concentrations. For example, optimal cell division in wild-type tobacco protoplasts occurred at defined auxin (NAA) concentration (Walden *et al.*, 1994). Salisbury and Ross (1992) reported that the auxin indolacetic acid (IAA) at concentrations of 10^{-7} to 10^{-13} M promoted root elongation, but root elongation was always inhibited at IAA of concentrationsof 1–10^{-6}M. Part of this inhibition was attributed to production of ethylene; since high concentrations of auxins are reported to produce ethylene (Salisbury and Ross, 1992). In experiment F, the reference cultures produced 0.03 mg/L taxol on day 10; taxol levels declined thereafter. Cultures in Experiment F produced less taxol than in experiments D and E; the reason for this behavior of the cultures is not known and can only be attributed to the instability of this cell line.

In order to be able to compare TC1 and TC2 cell lines, concentration of glucose consumed by the cells was calculated and taxol levels were normalized. These data are shown in the next Chapter.

3.3 Discussion

The effect of different concentrations and combinations of oxygen, carbon dioxide, and ethylene on taxol production in suspension cultures of *Taxus cuspidata* was studied using several factorial design experiments. Although carbon dioxide and ethylene can be important in plant cell culture systems, few studies have examined the role of these two gases quantitatively. In our experiments,

Table 3.13: Taxol data for Experiment F.

Treatment (O_2/CO_2/C_2H_4)	Taxol(day3) mg/L±SD	Taxol(day7) mg/L±SD	Taxol(day10) mg/L±SD
10/0.5/5	0.03±0.03	0.07±0.03	NA
10/0.5/50	0.02±0.03	0.017±0.01	0.058±0.03
21/0.03/0	0	0.016±0.01	0.03±0.01
Taxol(day14) mg/L±SD	Taxol(day21) mg/L±SD	Taxol(day24) mg/L±SD	Taxol(day28) mg/L±SD
0.08±0.03	NA	NA	NA
0.078±0.03	0	0	0
0	0	0	0

the suspension cultures were exposed to known and constant concentrations of oxygen, carbon dioxide, and ethylene for four weeks. The concentration of O_2, CO_2 and C_2H_4 in the medium is dependent on the concentration of these gases in the head space and on the solubilities of these gases in the medium (taken as water here). Equilibrium between the two phases for each gas is assumed according to the following.

Carbon dioxide has the highest solubility among the above mentioned gases. As carbon dioxide dissolves in water, the following reactions occur:

$$CO_2(g) \rightleftharpoons CO_2(d) \tag{3.6}$$

$$CO_2(d) + H_2O \overset{k_{CO_2}}{\rightleftharpoons} H_2CO_3(d) \rightleftharpoons HCO_3^-(d) + H^+ \rightleftharpoons CO_3^{2-}(d) + H^+ \tag{3.7}$$

The relative concentrations of the four dissolved species are a function of pH (Yegneswaran *et al.*, 1990). Due to the small volume of the samples, the pH of the *T. cuspidata* cultures were measured using pH paper (0–13 range); pH remained below 6.0 during all of the experiments. Below pH 8.0 the concentrations of CO_3^{-2} and H_2CO_3 are negligible, so the active form will be either CO_2 or HCO_3^- (Sisler and Wood, 1988). Yegneswaran *et al.* (1990) calculated the value

of k_{CO_2} to be 7200 hr^{-1} (or 20 s^{-1}) at room temperature. In our system, the flow rate of the gas mixture, the size of the flask and the volume of the cultured medium in each flask allowed equilibrium within 0.05 hours ($\leq$ 3 min) between head space gas and the liquid. The calculation of the mass transfer coefficient for our system was adapted from the derivation done by Haigh (1993). Because of the differences between the two systems, a brief summary of this calculation for our system is given below.

O'Connor and Dobbins (1966) have estimated the mass transfer coefficient for a flowing river using the average velocity and depth of the river (Equation 3.8).

$$k_l a = \left(\frac{D \times < u >}{\pi \times h^3}\right)^{0.5} \tag{3.8}$$

Where D is the diffusivity of the gas in water and u is the average velocity of the water. In our system, the average velocity of the liquid culture in the flask rotating at 125 rpm was 15.6 cm/s. The depth of 50 ml liquid in a 250 ml Erlenmeyer flask is 1.5 cm. The diffusivities of oxygen and carbon dioxide in water were estimated by the Wilke-Chang correlation (Perry and Chilton, 1973) to be 2.0×10^{-5} and 2.4×10^{-5} cm^2/s, respectively. The O'Connor-Dobbins correlation (O'Connor and Dobbins, 1966) then gives mass transfer coefficient values of 19.6 hr^{-1} for carbon dioxide and 21.4 hr^{-1} for oxygen. The mass transfer resistance at a liquid gas interface results from two resistances, the liquid boundary layer and the gas boundary layer. Since the gases used in our study are sparingly soluble in water, the liquid phase resistance is predominant (Bailey and Ollis, 1986). The rates of carbon dioxide production by *A. annua* and *C. roseus* suspension cultures are 0.25 and 0.30 mmol CO_2/hour/gram DWT, respectively (Haigh, 1993; Maurel and Pareilleux, 1985). The mass transfer coefficient for CO_2 is orders of magnitude less than the value for k_{CO_2}, and much larger than the rate of CO_2 formation. Hence, the rate at which carbon dioxide is transferred to the

cells is less limiting compared to the rate at which carbon dioxide is formed by the cells. Consequently, we assumed equilibrium between the head space and the dissolved gases throughout the experiments. The validity of this assumption was supported by measuring the head space gas concentrations biweekly throughout the experiment and was shown to be constant.

The dissolved concentration of O_2, CO_2 and C_2H_4 were then calculated using Henry's law. The oxygen concentration of 10% (v/v) corresponds to a liquid concentration of 0.1 mM. According to assumptions explained by Sisler and Wood (1988), the 0.5% (v/v) carbon dioxide used in the gas flow system is in equilibrium with a dissolved carbon dioxide concentration of 0.19 mM and 9.3 μM HCO_3^- (pH 5.5). The ethylene concentration of 5 ppm in the gas phase equates, using Henry's law, to a dissolved concentration of 0.033 μM.

It should be noted that similar dissolved concentrations of O_2, CO_2 and C_2H_4 are required in a bioreactor to achieve the same taxol concentrations observed in the shake flasks experiments. Two factors, gas composition and mechanical shear, were recognized as the potential origin of the differences between shake flask and bioreactor cultures (Schlatmann *et al.*, 1993). In the study by Schlatmann *et al.* (1993), recirculating a large part of the exhaust gas resulted in a comparable gas regime in the bioreactor as occurred in the shake flask cultures, which resulted in the absence of browning and a similar pattern of ajmalicine production as observed in shake flasks (Schlatmann *et al.*, 1993). ten Hoopen *et al.* (1994) also reported that the type of culture vessel affected production of ajmalicine, and that this production could be restored by creating a gas regime in the bioreactor comparable to that in a shake flask.

The reason our experiments were conducted in shake flasks is that eight different combinations of gases could be studied at one time, reducing the experiment-to-experiment variability. The variability in the inocula alone makes

interpretation of sequential experiments in bioreactors difficult (Tate and Payne, 1991) and increases the time and expense required to obtain the same results.

The statistical analysis showed that the interaction of carbon dioxide and ethylene had an important effect on production of taxol in 10% (v/v) oxygen. The combination of 10% (v/v) carbon dioxide and 2 ppm ethylene was inhibitory to taxol production, but low carbon dioxide (0.5% v/v) and 2 ppm ethylene had a positive effect on production of taxol. The combination of ethylene and carbon dioxide (21 ppm and 2%) also had an important factor on berberine production (Kim *et al.*, 1991). Under some conditions carbon dioxide is an antagonist of ethylene action. This inhibitory action of carbon dioxide and ethylene was reported under conditions of low ethylene; but was not observed when the gas phase concentration exceeded 1 ppm of ethylene (Yang, 1985).

Ethephon (2-chloroethylphosphonic acid), has been used in some plant cell culture studies (Cho *et al.*, 1988) to produce stoichiometric quantities of ethylene upon hydration. Although used widely in experiments by spraying dilute solutions on whole plants, recent results of d'Auzac *et al.* (1992) should be noted. Large amounts of ethephon reportedly resulted in an imbalance between toxic oxygen production (O_2^- and H_2O_2) and protective systems (superoxide dismutase and catalase) by changing the solution pH.

Figure 3.5 summarizes the effects of various head space concentrations of carbon dioxide and ethylene (with (10% v/v) oxygen) on taxol production in experiments A, B and C. Taxol concentrations increased as ethylene concentration was increased from 0 to 5 ppm; this trend was observed at carbon dioxide concentrations of 0.5% and 10%. The carbon dioxide concentration found to be the most effective in these studies was 0.5% (v/v), which is greater than that of ambient air, but this factor may not yet be optimized. The very high concentration of carbon dioxide (10% v/v) inhibited taxol production. Identical taxol

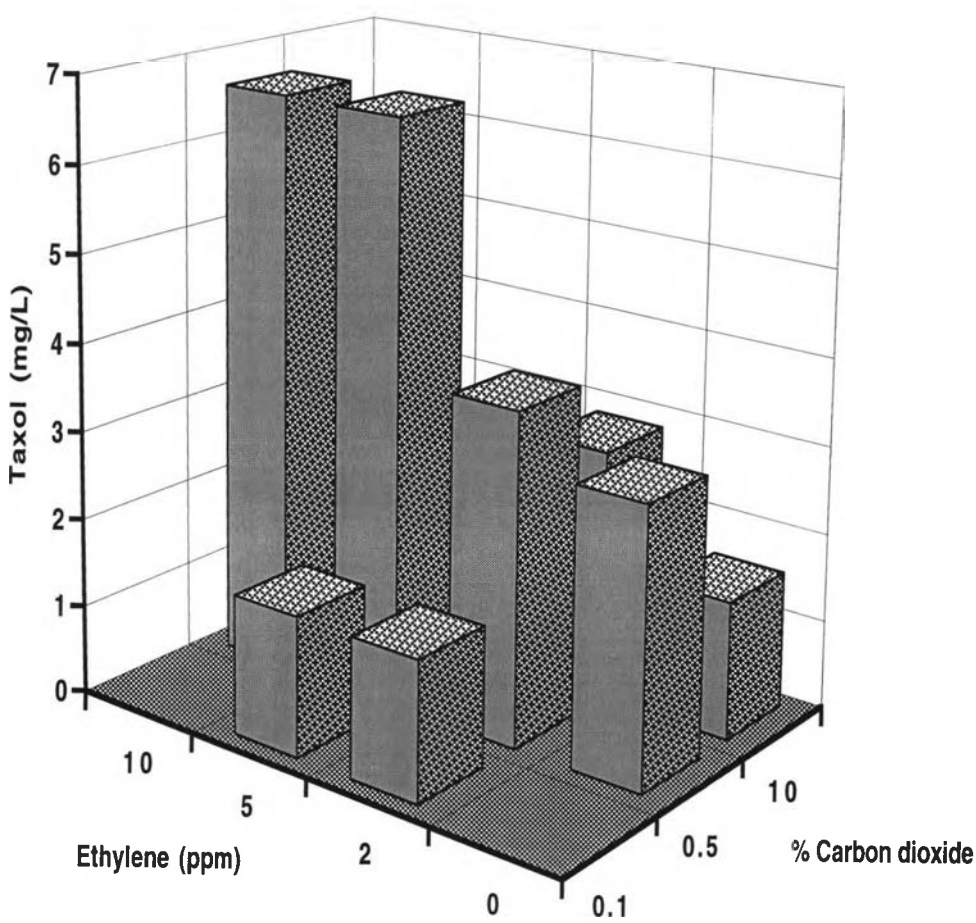

Figure 3.5: Highest concentration of taxol when cultures were equilibrated with 10% (v/v) oxygen and various headspace concentrations of carbon dioxide and ethylene.

productivities of 0.33 mg/L·day were observed for the 10% (v/v) oxygen, 0.5% (v/v) carbon dioxide and 5 ppm ethylene treatments in experiments B and C. After fructose addition (days 11–25) in experiment C, the average productivity doubled to 0.64 mg/L·day.

Fett-Neto *et al.* (1994b) have obtained 0.15 mg/L taxol during stationary phase (day 38) in suspension cultures of *Taxus cuspidata*; of which 66% was in the medium and 34% in the cells. Taxol concentration was improved by aromatic carboxylic acid and amino acid feeding from 2 to 10 μg/g (extracted dry weight) during the stationary phase (Fett-Neto *et al.*, 1994a). These values correspond to a volumetric productivity of 0.004 mg/L·day and a specific productivity of 0.075 μg/g·day. Successful production of taxol is also reported by Srinivasan *et al.* (1995). The highest yield of taxol (1.5 mg/L) was obtained in the pneumatically mixed bioreactor (Srinivasan *et al.*, 1995). In another study by Hirasuna *et al.* (1994), taxol levels of up to 13 mg/L by day 25 was obtained in shake flask cultures of *Taxus baccata.*

Using our gas apparatus, we were able to study the effects of volatile gases and their possible interactions quantitatively, without affecting shear stress of the cultures. Effects of oxygen, carbon dioxide, and ethylene on the metabolic activity of the cells are discussed in the next Chapter. Keeping those results in mind, our results may indicate that dissolved gas composition affects nutrient partitioning, which hypothetically may be linked to taxol productivity.

Chapter 4

PHYSIOLOGICAL RELATIONSHIPS BETWEEN TAXOL PRODUCTION AND CULTURE ACTIVITIES

Gas phase composition effects on taxol productivity were discussed in Chapter 3. This chapter explains the relationships between taxol production and culture activities. The same format as Chapter 3 is followed here. The results of the preliminary experiments are followed by the definitive experiments. The effect of head space gases on cell growth, sugar consumption, and calcium and phosphate uptake rates are explained for each experiment.

4.1 Why calcium and phosphate?

Plant cells vary in uptake rates of phosphate. Brodelius and Vogel (1985) observed phosphate was taken up very rapidly in suspension cultures of *Catharanthus roseus*, but was only transported into *Daucus carota* cells when required during growth. This was in spite of similar cytoplasmic pH values, similar vacuolar pH values and similar relative levels of the major intracellular phosphorylated metabolites in *C. roseus* and *D. carota* cell suspension cultures. Yamakawa *et al.* (1983) observed increased anthocyanin formation at low phosphate concentrations in suspension cultures of *Vitis* cells. In addition, calcium and phosphate are involved in the signal transduction in plants. Protein phosphorylation events are necessary for ethylene-activated signal transduction (Raz

and Fluhr, 1993). Calcium, as well, is involved in the ethylene-dependent induction of pathogenesis response (Raz and Fluhr, 1992). In our experiments, calcium and phosphate concentrations in the medium were measured throughout the 28 days of the experiments. The purpose of this was to find the relationship, if any, between taxol production and calcium and phosphate uptake rates by the cells. A decrease in the medium calcium concentration reflects uptake of calcium by cells. Whether intracellular calcium is then stored in the vacuole or endoplasmic reticulum or the cytosol is not known. Calcium uptake was somewhat erratic; phosphate uptake rate by the cells was used as a physiological handle; e.g., as a metabolic marker.

4.2 Preliminary experiments

4.2.1 Cell growth

In one preliminary experiment, the cultures were grown using the full factorial design for 14 days. Table 4.1 shows averages of the initial and final dry weights of cells along with the corresponding average growth rate for each treatment. These values were based on the dry weight measurements of the entire cellular content of each flask.

The data were subjected to statistical analysis. As can be seen in Equation 4.1, high carbon dioxide concentration (10% v/v) inhibited growth. Ethylene at 0 and 2 ppm did not change total dry weight of the cultures. Oxygen at 10% and 25% did not have a significant effect on the average growth rate of cultures.

$$DWT(day14) = 3.54 - 0.42[CO_2] - 0.14[CO_2 * C_2H_4] \qquad (4.1)$$

In another preliminary experiment, the cultures were grown under the full factorial design for 28 days; cell dry weight was not determined, but DNA

Table 4.1: Average growth rates when *Taxus cuspidata* suspension cultures were studied under full factorial design in preliminary experiments.

Treatment ($O_2/CO_2/C_2H_4$)	Initial Dry Wt. g/L±SD	Final Dry Wt.(14days) g/L±SD	Growth Rate Day^{-1}
25/10/2	0.86±0.08	2.75±0.25	0.23
25/10/0	0.86±0.08	3.24±0.21	0.27
25/0.5/2	0.86±0.08	3.91±0.32	0.32
25/0.5/0	0.86±0.08	4.02±0.31	0.33
10/10/2	0.86±0.08	3.66±0.16	0.31
10/10/0	0.86±0.08	2.83±0.25	0.24
10/0.5/2	0.86±0.08	3.79±0.22	0.31
10/0.5/0	0.86±0.08	4.09±0.21	0.34

content of samples was determined four times during the 28 days. In general, DNA content (μg DNA/g cells) increased until day 14, then gradually decreased. Presumably, there was no cell division after day 14, a period during which cells increased in size, increased in fresh weight and decreased in DNA concentration. The results of this preliminary experiment showed that high carbon dioxide concentration (10% v/v) and the interaction of carbon dioxide and ethylene had negative effects on DNA content (Equation 4.2). Equation 4.1 is similar to Equation 4.2 in that the signs of the coefficients are the same in both equations.

$$DNA(day14) = 4.5 - 0.6[CO_2] - 1.1[CO_2 * C_2H_4] \tag{4.2}$$

Measuring DNA was not ideal for quantifying growth, since cell size changed during growth, but we were limited to a finite volume of suspension cultures in each shake flask to achieve and maintain rapid equilibrium between gas and liquid throughout the experiment. As was shown by the value and sign of the coefficients in Equations 4.1 and 4.2, the effect of gases on dry weight and DNA content were of the same relative importance during the first 14 days of growth.

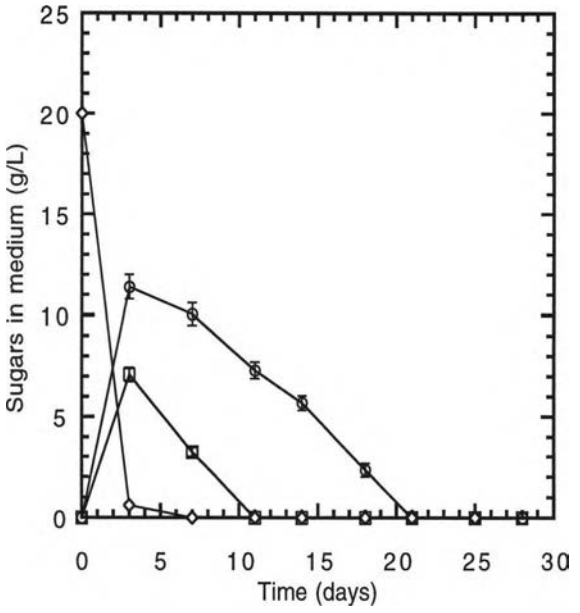

Figure 4.1: Kinetics of sugar consumption by TC1 cells. ◇ = sucrose; □ = glucose; ○ = fructose.

4.2.2 Sugar utilization

In all of the experiments, sugar utilization exhibited similar kinetics. In general, sucrose was hydrolyzed by the cells to glucose and fructose by day 7; glucose was consumed before fructose. An example of sugar uptake by these cells is presented in Figure 4.1. This mode of sugar uptake was observed in all the reference cultures as well as in the treated cultures, and it has been observed using *Taxus* and other plant cells in culture (Fowler, 1982; Hirasuna *et al.*, 1994; Nikolova *et al.*, 1991; Taticek *et al.*, 1990; Srinivasan *et al.*, 1995; Wickremesinhe and Arteca, 1994). The hydrolysis of sucrose can be attributed to an acid invertase, which may be associated with the cell wall, as has been found for cultured carrot cells (Ueda *et al.*, 1974) and autotrophic *Chenopodium*

rubrum cell cultures (Linden *et al.*, 1995). Intracellular sucrose degradation may also involve a pathway initiated by sucrose synthase (Huber and Akazawa, 1986).

4.2.3 Calcium and phosphate uptake

The reference cultures exhibited a slower uptake of phosphate and higher uptake of calcium than test cultures. High carbon dioxide concentration (10% v/v) and high ethylene concentration (2 ppm) inhibited calcium uptake whereas high oxygen concentration (25% v/v) promoted calcium uptake.

In contrast, 25% (v/v) oxygen had a negative, and 10% (v/v) carbon dioxide a positive effect on uptake of phosphate from the medium.

4.3 Definitive experiments

4.3.1 Cell growth

In experiment A, ethylene concentration at 2 ppm had a positive effect on DNA content during the first 14 days of the experiment; i.e. the time during which the cells were dividing. Carbon dioxide concentration at 10% (v/v) had a negative effect throughout the experiment. The greatest DNA change in 14 days (14 μg/g) was observed when cultures were equilibrated with 10% (v/v) oxygen, 0.5% (v/v) carbon dioxide and 2 ppm ethylene.

Experiment B, under conditions of relatively lower carbon dioxide and greater ethylene concentrations showed growth was again dependent on ethylene. Maximum DNA content change in 14 days (26 μg/g) was observed when ethylene concentration was increased to 5 ppm (10% (v/v) oxygen and 0.5% (v/v) carbon dioxide). During the first two weeks of the experiment, high ethylene concentration was a positive and carbon dioxide was a negative factor, i.e. 5 ppm ethylene promoted and 0.5% (v/v) carbon dioxide inhibited DNA accumulation. Ethylene at levels of 2 and 5 ppm and carbon dioxide at levels of 0.1

Table 4.2: Changes in DNA content of suspension cultures of *Taxus cuspidata* in experiments A and B.

Treatment ($O_2/CO_2/C_2H_4$)	DNA(day0) μg/g±SD	DNA(day14) μg/g±SD	Max. change μgDNA/g	DNA (μg/g.Day)
Expt. A				
10/10/2	4.3±0.8	15.1±0.6	10.8±1.0	0.25
10/10/0	4.3±0.8	13.0±0.5	8.7±0.9	0.22
10/0.5/5	4.3±0.8	30.7±0.8	26.4±1.1	0.51
10/0.5/2	4.3±0.8	18.7±0.8	14.4±1.1	0.31
Expt. B				
10/0.5/2	4.3±0.8	17.5±0.9	13.2±1.2	0.31
10/0.5/0	4.3±0.8	14.7±0.5	10.4±0.9	0.24
10/0.1/5	4.3±0.8	18.4±0.6	14.1±1.0	0.31
10/0.1/2	4.3±0.8	17.3±0.7	13.0±1.1	0.29

and 0.5% (v/v) were not significant factors on growth during the last two weeks of the experiment, during which cells expand in size. Table 4.2 shows the DNA values and the change for experiments A and B. The treatment 10% (v/v) O_2, 0.5% (v/v) CO_2, 2 ppm C_2H_4 was repeated and yielded similar results in both A and B experiments.

No DNA data was obtained during experiment C thru F because extractions to determine intracellular taxol were conducted.

4.3.2 Sugar utilization

Consumption rates of glucose and fructose were similar in both reference and treated cultures, but the onset of glucose and fructose utilization in the reference cultures were delayed by 4 days (Figure 4.2). The uptake rate of fructose is reportedly affected by the oxygen supply rate; cells grown at the lower oxygen transfer rate were reportedly unable to utilize fructose (Taticek *et al.*, 1990). In our work, the reference cultures exposed to 21% O_2 exhibited delayed fructose uptake compared to treatments exposed to 10% (v/v) O_2.

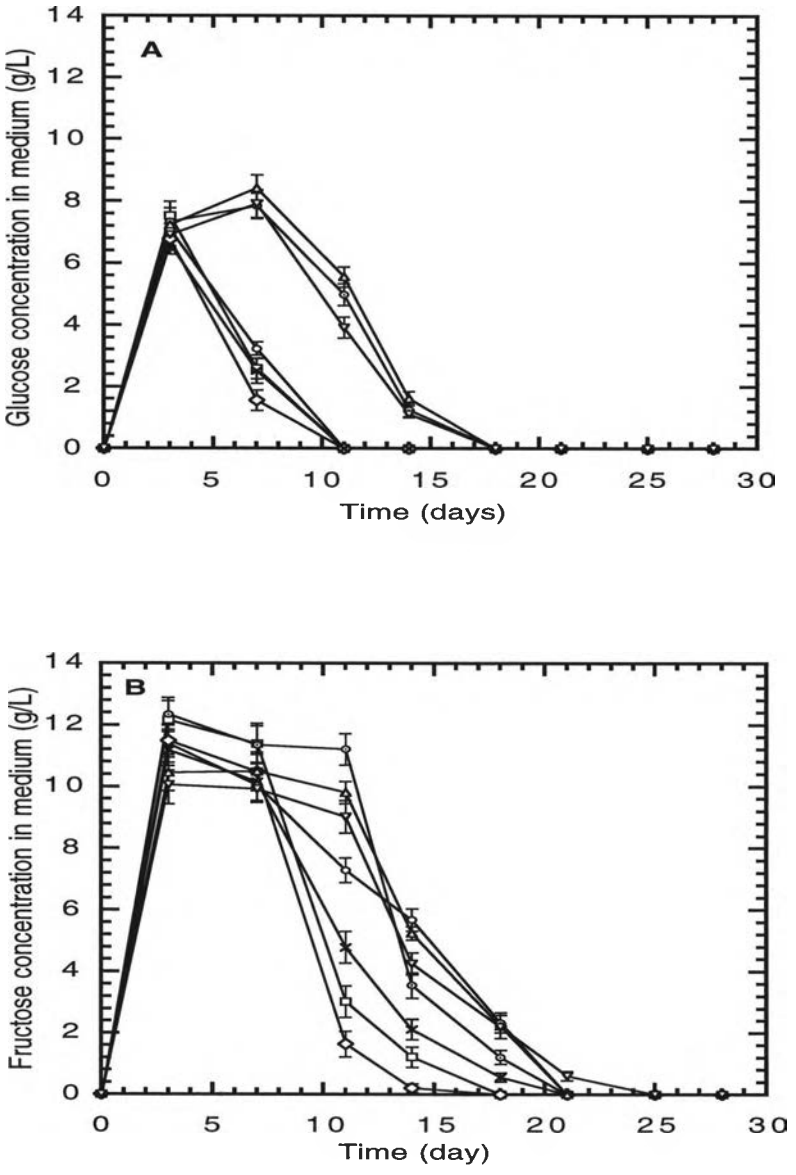

Figure 4.2: Kinetics of glucose and fructose utilization when TC1 suspension cultures were equilibrated with various head space gas compositions (Expt. B). **A:** glucose uptake patterns; **B:** fructose uptake patterns. $\circ$ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 5 ppm C_2H_4; $\square$ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 2 ppm C_2H_4; $\diamond$ = 10% (v/v) O_2, 0.1% (v/v) CO_2, 5 ppm C_2H_4; $\times$ = 10% (v/v) O_2, 0.1% (v/v) CO_2, 2 ppm C_2H_4; $\odot$ = 125 ml Bellco capped controls; $\triangle$ = 250 ml Bellco capped controls (turned red after day 21); $\bigtriangledown$ = Humidified air (turned red after day 25).

In experiment B, the gas treated cultures were depleted of glucose during the second week and of fructose during the third week of the experiments (Figure 4.2). Fett-Neto *et al.* (1994b) have reported similar uptake patterns for the *T. cuspidata* cell line; however, fructose and glucose were exhausted at approximately the same time. Similar observations on the depletion and utilization of sugars by *Taxus* cells were made by Wickremesinhe and Arteca (1994).

The cultures treated with 10 ppm ethylene in Experiment C had a slower rate of glucose and fructose utilization than did the 5 ppm treatments (Figure 4.3). When more fructose was added to the cultures on day 11, the consumption rate of glucose was reduced compared to those in which no fructose was added (Figure 4.3). Cultures to which 10 g/L fructose was added, contained 2.5 g/L fructose when the experiment was stopped (day 28). Srinivasan *et al.* (1995) reported carbohydrate utilization after fructose addition occurred at a much faster rate than before. Also glucose was utilized completely but a substantial amount of fructose remained unused on day 30 of the experiments. Wickremesinhe and Arteca (1994) have also reported that the growth of *Taxus x media* suspensions was faster during a period of increased fructose availability and they suggest that this phenomenon may be caused by the increased entry of fructose into the cells coupled with increased fructokinase activity.

In experiment D, the patterns of sugar uptake were similar to experiments A-C, but the kinetics were slower as is shown in Figure 4.4. Both glucose and fructose had not been depleted by day 28 of the study. This was another indicator that this cell line (TC2) grew more slowly than the previous cell line (TC1). Hence, the g/L glucose consumed by the cells was calculated according to Equation 4.3 for all the experiments as a way to normalize the data.

$$(20 - Suc_m) = (Glc_m + Glc_c) + (Fruc_m + Fruc_c) \tag{4.3}$$

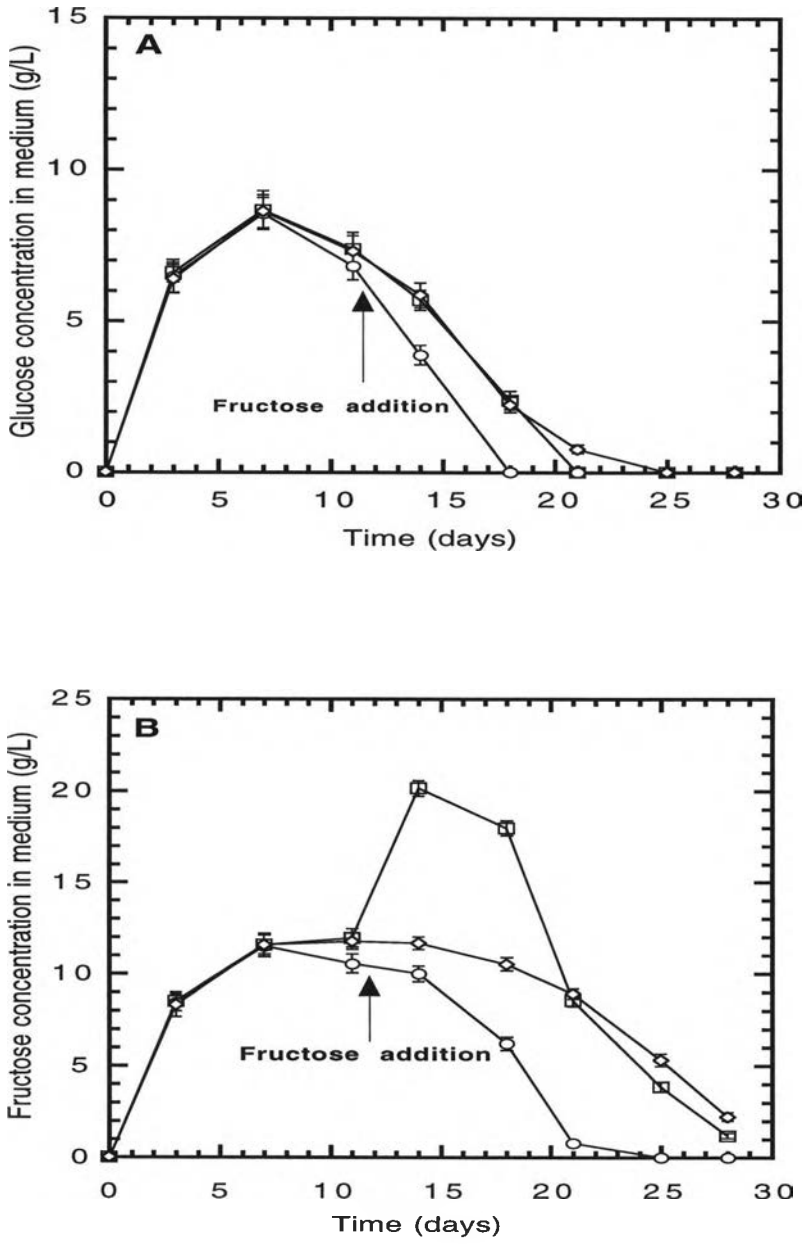

Figure 4.3: Kinetics of glucose and fructose utilization when TC1 suspension cultures were equilibrated with various head space gas compositions (Expt. C). **A:** glucose uptake uptake patterns; **B:** fructose uptake patterns. $\circ$ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 5 ppm C_2H_4; $\square$ = 10 g/L fructose added to cultures equilibrated with 10% (v/v) O_2, 0.5% (v/v) CO_2, 5 ppm C_2H_4; $\diamond$ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 10 ppm C_2H_4; $\odot$ = 125 ml Bellco capped controls; $\triangle$ = 250 ml Bellco capped controls (turned red after day 18).

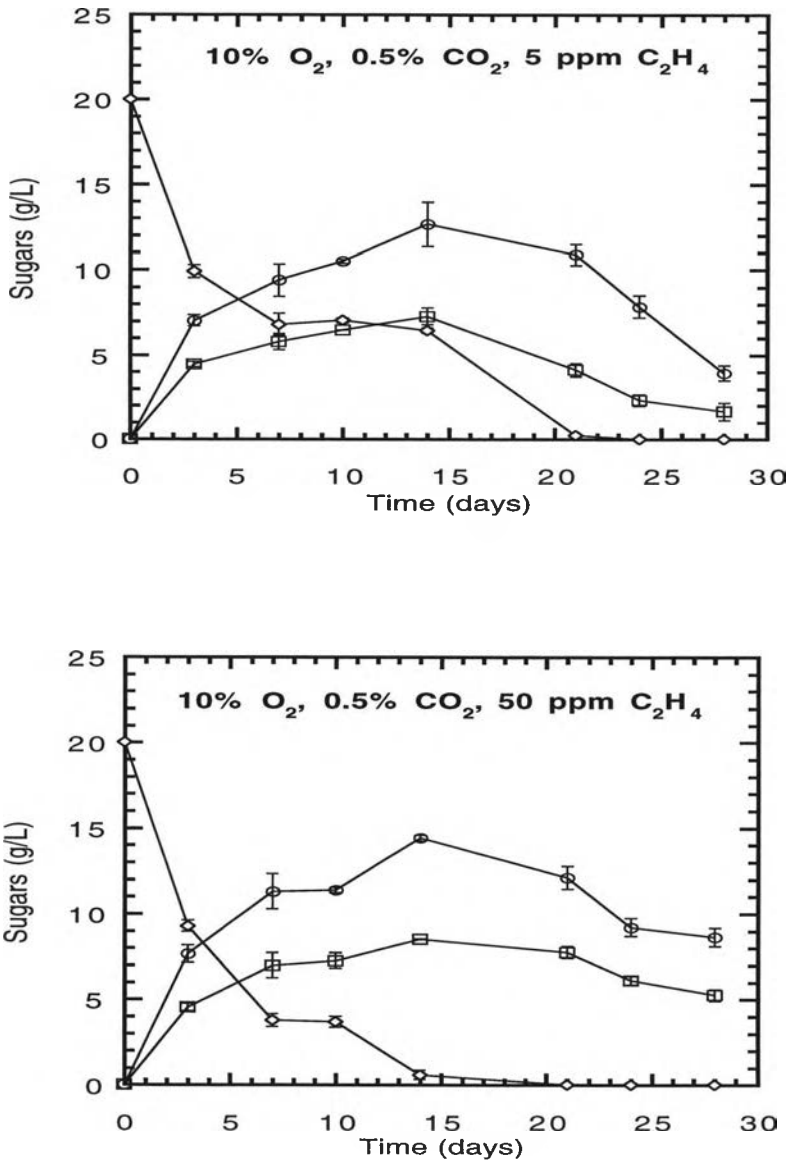

Figure 4.4: Kinetics of sugar utilization by TC2 cells in Experiment D. ◇ = sucrose; □ = glucose; ○ = fructose.

Where the subscript "m" signifies medium concentration of sugars and "c" represents the consumed concentration of sugars. All the flasks contained 20 g/L sucrose on day 0. If there is no consumption of fructose until glucose concentration in the media is reduced to 3 g/L, then the glucose consumed can be calculated following Equation 4.4. This assumption is based on the observation that the concentration of fructose in the medium starts declining when glucose concentration in the medium is approximately 3 g/L.

$$Glc_c = 20 - Suc_m - Glc_m - Fruc_m \tag{4.4}$$

The glucose consumption rates were used to normalize taxol productivities. Phosphate uptake per gram glucose consumed was also calculated. These will be discussed in detail in the next chapter, where an attempt is made to understand ethylene action.

4.3.3 Calcium uptake rate

In experiment A, the reference cultures and the cultures exposed to 10% (v/v) CO_2 and 2 ppm C_2H_4 showed an increase in the concentration of calcium in the medium on day 14 that was not observed in the other cultures (Figure 4.5). Both ethylene and carbon dioxide had negative effects on calcium uptake from the medium.

Similar observations were made in experiment B, that is both carbon dioxide and ethylene had negative effects on calcium uptake. Cells picked up less calcium from the medium as the concentrations of carbon dioxide and ethylene were increased to 0.5% (v/v) and 5 ppm respectively (Figure 4.6). The regression model equations describing the effects of each gas on calcium uptake are presented in Appendix C.

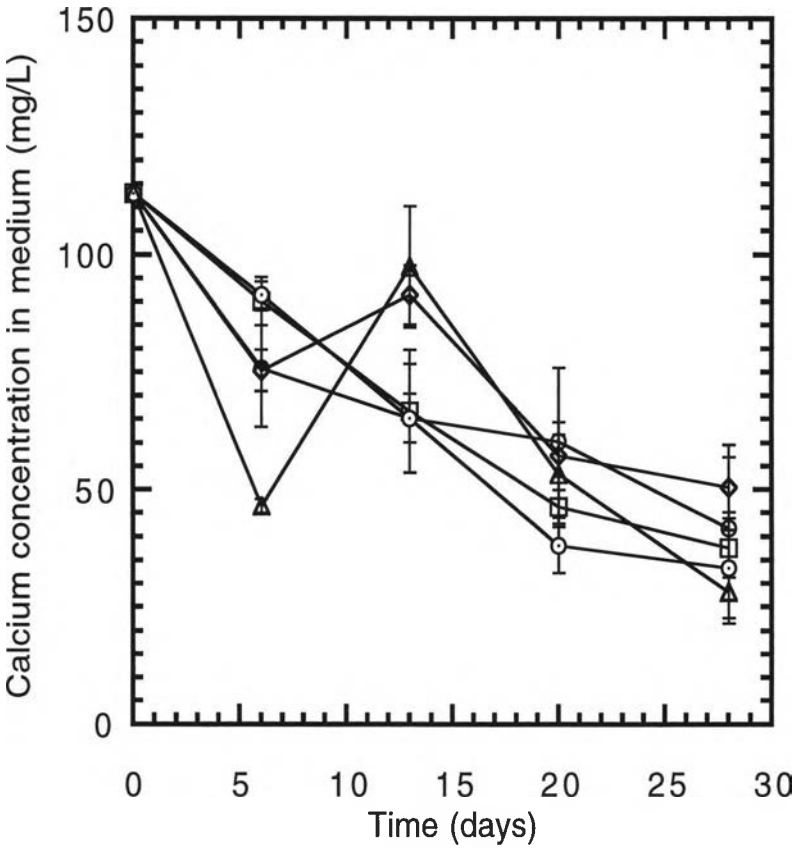

Figure 4.5: Kinetics of calcium uptake in TC1 suspension cultures equilibrated with various head space gas compositions (Expt. A). □ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 2 ppm C_2H_4; ○ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 0 ppm C_2H_4; ◇ = 10% (v/v) O_2, 10% (v/v) CO_2, 10 ppm C_2H_4; ⊙ = 10% (v/v) O_2, 10% (v/v) CO_2, 0 ppm C_2H_4; △ = Humidified air.

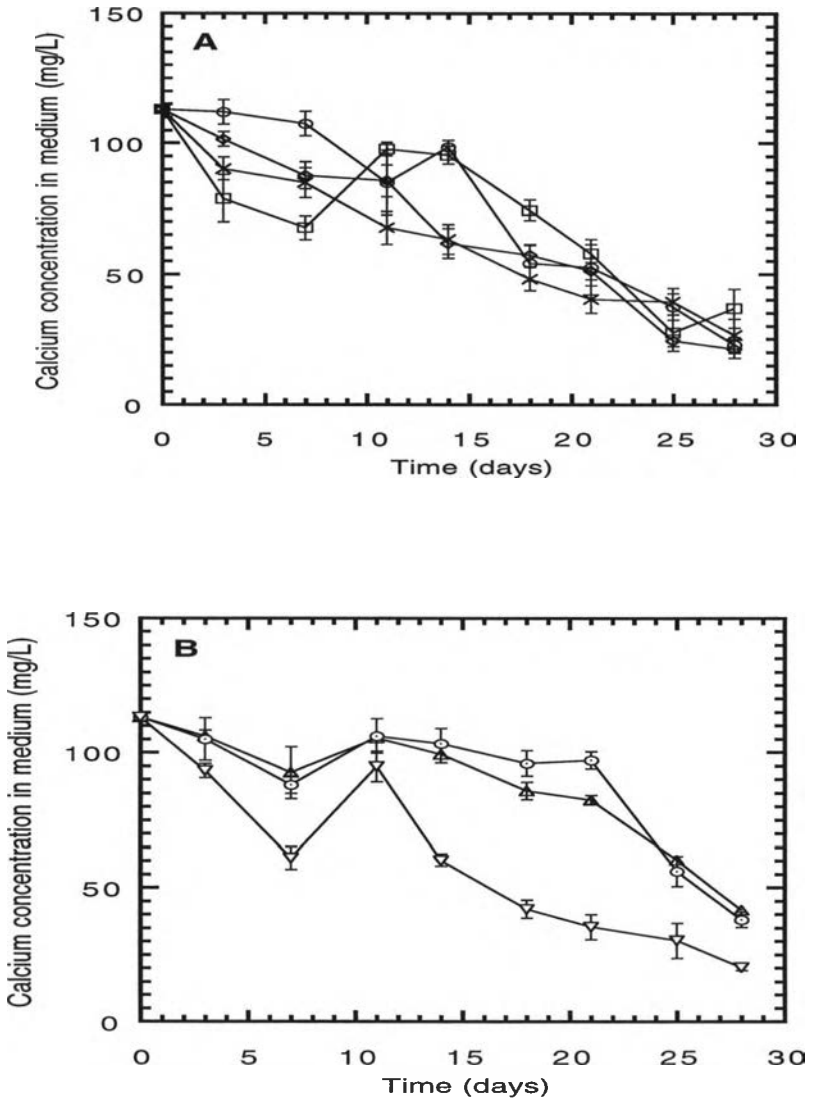

Figure 4.6: Kinetics of calcium uptake when TC1 suspension cultures were equilibrated with various head space gas compositions (Expt. B). **A:** gas treated cultures; $\circ$ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 5 ppm C_2H_4; $\square$ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 2 ppm C_2H_4; $\diamond$ = 10% (v/v) O_2, 0.1% (v/v) CO_2, 5 ppm C_2H_4; $\times$ = 10% (v/v) O_2, 0.1% (v/v) CO_2, 2 ppm C_2H_4; **B:** reference cultures; $\odot$ = 125 ml Bellco capped controls; $\triangle$ = 250 ml Bellco capped controls (turned red after day 21); $\bigtriangledown$ = Humidified air (turned red after day 25).

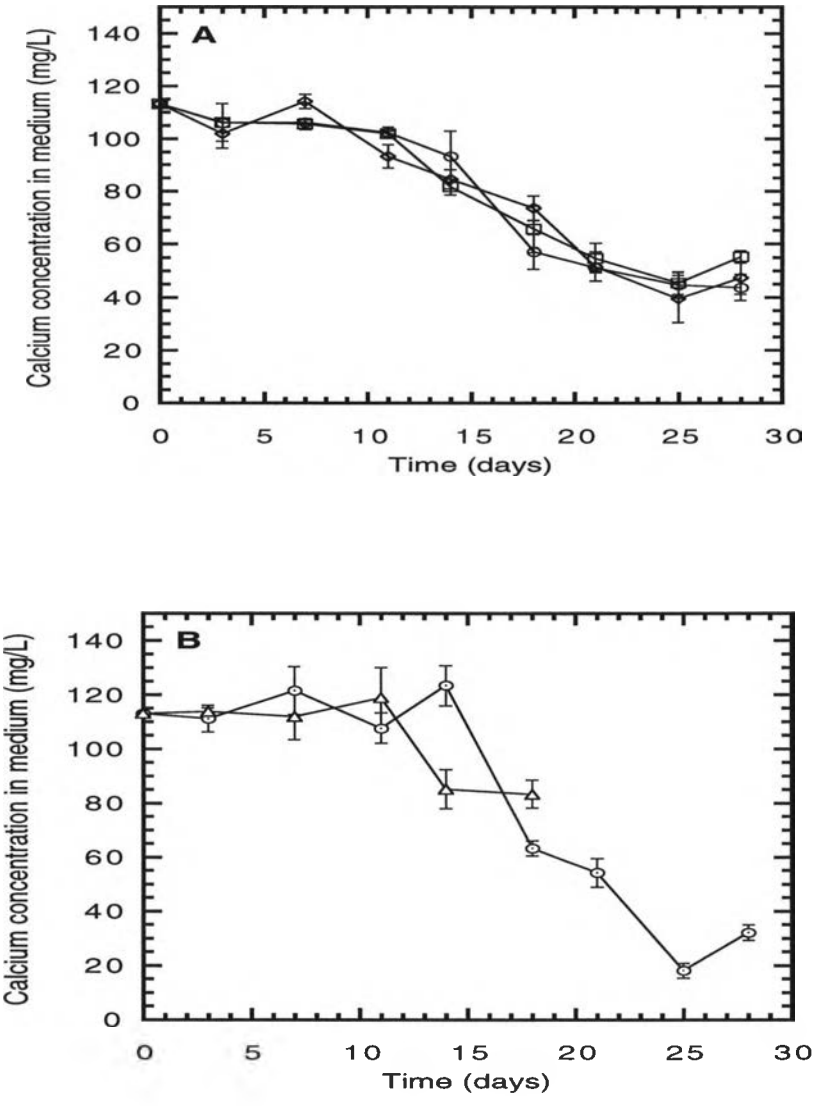

Figure 4.7: Kinetics of calcium uptake when TC1 suspension cultures were equilibrated with various head space gas compositions (Expt. C). **A:** gas treated cultures; $\circ$ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 5 ppm C_2H_4; $\square$ = 10 g/L fructose added to cultures equilibrated with 10% (v/v) O_2, 0.5% (v/v) CO_2, 5 ppm C_2H_4; $\diamond$ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 10 ppm C_2H_4; **B:** reference cultures; $\odot$ = 125 ml Bellco capped controls; $\triangle$ = 250 ml Bellco capped controls (turned red after day 18).

Table 4.3: Average calcium uptake rates for Experiments A, B, and C using TC1 suspension cultures.

Treatment ($O_2/CO_2/C_2H_4$)	Avg. calcium uptake rate (mg/L·day)
21/0.03/0 ppm	7.5
10/0.5/0 ppm	6.3
10/0.5/2 ppm	5.2
10/0.5/5 ppm	4.2
10/0.5/10 ppm	3.9
10/10/0 ppm	3.7
10/10/2 ppm	1.1
10/0.1/2 ppm	5.6
10/0.1/5 ppm	4.6
10/0.5/5 ppm+F	4.4

The uptake rate of calcium was greatest under ambient conditions in Experiment C as well. Cultures equilibrated with 10 ppm ethylene had a lower calcium uptake rates than did the 5 ppm cultures (Figure 4.7).

The average calcium uptake rates in cultures equilibrated with different concentrations of gases are presented in Table 4.3. Use of the low oxygen concentration (10% v/v) reduced the uptake rate of calcium from the medium. Reference cultures (21% oxygen) had the highest calcium uptake rates in experiments A–C. Calcium uptake from the medium decreased as the concentration of ethylene was increased from 0 to 10 ppm (Figure 4.8). Carbon dioxide at levels of 0.5–10% (v/v) was also inhibitory to calcium uptake compared to ambient conditions (0.03% v/v).

Calcium in the medium was not depleted during the 28 days of the study in Experiment D, for which the cell line TC2 was used (Figure 4.9). Calcium uptake at 5 and 50 ppm ethylene were not significantly different.

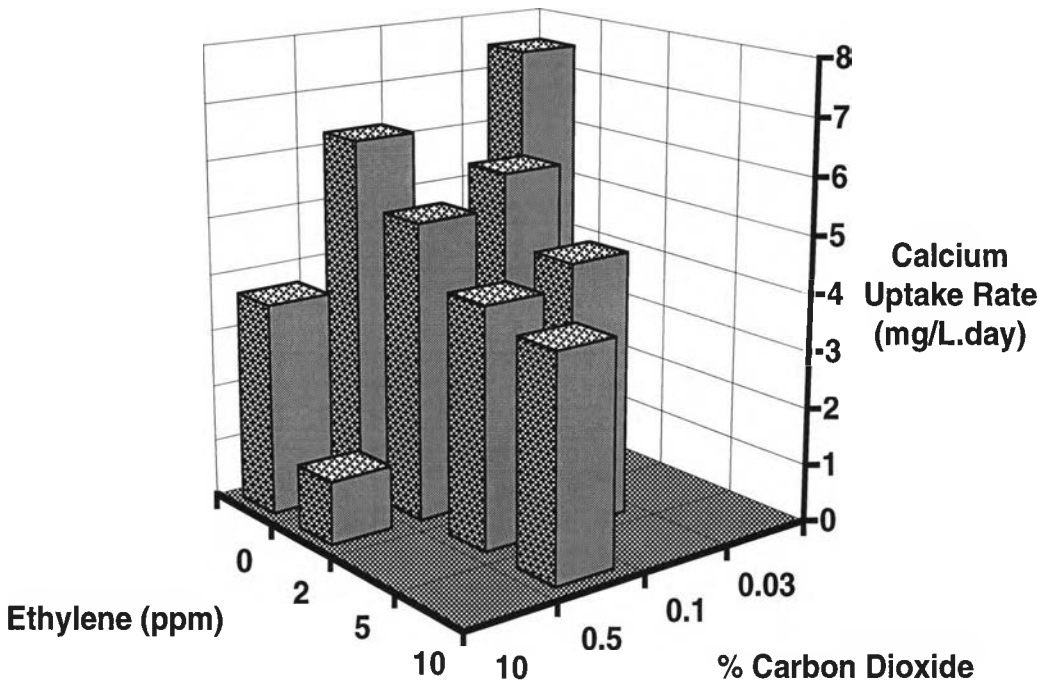

Figure 4.8: Average calcium uptake rates (Experiments A-C), when TC1 suspension cultures were equilibrated with 10% (v/v) oxygen and various compositions of ethylene and carbon dioxide.

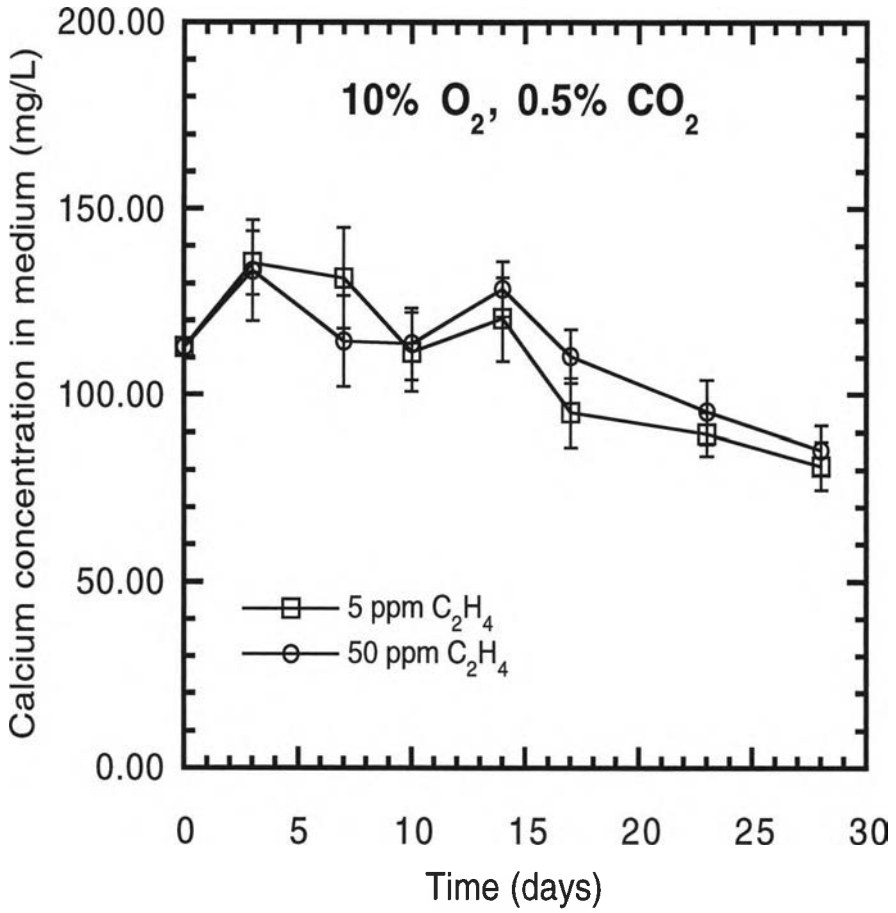

Figure 4.9: Kinetics of calcium uptake in TC2 suspension cultures in Experiment D. □ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 5 ppm C_2H_4; ○ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 50 ppm C_2H_4.

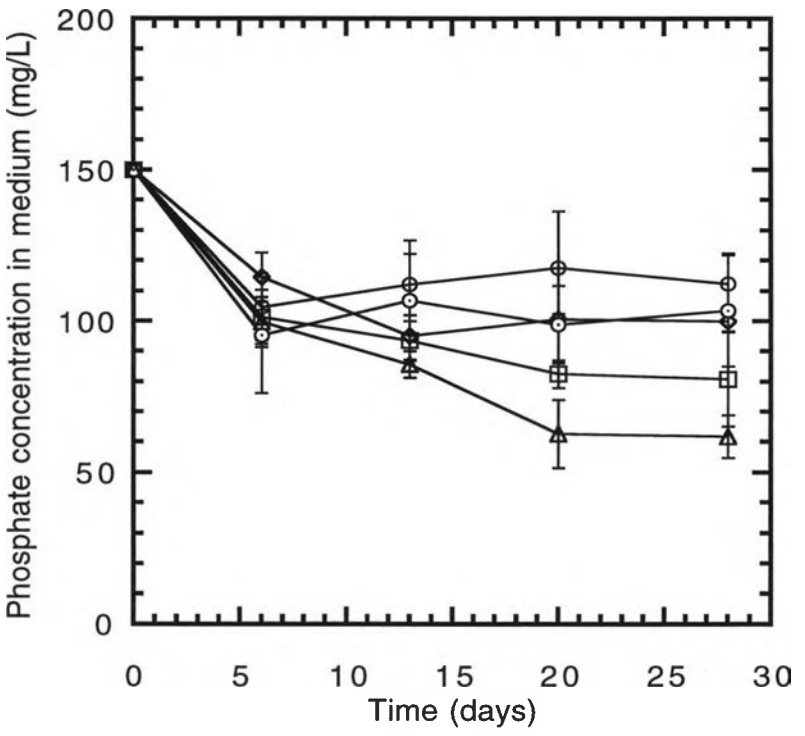

Figure 4.10: Kinetics of phosphate uptake in TC1 suspension cultures equilibrated with various head space gas compositions (Expt. A). □ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 2 ppm C_2H_4; ○ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 0 ppm C_2H_4; ◇ = 10% (v/v) O_2, 10% (v/v) CO_2, 10 ppm C_2H_4; ⊙ = 10% (v/v) O_2, 10% (v/v) CO_2, 0 ppm C_2H_4; △ = Humidified air.

4.3.4 Phosphate uptake rate

The significant factors affecting uptake of phosphate from the medium by the cells in Experiment A are shown in the regression model equations in Appendix C. Phosphate uptake was enhanced as ethylene concentration was increased to 2 ppm and carbon dioxide was reduced to 0.5% (v/v) (Figure 4.10).

In Experiment B, an increase in phosphate level in the medium was observed on day 7 for reference cultures (Figure 4.11); the treated cultures were

Table 4.4: Average phosphate uptake rates for Experiments A, B, and C.

Treatment ($O_2/CO_2/C_2H_4$)	Avg. phosphate uptake rate (mg/L·day)
21/0.03/0 ppm	0.9
10/0.5/0 ppm	1.5
10/0.5/2 ppm	4.2
10/0.5/5 ppm	8.2
10/0.5/10 ppm	8.1
10/10/0 ppm	1.6
10/10/2 ppm	2.4
10/0.1/2 ppm	3.5
10/0.1/5 ppm	5.9
10/0.5/5 ppm +F	8.8

depleted of phosphate by day 11. Carbon dioxide had a negative effect during the first week, and a positive effect thereafter, that is phosphate uptake was higher at 0.5% (v/v) carbon dioxide from days 7 to 21. Ethylene had a negative effect during the first two weeks of the experiment thereafter it was positive.

Cultures exposed to 10 ppm ethylene had a greater uptake rate of phosphate than ones given 5 ppm ethylene in experiment C. Reference cultures had the lowest rate of phosphate uptake from the medium in experiment C as well. Cultures to which fructose was added on day 11, exhibited similar phosphate uptake kinetics to the cultures equilibrated with 10 ppm ethylene.

Table 4.4 shows the average phosphate uptake rates for experiments A, B and C.

In contrast to calcium uptake, phosphate uptake was enhanced as the ethylene concentration was increased (Figure 4.13). Also when the carbon dioxide concentration was 0.5% (v/v) compared to 0.03%, phosphate uptake was enhanced. In contrast to these results where phosphate was depleted in approximately 14 days, Srinivasan *et al.* (1995) reported the uptake of 90% of the initial phosphate by day 25. Their cultures were exposed to ambient concentrations of

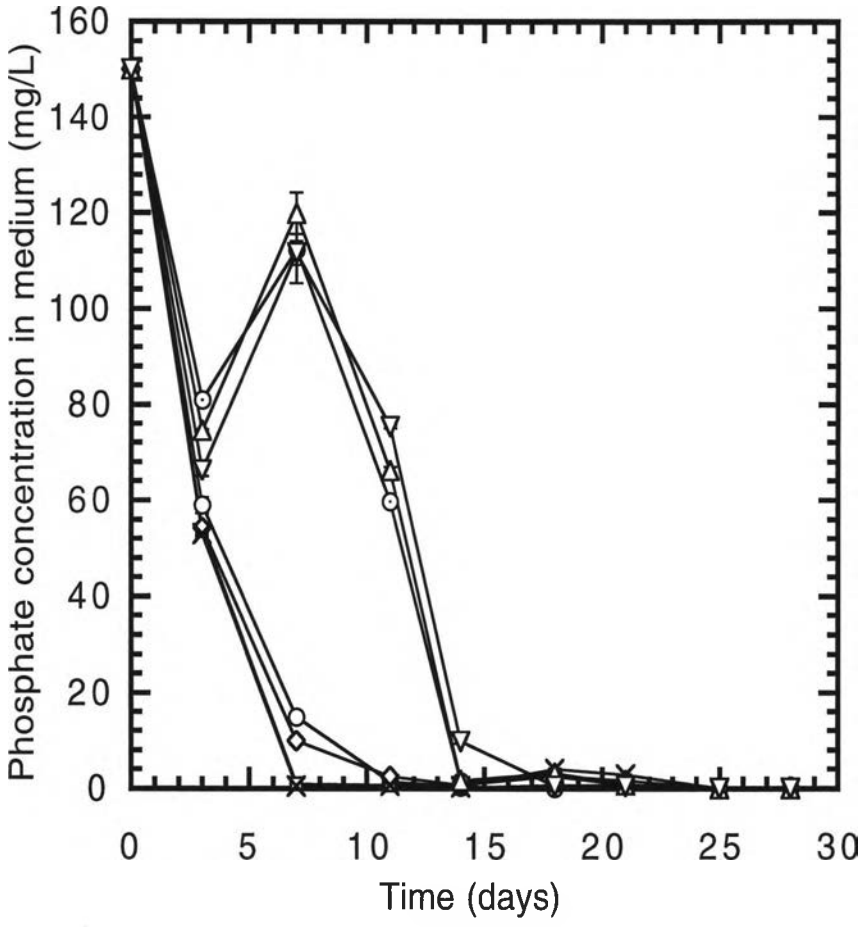

Figure 4.11: Kinetics of phosphate uptake when cultures were equilibrated with various composition of head space gases (Expt. B): $\circ$ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 5 ppm C_2H_4; $\square$ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 2 ppm C_2H_4; $\diamond$ = 10% (v/v) O_2, 0.1% (v/v) CO_2, 5 ppm C_2H_4; $\times$ = 10% (v/v) O_2, 0.1% (v/v) CO_2, 2 ppm C_2H_4; $\odot$ = 125 ml Bellco capped controls; $\triangle$ = 250 ml Bellco capped controls (turned red after day 21); $\triangledown$ = Humidified air (turned red after day 25).

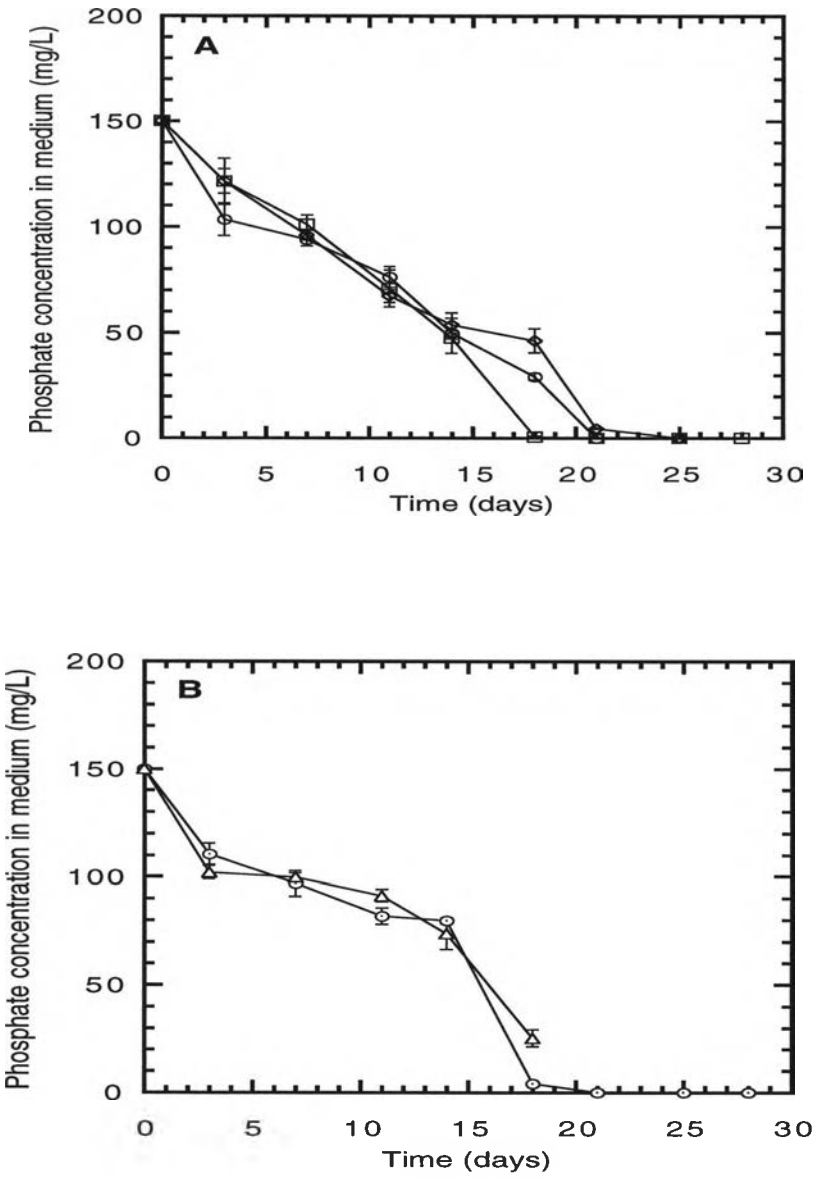

Figure 4.12: Kinetics of phosphate uptake when TC1 suspension cultures were equilibrated with various head space gas compositions (Expt. C). **A:** gas treated cultures; $\circ$ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 5 ppm C_2H_4; $\square$ = 10 g/L fructose added to cultures equilibrated with 10% (v/v) O_2, 0.5% (v/v) CO_2, 5 ppm C_2H_4; $\diamond$ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 10 ppm C_2H_4; **B:** reference cultures; $\odot$ = 125 ml Bellco capped controls; $\triangle$ = 250 ml Bellco capped controls (turned red after day 18).

oxygen and carbon dioxide (similar to our reference cultures). Figure 4.14 shows the relationship between taxol concentration and calcium and phosphate uptake rates. The greatest concentrations of taxol were observed when phosphate uptake rates ranged between 8.1 and 8.2 mg/L·day and calcium uptake rates between 3.9 and 4.2 mg/L·day.

In experiment D, the phosphate uptake rates for both 5 and 50 ppm were similar (Figure 4.15); they were both lower than the reported ones in the previous experiment (3.61±0.55 and 3.55±0.44 respectively). These results will be discussed further in the following chapter.

4.4 Discussion

The gas phase composition affects growth and uptake rates of nutrients in suspension cultures of *Taxus cuspidata*. Most often, reports have stated that growth of plant cell suspension cultures is inhibited under low concentration of oxygen (Pareilleux and Vinas, 1983; Gao and Lee, 1992; Tate and Payne, 1991; Kobayashi *et al.*, 1989; Leckie *et al.*, 1991). In our experiments, cell growth was evaluated as the DNA content per gram fresh weight. Oxygen at levels of 10% and 25% (v/v) did not have a significant effect on DNA content of the cultures. Carbon dioxide at concentrations of 0.5 and 10% (v/v) reduced the specific DNA content of the cultures. Ethylene concentrations of 2 and 5 ppm increased the DNA content of the cells. The effect of carbon dioxide and ethylene on DNA was significant only during the first two weeks of the experiments, during the time that cell division occurs. Cho *et al.* (1988) observed a 20–30% reduction in growth of *Nicotiana tabacum* cell cultures with 560 ppm ethylene (supplied as ethephon), but 25 ppm ethylene stimulated growth 3-fold; the ratio of packed cell volume to cell dry weight indicated ethylene makes tobacco cells more compact.

Some studies have suggested that high aeration rates may be detrimental to cell growth in air sparged bioreactors, possibly due to removal of carbon

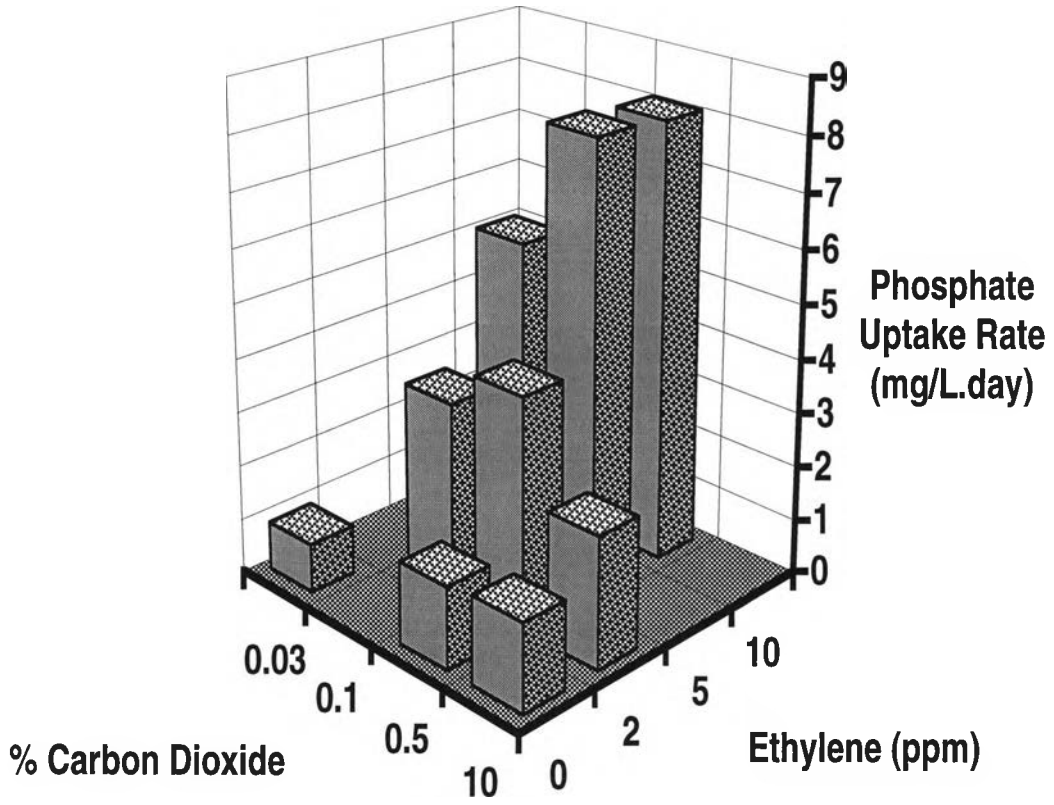

Figure 4.13: Average phosphate uptake rates (Experiments A-C), when TC1 suspension cultures were equilibrated with 10% (v/v) oxygen and various compositions of ethylene and carbon dioxide.

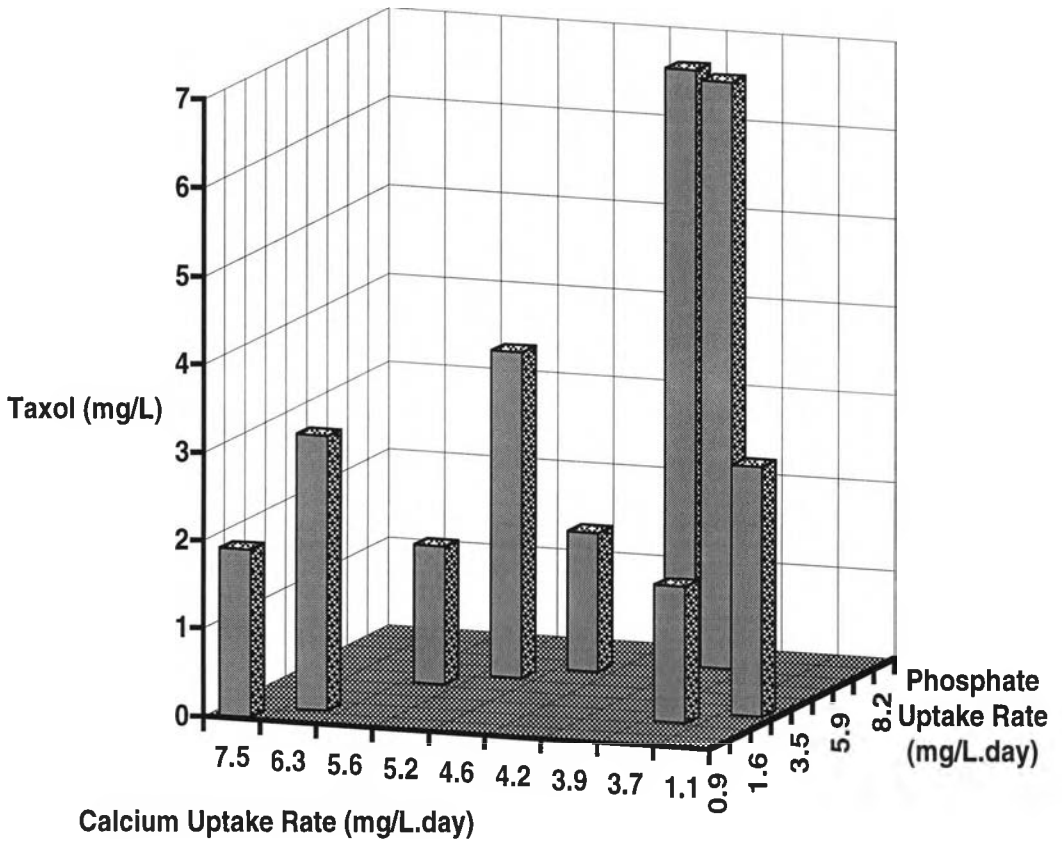

Figure 4.14: Taxol concentration in the medium relative to uptake rates of calcium and phosphate using TC1 cells.

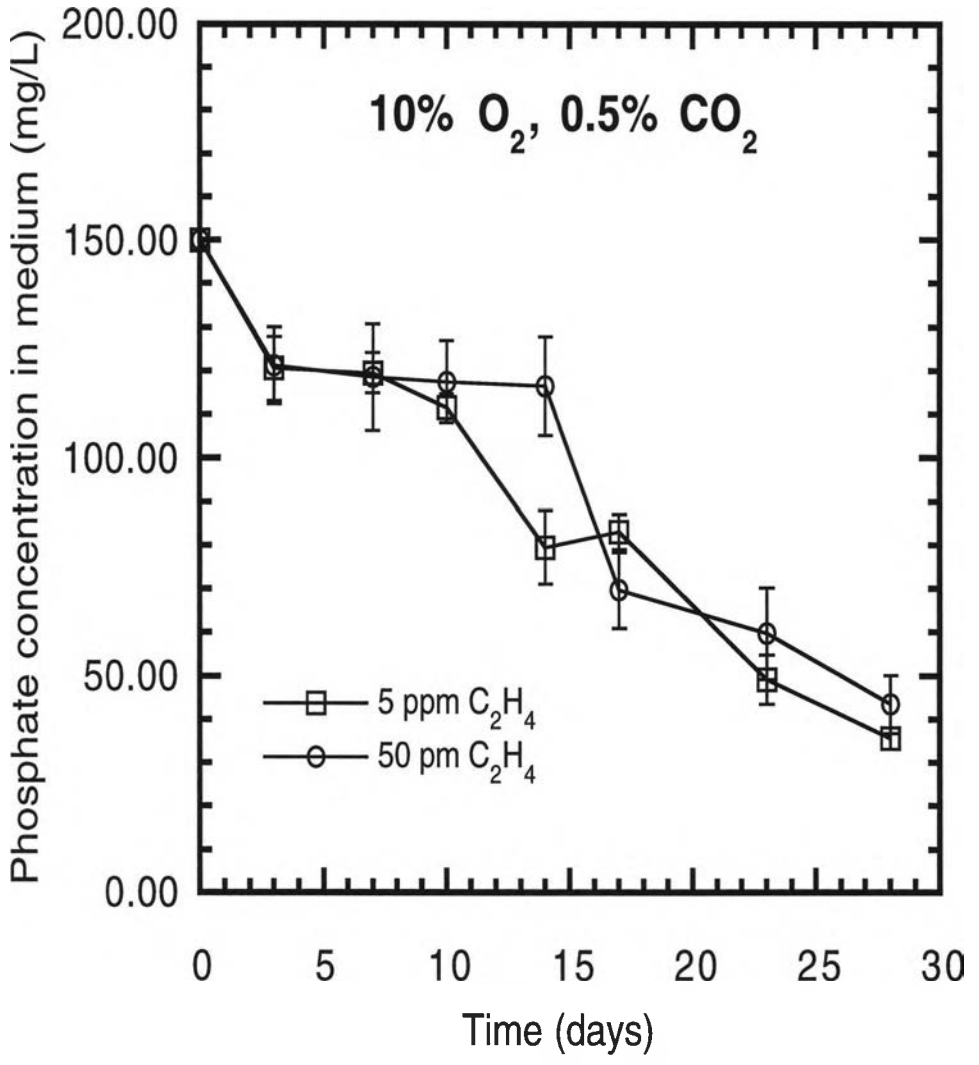

Figure 4.15: Kinetics of phosphate uptake in TC2 suspension cultures in Experiment D. □ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 5 ppm C_2H_4; ○ = 10% (v/v) O_2, 0.5% (v/v) CO_2, 50 ppm C_2H_4.

dioxide (Ducos and Pareilleux, 1986; Maurel and Pareilleux, 1985; Smart and Fowler, 1981; Hegarty *et al.*, 1986). Carbon dioxide has been considered essential for the activity of phosphoenolpyruvate carboxylase (PEPC); the decrease in PEPC activity coincided with a decrease in growth rate the cells (Hegarty *et al.*, 1986).

Low oxygen concentration (10% v/v) reduced the uptake of calcium from the medium compared to the reference cultures (21% oxygen) in Experiments A-C with the highest calcium uptake rates. Calcium uptake from the medium decreased as the concentration of ethylene was increased from 0 to 10 ppm. Carbon dioxide at levels of 0.5–10% (v/v) was also inhibitory to calcium uptake compared to ambient conditions (0.03% v/v). A general observation evolved that nearly constant rates of calcium uptake were correlated to higher rates of taxol production. Conversely, transient influx and efflux of calcium appeared to be detrimental to taxol production.

When the plant is stressed a series of signal transduction events begins. Cytosolic calcium increases due to release from internal stores in the endoplasmic reticulum, vacuole and mitochondria or extracellularly from the cell wall or freespace (the external medium in the case of plant cell cultures) (Asaoka *et al.*, 1992). Calcium, known as a second messenger, is able to transfer an external signal to internal events of the cell such as protein phosphorylation and regulation of gene transcription (Drobak and Bjorn, 1991). In some cases ethylene is thought to be an end product of these events in what Fluhr calls an ethylene-dependent pathway (Raz and Fluhr, 1992).

Average phosphate uptake rates increased as ethylene concentration increased. The reference cultures showed the lowest rate of phosphate uptake. Carbon dioxide at levels of 0.5%–10% (v/v) had a negative effect on phosphate

uptake. Phosphate has been shown to be an important regulatory factor of microbial cell metabolism (Prescott *et al.*, 1993). It has been reported that phosphate concentrations that are optimal for growth suppressed the biosynthesis of secondary metabolites. A similar regulatory behavior has also been found in cell suspension cultures of plants, where modified phosphate free media stimulated the accumulation of indole alkaloids in *C. roseus* cultures (Knobloch and Berlin, 1981) and cinnamoyl putrescines in *N. tabacum* suspension cultures (Knobloch, 1982). In phosphate limited cultures growth was reduced and the activities of the enzymes tryptophan decarboxylase and phenylalanine ammonia lyase were enhanced; this was correlated with the synthesis of proteins, which competes with the common precursor phenylalanine (Knobloch, 1982). It was suggested by Margna (1977) that substrate availability and enzyme activity may be involved in a balanced regulation diverting the flow of carbon from protein synthesis to secondary product formation under phosphate limited conditions. In our studies in an effort to understand the physiological activity of the cell when taxol is produced, phosphate uptake was used as a metabolic marker.

Ethylene has been shown to have many physiological effects, including inhibition of growth and stimulation of production of secondary metabolites from various plant tissue and cell cultures (Chi and Pua, 1989; Cho *et al.*, 1988; Songstad *et al.*, 1989). As discussed in Chapter 3, taxol production increased as ethylene concentration was increased from 0 to 5 ppm. Low oxygen concentration (10% v/v) induced early taxol production and increased phosphate uptake from the medium. At 0.5% carbon dioxide, cultures had a reduced growth but higher taxol concentration compared to cultures equilibrated with lower carbon dioxide concentrations. Cultures also had a lower uptake of calcium and higher uptake of phosphate at 0.5% carbon dioxide concentration compared to ambient conditions. Higher taxol concentration was observed when 0.5% carbon dioxide was supplied

with 2 and 5 ppm ethylene. Ethylene concentration at 5 ppm increased DNA content during the first two weeks of the experiment, hence enabled cultures to continue taxol production until day 25. Cultures to which 10 g/L fructose was added on day 11 (Figure 4.16), had a similar calcium uptake rate to cultures with no fructose addition (compare with Figure 4.17). The fructose spike increased the phosphate uptake rate and prolonged the carbohydrate supply for the cells.

Kinetics of growth, calcium and phosphate uptake along with concentration of taxol in cultures for the best treatments of Experiments A, B, and C are compared in Figures 4.18, 4.17, and 4.16. Cultures equilibrated with 10% (v/v) oxygen, 0.5% (v/v) carbon dioxide and 5 ppm ethylene continued production of taxol until day 21 (6.5 mg/L) and reached a maximum DNA content change of 26 μg/g in 14 days. Cultures that were exposed to 2 ppm ethylene (10% v/v O_2, 0.5% v/v CO_2) produced 3.5 mg/L taxol on day 7; taxol concentration declined thereafter (Figure 4.18). Maximum DNA change for these cultures was 12 μg/g in 14 days. Comparison of the Figures 4.18, 4.17 and 4.16 show that head space gas concentrations affect nutrient partitioning, which is linked to growth and taxol productivity. ten Hoopen *et al.* (1994) reported that the different pathways involved in growth and secondary metabolite production may be differently influenced by the gas regime in the cultured plants. Dependence of the transport physiology of the cells upon the gaseous phase composition was also observed in *C. roseus* cells (Maurel and Pareilleux, 1986). Cultures had a shorter lag period and higher specific growth rate using 2% (v/v) carbon dioxide compared to 0.03% (v/v). Maurel and Pareilleux (1986) attributed enhanced operation of the Krebs cycle function to more rapid replenishment of TCA cycle intermediates. Our results may indicate that dissolved gas composition affects nutrient partitioning, which is linked to growth and taxol productivity.

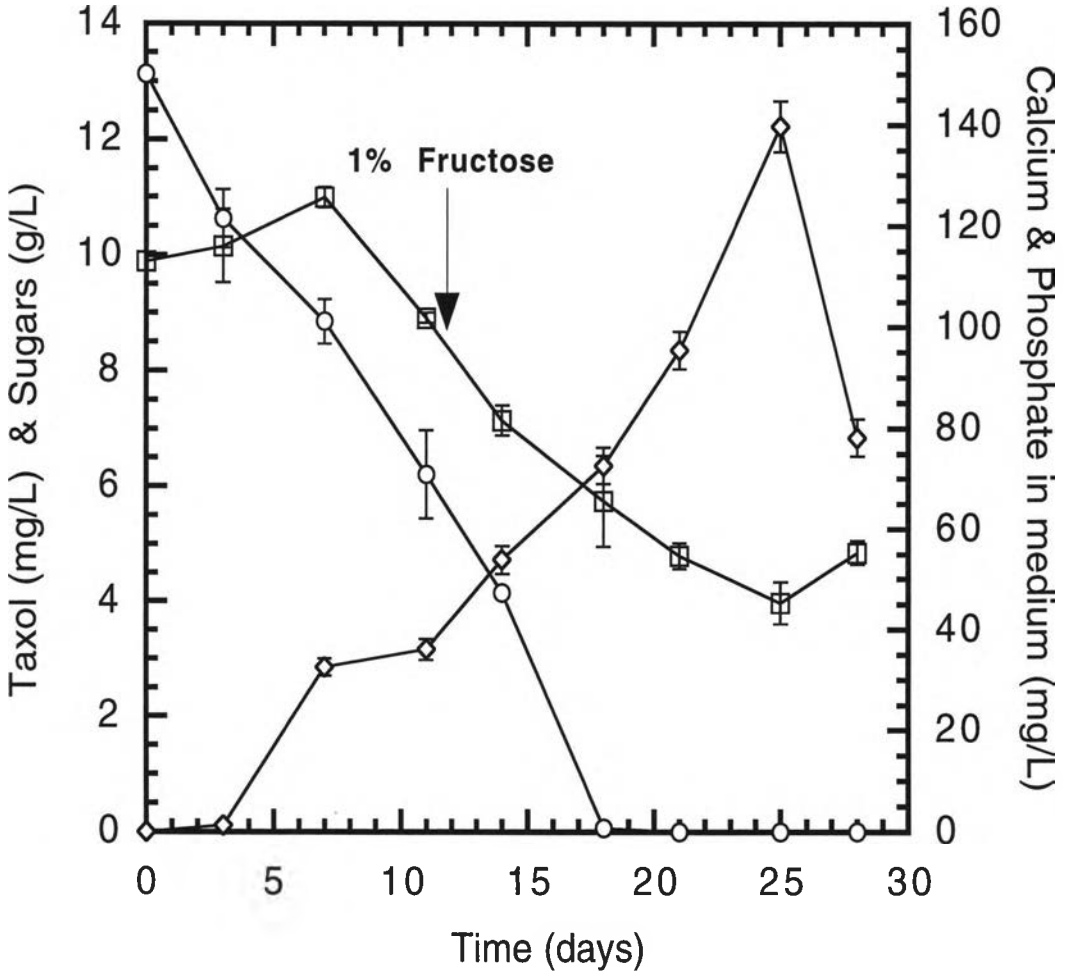

Figure 4.16: Kinetics of nutrient uptake and taxol production when cultures were equilibrated with 10% (v/v) O_2, 0.5% (v/v) CO_2 and 5 ppm C_2H_4, 10 g/L fructose was added on day 11 : □ = calcium (mg/L); ◯ = phosphate (mg/L); ◇ = taxol (mg/L).

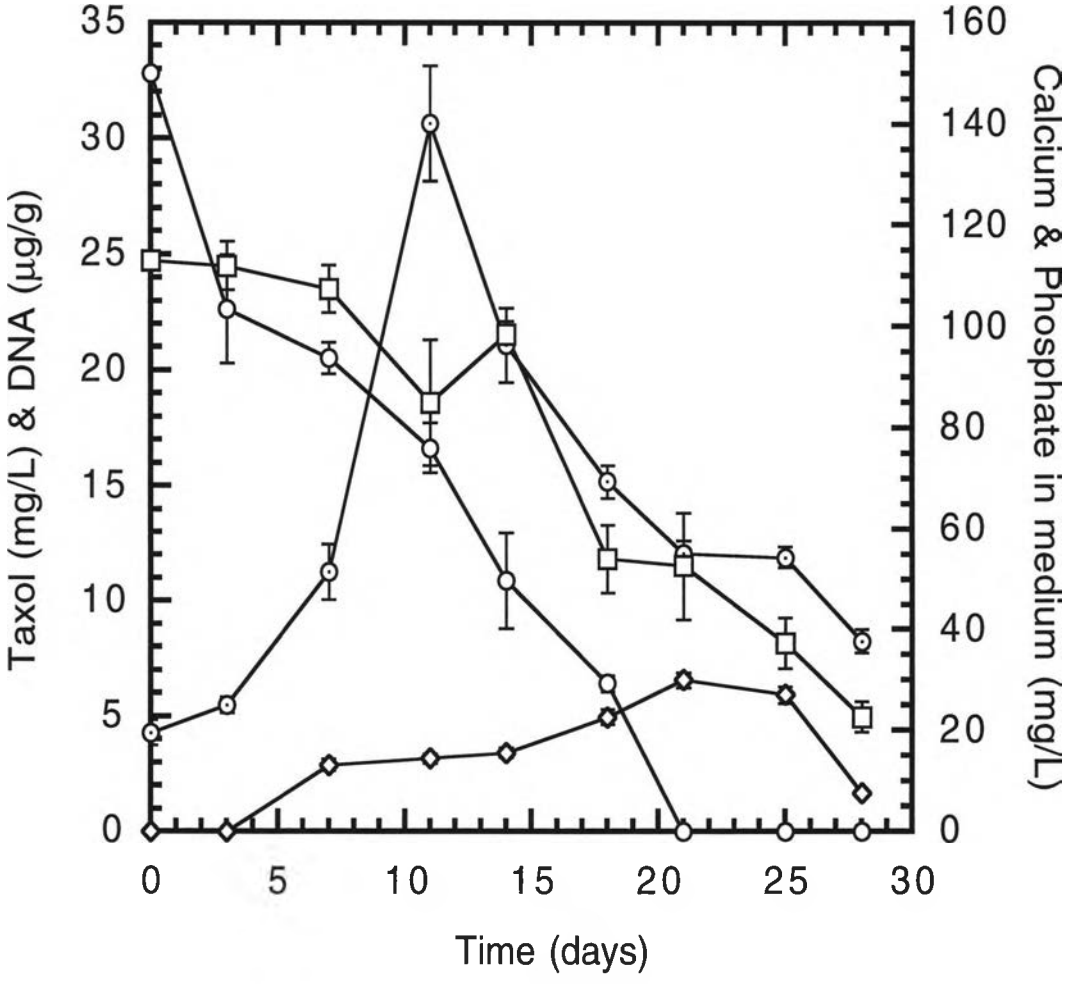

Figure 4.17: Kinetics of growth, nutrient uptake and taxol production when cultures were equilibrated with 10% (v/v) O_2, 0.5% (v/v) CO_2 and 5 ppm C_2H_4 : $\odot$ = DNA (μg/g); $\square$ = calcium (mg/L); $\bigcirc$ = phosphate (mg/L); $\diamond$ = taxol (mg/L).

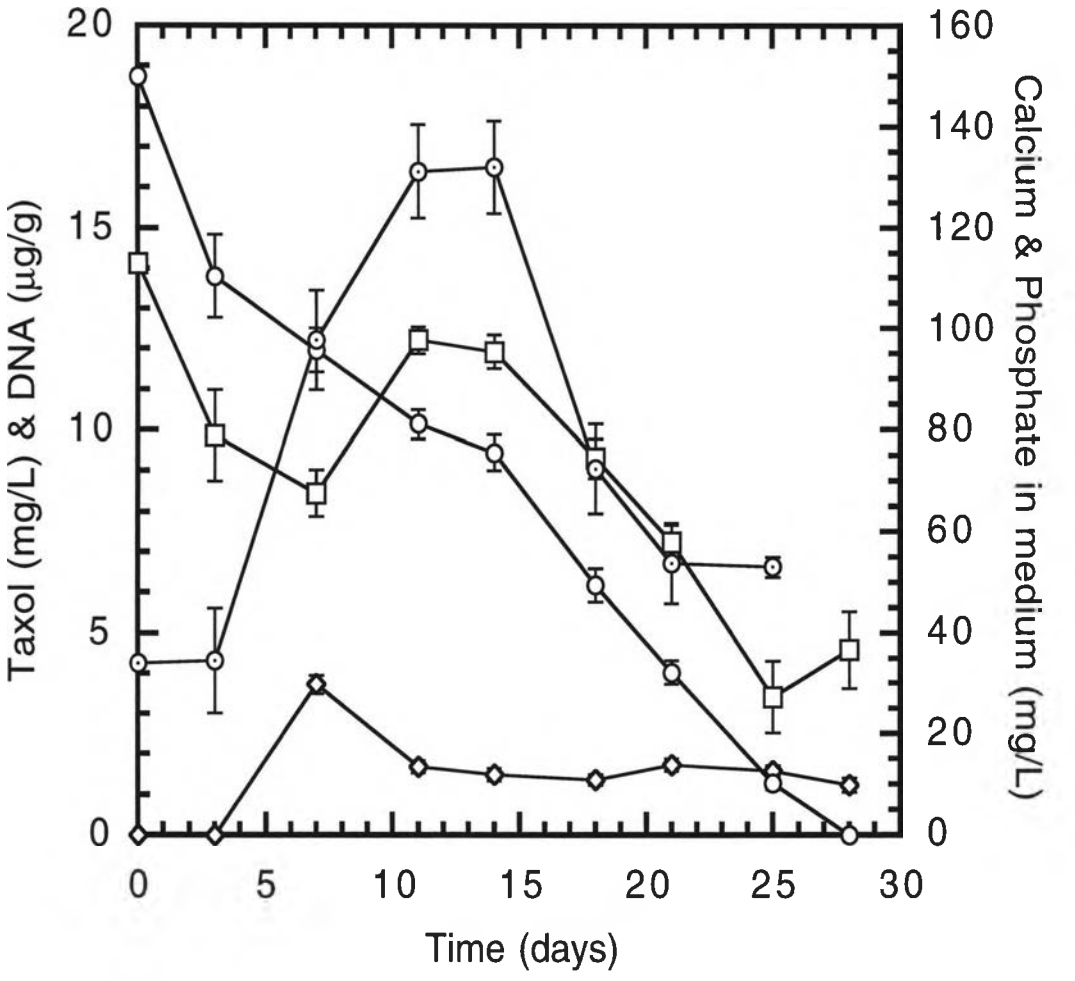

Figure 4.18: Kinetics of growth, nutrient uptake and taxol production when cultures were equilibrated with 10% (v/v) O_2, 0.5% (v/v) CO_2 and 2 ppm C_2H_4 : $\odot$ = DNA (μg/g); $\square$ = calcium (mg/L); $\bigcirc$ = phosphate (mg/L); $\diamond$ = taxol (mg/L).

Chapter 5

DIRECT/INDIRECT MODEL FOR ETHYLENE BINDING

In this chapter the binding kinetics of ethylene are investigated. The data from the previous chapters on taxol production and phosphate uptake rates are used to explore the direct or indirect binding mechanisms of ethylene.

5.1 Recapitulation from previous chapters

In Chapters 2 and 3, the effect of headspace ethylene at constant concentrations of 0, 2, 5, 10, and 50 ppm was studied on production of taxol and uptake of nutrients. Taxol concentration in the medium 21 days after inoculation increased as ethylene concentration was increased from 0 to 5 ppm and leveled out at 10 ppm (Figure 5.1A). With the cell line TC2, taxol productivity with 5 ppm ethylene (0.2 mg/L·Day) was lower than the productivity in the original (TC1) cell line (0.31 mg/L·Day). Taxol productivity (with TC2 cell line) under 50 ppm ethylene was 0.17 mg/L·Day. Even when taxol concentrations were normalized using the glucose consumption rates, taxol productivity in the 50 ppm ethylene treatments were less than the maximum obtained using 5 ppm (Figure 5.1B). Saturation kinetics are expected for ethylene action (Sisler, 1991). However, in our studies, 5 and 10 ppm ethylene resulted in the greatest taxol productivities with both TC1 and TC2 cell lines.

The importance of phosphate esters and anhydrides to cellular metabolism are presented in detail by Westheimer (1987); no other compound appears to

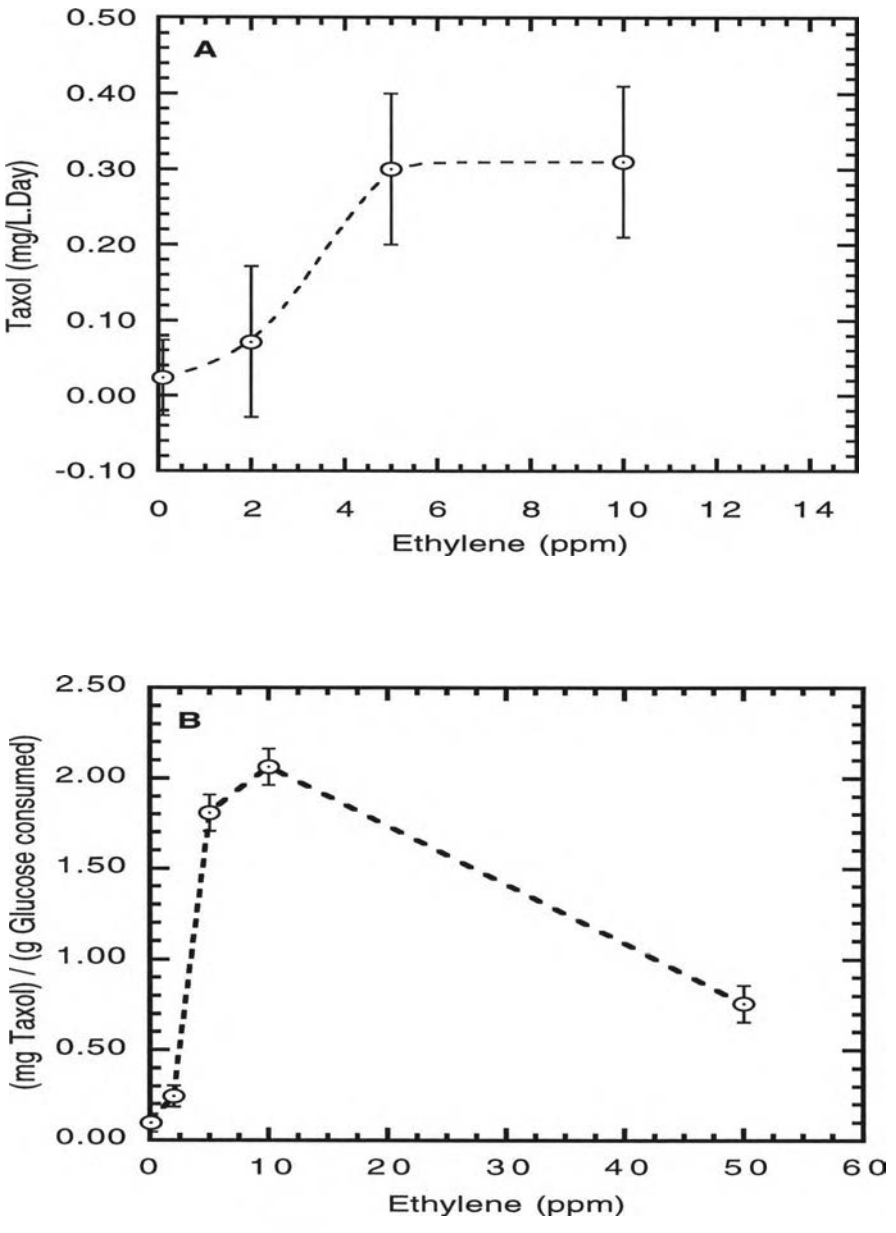

Figure 5.1: Taxol productivity at various gaseous concentrations of ethylene in the head space. **A:** Data from experiments A-C using TC1; **B:** Normalized data from experiments A-F using both TC1 and TC2.

fulfill the multiple roles of phosphate in biochemistry. Protein phosphorylation events are necessary for ethylene-activated signal transduction (Raz and Fluhr, 1993) and protein phosphorylation of cultured plant cells is affected by fungal elicitor treatments (Dietrich *et al.*, 1990). In our studies, phosphate uptake rate (mg/L·Day) increased as the exogenous ethylene concentration increased from 0 to 5 ppm and reached saturation limits at 10 ppm (Figure 5.2A). Phosphate uptake rates per glucose consumed also demonstrated saturation kinetics (Figure 5.2B). Keep in mind that the 0, 5, and 10 ppm treatments were repeated in the second series of experiments; these data fit with the previous data when normalized in this manner. Since ethylene concentration at 50 ppm had an inhibitory effect on taxol production but not on phosphate uptake rate; independent regulation of taxol and physiological functions of the cell was investigated with regard to ethylene action. Calcium data were not considered using these models because the erratic behavior of calcium concentration in the medium and its unknown distribution in internal space make interpretation difficult.

5.2 Direct/indirect model

Ethylene production by plants is associated with stress. Application of ethylene to the plant cell culture may have physiological ramifications that result in normal stress responses. In the case of the *Taxus* spp. cell lines, the stress response conceivably could be production of secondary metabolites, i.e. taxol and other taxanes. The action of ethylene in signal transduction is considered as independent, or at least secondary to, the phenomena leading to expression of defense response genes in plants. The relationship between elicitation and ethylene action is simply not understood; there is great interest in understanding the factors responsible for gene activation by ethylene and elicitors and the mechanism by which these signals are perceived by plant cells.

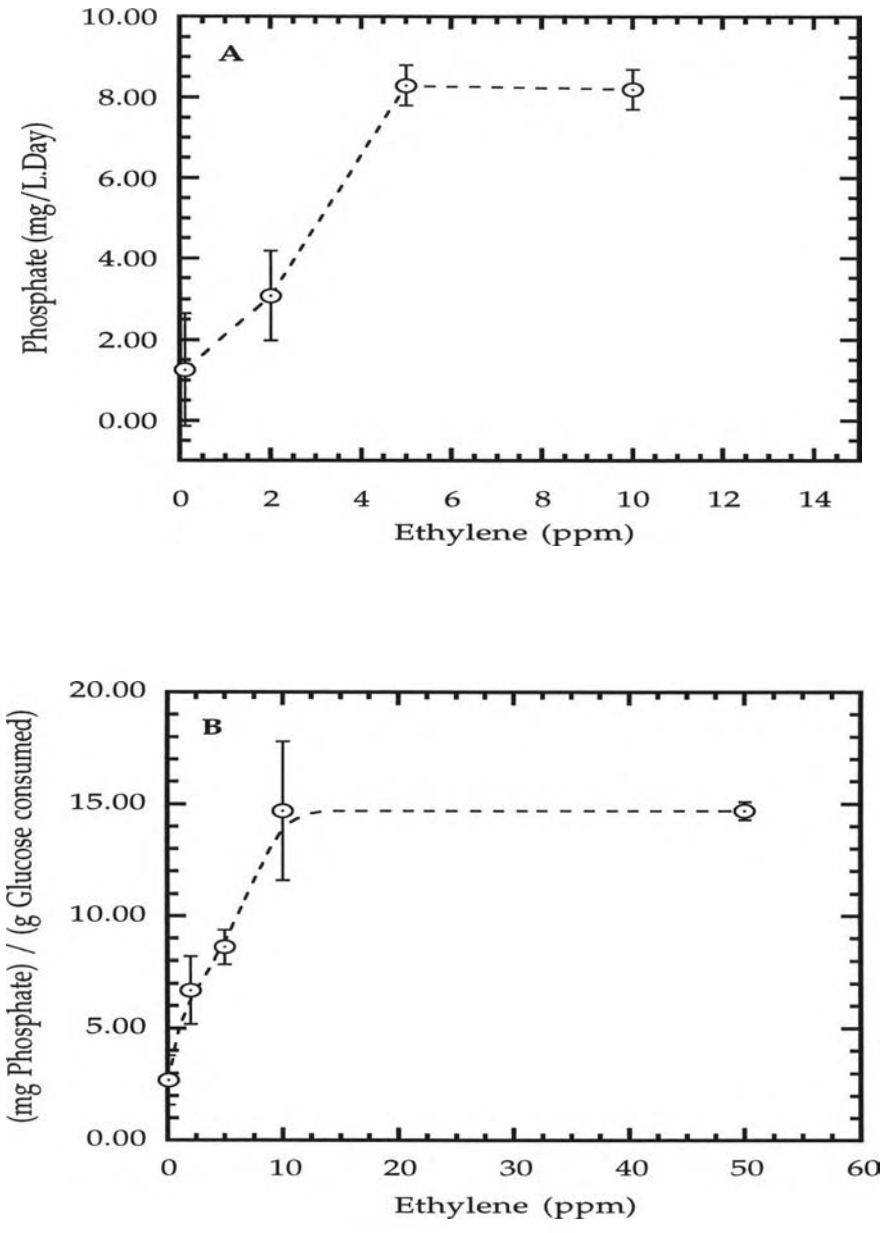

Figure 5.2: Phosphate uptake rate at various gaseous concentrations of ethylene in the head space. **A:** Data from experiments A-C using TC1; **B:** Normalized data from experiments A-F using both TC1 and TC2.

Exogenously applied ethylene induces biochemical and structural changes that are thought to play a role in defense against pathogens (Boller, 1991). According to Sisler (1991), there are three ways that ethylene might act: 1) by serving as a co-factor in some reaction, 2) by being oxidized to some component and being incorporated into tissue, 3) by binding to a receptor, providing some function and then either diffusing away or being destroyed as is the case with other hormones. The first two methods of action of ethylene are unlikely (see Sisler (1991) and references therein). The third one is under extensive research (Kieber *et al.*, 1993; Hall *et al.*, 1990; Chang *et al.*, 1993).

Two types of ethylene binding sites (receptors) have been characterized based on high and low values of association/dissociation constants. The fast-action receptor has a $t_{1/2}$ of 0.1–0.5 h and the slow one a $t_{1/2}$ of 13–22 h (Hall *et al.*, 1990). The responses from exposing plants to ethylene occur with short response times; and when the ethylene is removed, the response ceases quickly (Sisler, 1991). Also the synthesis of the enzymes depends on a continued exposure to ethylene. A withdrawal of ethylene greatly reduces the synthesis and would not represent the action of the long-term binding component.

Chang *et al.* (1993) and Kieber *et al.* (1993) have identified the genes for several proteins involved in ethylene signal transduction. These appear to perceive ethylene and transduce the signal through phosphate transfer reactions to proteins that act directly or indirectly as DNA-binding transcriptional activators after phosphorylation.

Saturable binding sites for ethylene have been demonstrated in plant tissues and extracts (Abeles *et al.*, 1992). Mutations in at least three different genes, one of which is ETR1, cause insensitivity to ethylene. This gene acts early in the ethylene signal transduction pathway, possibly as an ethylene receptor, or as a regulator of the pathway (Chang *et al.*, 1993). The amino-terminal

half of the ETR1 protein shows no sequence similarities to the available protein sequence databases, whereas the carboxyl-terminal portion contains a high degree of sequence identity with the family of prokaryotic signal transducers known as the two-component systems (Chang *et al.*, 1993). Potential ETR1 targets include components represented by the other ethylene response mutants in *Arabidopsis*. One of these is CTR1, which acts downstream of ETR1 and is suggested to be a negative regulator of ethylene response (Kieber *et al.*, 1993). In bacteria the two protein components function together to regulate adaptive responses to a broad range of environmental stimuli such as chemotaxis, motility, sporulation and virulence. This two-component regulatory system of bacteria usually involves a transmembrane protein (sensor) which senses external conditions and changes its conformation to signal a cytoplasmic protein (activator). Many activators regulate transcription of specific sets of genes. The supposed pairing of ETR1 and CTR1, a two component system homolog in the signal transduction pathway is plausible (Chang *et al.*, 1993). Whether ETR1 binding is to ethylene directly or indirectly would be analogous to the chemotaxis system in which receptors either bind directly ligands such as aspartate and serine or bind indirectly via binding protein ligands such as maltose, ribose and galactose.

The direct binding of ethylene (E) to the receptor (R) can be described by the Michaelis-Menten equation as following :

$$E + R \rightleftharpoons ER \rightarrow Response \tag{5.1}$$

$$Response = \frac{V_m[E]}{K_m + [E]} \tag{5.2}$$

For a large number of gases soluble in water, Henry's Law can be used to relate the gaseous concentration of the gas to its equilibrium concentration in water. The design of the gas mixing apparatus in our studies allowed equilibrium between head space gas phase and the liquid throughout the experiments. Hence,

the dissolved concentration of ethylene (E) in the culture medium was calculated using Henry's law. For phosphate uptake as the response, $K_m = 4 \times 10^{-8}$M ethylene and V_{max} = 15 (mg phosphate) / (g glucose). For taxol productivity $K_m = 4 \times 10^{-8}$M ethylene and V_{max} = 0.35 mg/L·Day taxol.

The mathematical model of dose-response relationships for indirect binding of ligand to receptor in the chemotaxis system is given by Yaghmai and Hazelbauer (1993). By analogy, the indirect binding of ethylene (E) to receptor (R) by intermediate binding to a binding protein (B) has the following model :

$$E + B + R \overset{K_1}{\rightleftharpoons} EB + R \overset{K_2}{\rightleftharpoons} EBR \rightarrow Response$$

$$Response = pF_o = p\frac{K_1K_2[B][E]}{1 + K_1[E](1 + K_2[B])} \tag{5.3}$$

The two rate constants can be related as: $K_r = \frac{1}{K_m} = K_1 (1 + K_2[B])$. The binding site concentration [B] for various plant vegetative tissue is reported by Sisler (1991). In our studies, the average value reported for the binding site concentration ([B] = 4×10^{-9} M) was used. From Sisler (1991), $K_1 = 4 \times 10^3$ ($M^{-1}S^{-1}$). Therefore, with $K_m = 4 \times 10^{-8}$ M, $K_2 = 6.3 \times 10^{12}$ M^{-1}. Sisler's high and low values of [B] were used to evaluate the sensitivity of this parameter. The proportionality constant (p) relates the fraction of occupied binding sites (F_o) to the response. The value for (p) was calculated using the maximal value of the response and F_o at saturation (100%), i.e. the V_{max} from the Michaelis-Menten analysis.

Figure 5.3 shows Equations 5.2 and 5.3 applied to our system. The specific phosphate uptake and specific and volumetric taxol productivity have been used as responses. The limits of sensitivity of the indirect model is shown by the dotted lines on Figure 5.3. The dotted lines are obtained using our data and the high and low values of binding site concentrations cited in the

literature; the solid line represents the average of these values. Figure 5.3A shows that specific phosphate uptake exhibits saturation kinetics and that the indirect binding model fits these data better than the direct binding model. However, the taxol productivities show a decline at the high concentration of ethylene (50 ppm). The curves are drawn using parameters calculated by disregarding the 50 ppm ethylene. Again, the indirect binding model fits the data better than the direct binding model at the physiological concentrations of ethylene. Because taxol productivity (Figures 5.3B & C) relationships are different than that of phosphate uptake (Figure 5.3A), taxol biosynthesis may be under regulation of ethylene effects on gene transcription that are not related to physiological functions of the cell.

The biosynthetic precursor to ethylene, ACC, glycopeptide elicitors and a chitin heptamer all caused rapid and significant expression of a reported gene under the control of a bean chitinase promoter (Brodelius and Vogel, 1985). Broglie *et al.* (1986) demonstrated a 75- to 100-fold increase in translatable chitinase mRNA seen upon ethylene treatment of bean seedlings. The proteins involved in ethylene signal transduction and the chitosan signal transduction pathway are possibly different; the genes for these proteins are just now being elucidated. Alternatively, it is possible that ETR1 is not an intermediate component of the ethylene signal transduction pathway, but rather a regulator of the pathway (Chang *et al.*, 1993). In our studies phosphate uptake rate is considered as a physiological handle, i.e. an indicator of culture activity.

5.3 Conclusion

The results and modelling presented here could be summarized as follows: The effect of ethylene on the activity of the cell, as indicated by the phosphate uptake, a metabolic marker, is independent of the effect on secondary

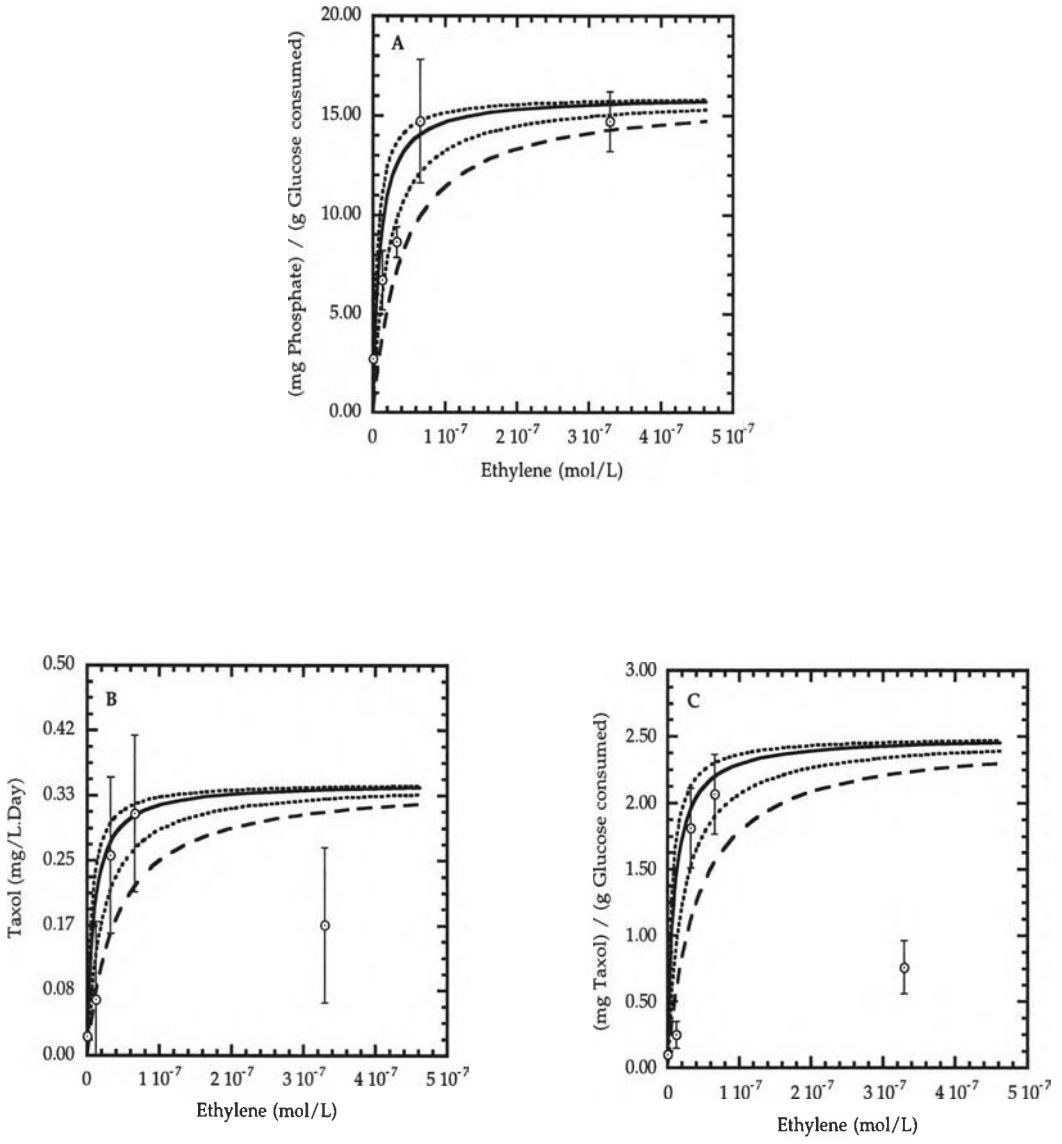

Figure 5.3: The mathematical models of direct and indirect binding of ethylene to receptor, applied to our data. $\odot$ = Data points; - - - - = Direct binding; ——— = Indirect binding; ..... = error limits on indirect binding model.

metabolism. Taxol productivity appears to be under ethylene control by regulation of transcription of biosynthetic enzymes. While these conclusions give no physical evidence of the mechanism of ethylene action, it is frequently useful when the available information is condensed for discussion. A drawing is presented in Figure 5.4 which shows ETR1 receptors as integral components of the endoplasmic reticulum membranes. Hall *et al.* (1990) isolated receptor on intracellular membrane systems rather than cytoplasmic membrane localization as normally considered for hormone receptors. Ethylene is synthesized internally; exogenous ethylene diffuses readily. Precedent for intracellular receptors of the two-component regulatory system comes from the receptors for phosphate regulation of bacteria appearing on the cytoplasmic region (Stock *et al.*, 1992). As mentioned above, indirect binding of maltose in the chemotaxis model serves as precedence of CTR1 involvement. The data presented here allows better agreement for the indirect binding model than for direct binding of ethylene to ETR1. ETR1 is not directly involved in DNA binding, but a transphosphorylation product such as EIN1, that has been shown to genetically linked downstream to ETR1, may be responsible for promoter interaction (Guzman and Ecker, 1990).

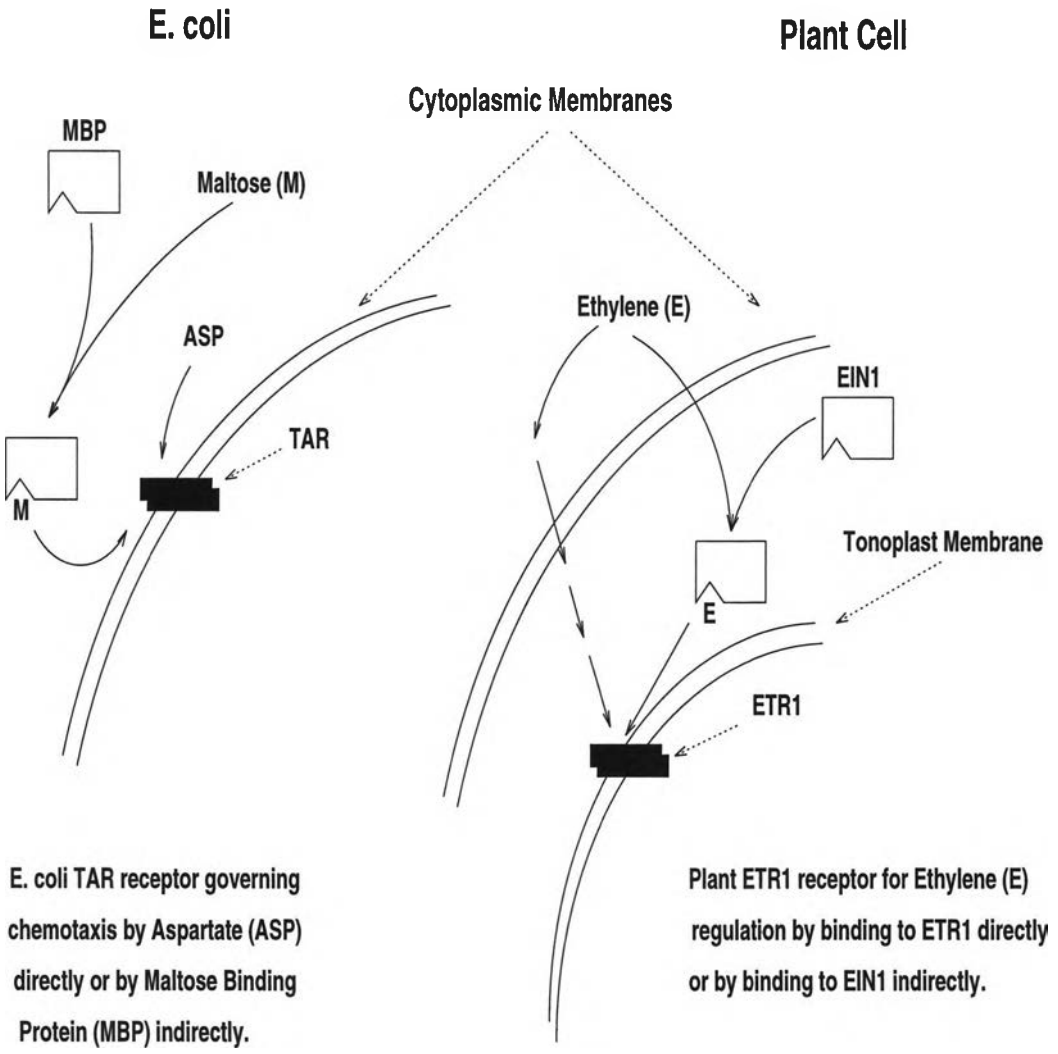

Figure 5.4: Hypothetical mechanism of action of ethylene; compared to the *E. coli* chemotaxis system.

Chapter 6

ELICITATION STUDIES

As explained in Chapter 2, jasmonic acid and methyl jasmonate play important roles in the signal transduction cascade of plants which is initiated as a result of pathogen attack. Elicitation activity of methyl jasmonate in different plant cells has been documented (Tamari *et al.*, 1995; Creelman *et al.*, 1992; Dittrich *et al.*, 1992; Mizukami *et al.*, 1993; Kauss *et al.*, 1992). Also methyl jasmonate can induce ethylene production in plants (Saniewski *et al.*, 1987). Plant defense genes were reported to be synergistically induced by ethylene and methyl jasmonate (Xu *et al.*, 1994). In this chapter the effect of methyl jasmonate and ethylene on production of taxol is discussed. Since taxol is a phytoalexin and is presumed to be produced by the plant as a response to pathogenic attack, understanding the effect of ethylene/methyl jasmonate on production of taxol may help elucidate the mechanism of induction.

6.1 Background information

To deter pathogenic microorganisms and herbivores, plants produce pathogen related (PR) proteins as an inducible chemical defense system (Gundlach *et al.*, 1992). These proteins are involved in the reinforcement of plant cell walls, formation of callose, biosynthesis of antimicrobial hydrolytic enzymes and biosynthesis of phytoalexins (Harborne, 1988; Lindsay *et al.*, 1993). Phytoalexins serve

as antibiotics in the defense system of plants. The two major classes of phytoalexins are terpenoids and isoflavonoids. Biosynthesis of the isoflavonoids *via* the phenylpropanoid pathway has been under extensive research (Brooks and Watson, 1991). Taxol is a natural diterpenoid with strong anticancer activity.

Elicitors are compounds that stimulate synthesis of phytoalexins in cultured plant cells. Biotic elicitors include glucan polymers, glycoproteins, low molecular weight organic acids and fungal cell wall materials; abiotic elicitors include ultraviolet or far-red radiation, salts of heavy metals, and chemicals (DiCosmo and Misawa, 1985). Environmental stresses such as wounding, temperature and pathogenic attack elicit PR responses and enhance ethylene biosynthesis (Chang *et al.*, 1993). Treating plants with ethylene induces defense gene expression, as demonstrated for chitinase (Broglie *et al.*, 1986), hydroxyproline-rich glycoproteins (HRGPs) (Ecker and Davis, 1987), and the enzymes involved in the synthesis of the flavonoid phytoalexins (Rumeau *et al.*, 1988). Exogenously applied gaseous ethylene has been closely correlated as part of the signal transduction pathway initiated by some, but not all, elicitors (Raz and Fluhr, 1992; Roby *et al.*, 1991; Seifert *et al.*, 1994). A number of ethylene induced genes have been isolated and characterized (Abeles *et al.*, 1992). In Chapter 3, taxol production was related to exogenous ethylene concentration (Mirjalili and Linden, 1995a).

Jasmonic acid may be part of a general signal transduction system regulating inducible defense genes in plants (Farmer and Ryan, 1990). Kutchan (1991) observed that the maximum jasmonate accumulation preceded the maximum defense gene transcript accumulation, which suggested an integral role for this molecule in the signal transduction system of plants. Endogenous jasmonic acid and its methyl ester (MeJA) accumulated rapidly and transiently after treatment of plant cell suspension cultures of *Rauvolfia canescens* and

Eschscholtzia californica with a yeast elicitor (Gundlach *et al.*, 1992). Thirty six plant species tested in cell suspension culture could be elicited by exogenously supplied methyl jasmonate (MeJA) to accumulate secondary metabolites (Gundlach *et al.*, 1992). Gundlach *et al.* (1992) suggested that addition of methyl jasmonate initiates *de novo* transcription of genes, such as phenylalanine ammonia lyase (PAL). This enzyme is a key regulator of the phenylpropanoid pathway that yields a diverse range of phenolics with defense-related functions. The elicitation activity of MeJA in suspension cultures of *Lithospermum erythrorhizon* was much higher than that of yeast extract in terms of the induction of PAL and 4-hydroxyphenylpyruvate reductase (HPR) activities and rosmarinic acid (RA) accumulation (Mizukami *et al.*, 1993).

Xu *et al.* (1994) observed the synergistic induction of plant defense genes by ethylene and MeJA; ethylene/MeJA resulted in osmotin protein accumulation to levels similar to those induced by osmotic stress. Many genes are induced by several unrelated signals, such as osmotic stress and pathogen invasion, suggesting a relationship between inductive signals and gene function (Xu *et al.*, 1994). Particular signal combinations may synergistically hyperinduce plant defense genes and be more specifically related to gene function than to any single inductive signal (Xu *et al.*, 1994). The responsive sequences of the osmotin promoter to combinations of ethylene and MeJA are present on the same DNA fragment where responsiveness to other signals has been mapped (Xu *et al.*, 1994).

This Chapter provides the results of the elicitation Experiments, G and H. Experiment G was a full factorial design with the two factors ethylene and methyl jasmonate. The treatment that resulted in the highest taxol production in Experiment G, was repeated in Experiment H. A simple induction model is proposed in Chapter 7; the results of the ethylene and elicitation experiments are analyzed using this model.

6.2 Experimental design

In Experiment G, the suspension cultures of *Taxus cuspidata* (TC2 cell line) were challenged with various concentrations and combinations of methyl jasmonate and ethylene. Each factor had three levels; hence a full factorial design required 9 treatments, and duplicating each resulted in 18 treatments. Since the gas mixing apparatus allowed parallel experimentation of 15 treatments; we were able to duplicate all but three treatments. The full experimental design containing these 15 treatments was performed in Experiment G. As will be discussed in later sections, the combination of 5 ppm ethylene and 10 μM MeJA resulted in the highest taxol production. Consequently, this treatment was repeated in Experiment H as duplicates. Table 6.1 shows the experimental design used for Experiments G and H. Since taxol production was observed during the first week of the experiments, elicitation was started seven days after growth was initiated under 10% (v/v) oxygen and 0.5% (v/v) carbon dioxide and variable headspace ethylene concentrations. The headspace gas concentrations were those that resulted in the highest taxol production, as discussed in Chapter 3. Both experiments were run for 14 days.

6.3 Results

6.3.1 Growth analysis

Table 6.2 shows the dry weight data when the TC2 cell cultures were exposed to various concentrations and combinations of ethylene and MeJA in Experiment G. Content of the whole flasks were used to measure the dry weight. Statistical analysis showed that MeJA was the only factor that had a significant effect ($p \leq 0.05$) on dry weight of the cultures (Equation 6.1).

$$DWT(g/L) = 1.56 + 0.33[MeJA] \tag{6.1}$$

Table 6.1: Experimental designs used in Experiments G and H (TC2 cell line).

# of replicates	O_2% (v/v)	CO_2% (v/v)	C_2H_4 (ppm)	MeJA (μM)
Expt. G				
2	10	0.5	0	0
2	10	0.5	0	10
2	10	0.5	0	100
2	10	0.5	5	0
2	10	0.5	5	10
2	10	0.5	5	100
1	10	0.5	10	0
1	10	0.5	10	10
1	10	0.5	10	100
Expt. H				
2	10	0.5	5	10

Growth rates of the cultures increased as the concentration of MeJA increased; from 0.15 d^{-1} to 0.19 d^{-1} to 0.22 d^{-1} at 0, 10 and 100 μM MeJA, respectively. This was independent of the ethylene concentration flow into the cultures. This phenomenon was in accord with the fact that the rate of glucose consumption increased as the concentration of MeJA was increased (next section). The highest growth rate was observed with 5 ppm ethylene and 100 μM MeJA (0.29 d^{-1}). The average growth rate with this cell line (0.19 d^{-1}) was 67% that of the TC1 cell line.

The growth rates of the cultures exposed to 5 ppm ethylene and 10 μM MeJA (with 10% (v/v) O_2 and 0.5% (v/v) CO_2), in Experiment H were lower compared to Experiment G (Table 6.2). These cultures also produced less taxol compared to the cultures in Experiment G (Section 6.3.3).

6.3.2 Sugar utilization

The patterns of sugar utilization in Experiments G and H were similar to those of Experiments A-F; however, the kinetics were slower. Figure 6.1 shows

Table 6.2: Average growth rates when TC2 suspension cultures were treated with various concentrations and combinations of methyl jasmonate and ethylene in Experiments G and H.

C_2H_4 ppm	MeJA μM	Initial Dry Wt. g/L±SD	Final Dry Wt.(14days) g/L±SD	Growth Rate Day^{-1}
Expt. G				
0	0	0.58±0.08	1.29±0.08	0.16
0	10	0.58±0.08	1.57±0.08	0.19
0	100	0.58±0.08	1.59±0.08	0.19
5	0	0.58±0.08	1.12±0.03	0.14
5	10	0.58±0.08	1.41±0.3	0.17
5	100	0.58±0.08	2.41±0.2	0.29
10	0	0.58±0.08	1.27	0.16
10	10	0.58±0.08	1.75	0.22
10	100	0.58±0.08	1.60	0.19
Expt. H				
5	10	0.55±0.09	1.01±0.2	0.13

the kinetics of sucrose hydrolysis at various concentrations and combinations of ethylene and methyl jasmonate. Sucrose hydrolysis was faster when cultures were exposed to 5 and 10 ppm ethylene compared to 0 ethylene (compare Figures 6.1B & C with A). With 0, 5, and 10 ppm ethylene, sucrose hydrolysis was completed by days 14, 10 and 8, respectively.

The glucose and fructose in the medium were not depleted during the 14 days of the experimentation (Figures 6.2 and 6.3). According to the statistical regression analysis (Appendix C), methyl jasmonate had a positive effect on utilization of glucose; this effect was independent of ethylene concentration in the head space and the time of sampling. However, fructose utilization was affected by the concentration of ethylene only during the 8 hours after elicitation, this effect was positive. Methyl jasmonate, on the other hand, had a significant effect on the 14th day of the experiment. The statistical regression equations are listed in Appendix C.

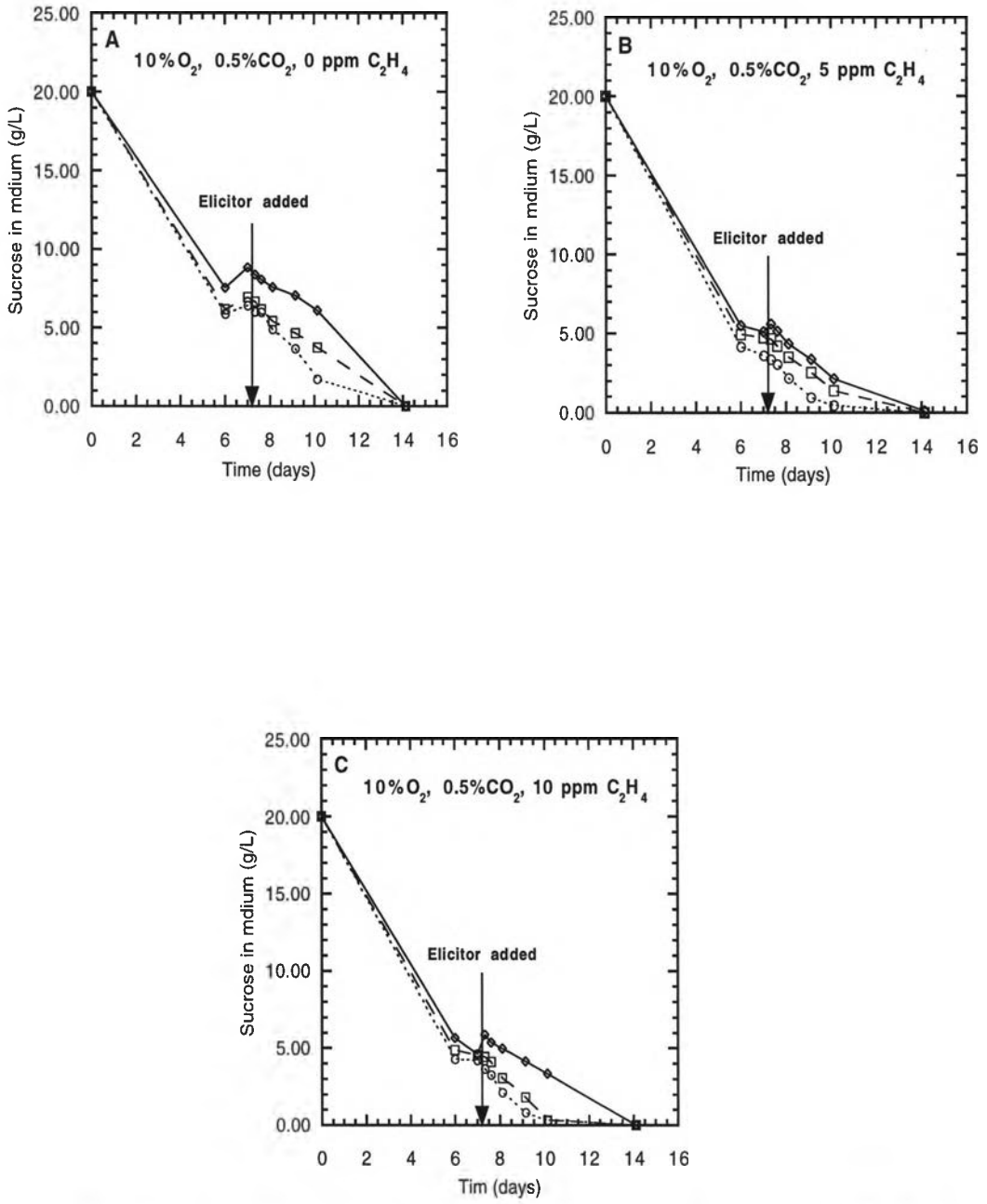

Figure 6.1: Kinetics of sucrose hydrolysis in Experiment G. $\diamond$ = 0 μM MeJA; $\square$ = 10 μM MeJA; $\circ$ = 100 μM MeJA.

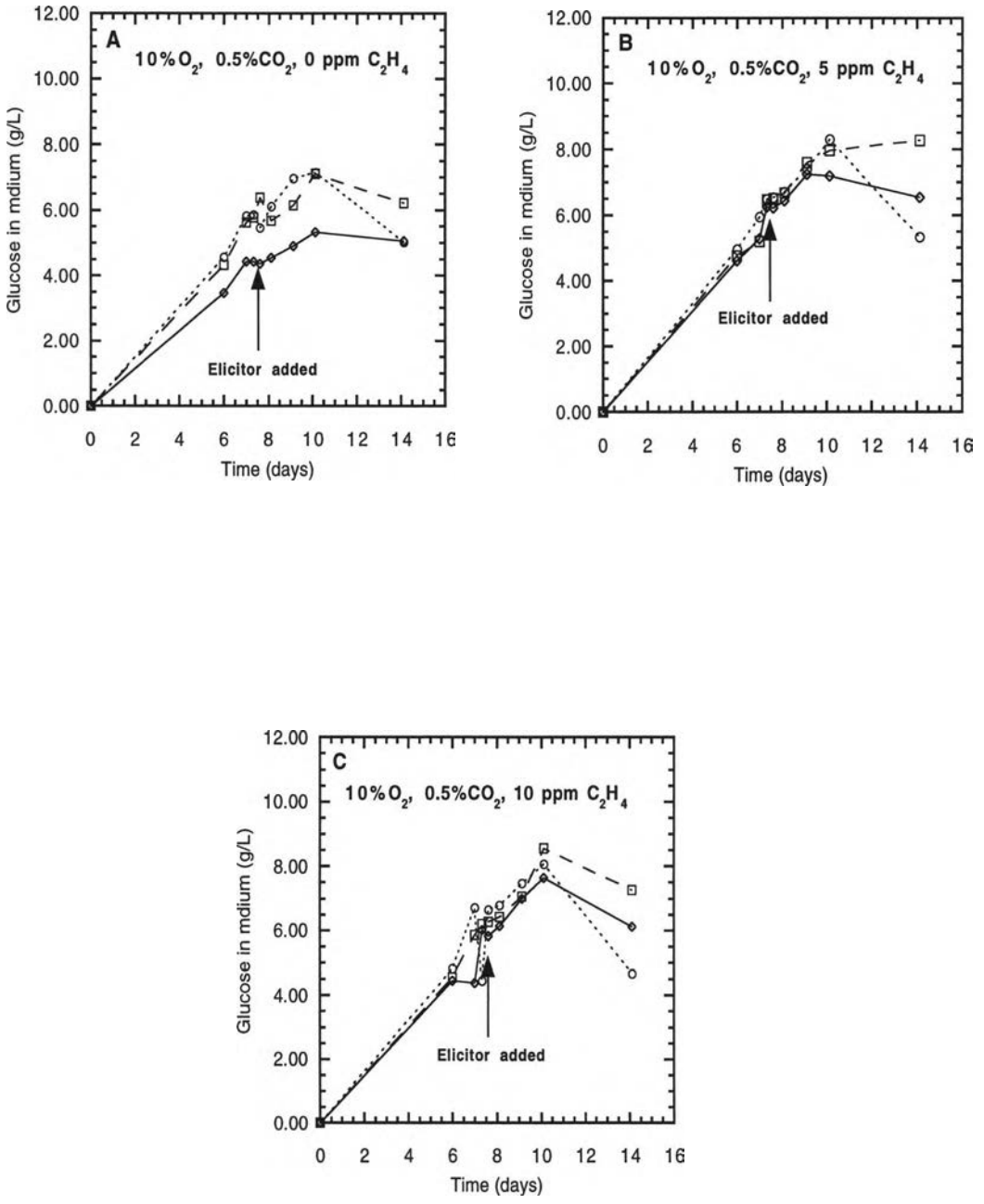

Figure 6.2: Kinetics of glucose utilization in Experiment G. $\diamond$ = 0 μM MeJA; $\square$ = 10 μM MeJA; $\circ$ = 100 μM MeJA.

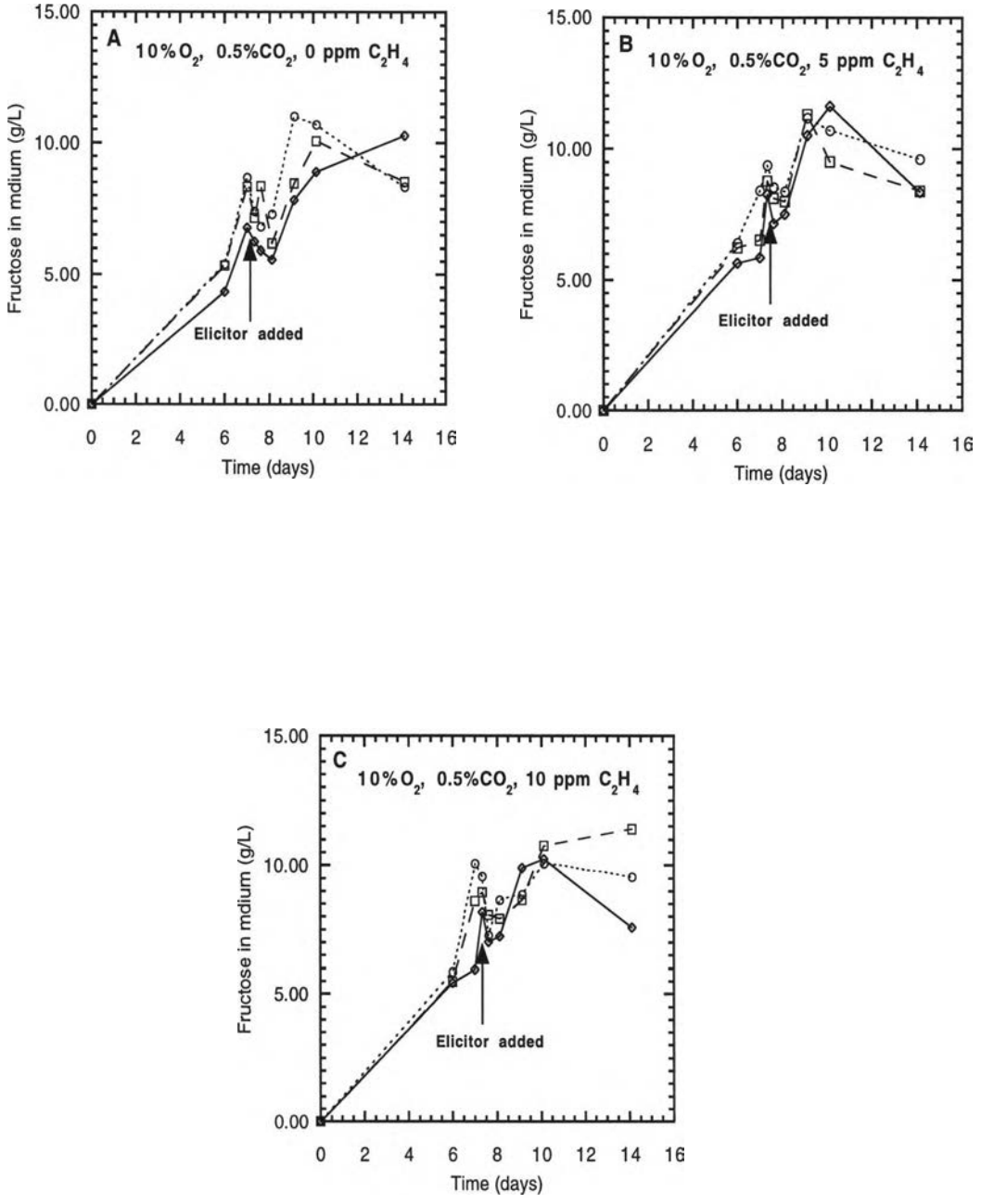

Figure 6.3: Kinetics of fructose utilization in Experiment G. $\diamond$ = 0 μM MeJA; $\square$ = 10 μM MeJA; $\circ$ = 100 μM MeJA.

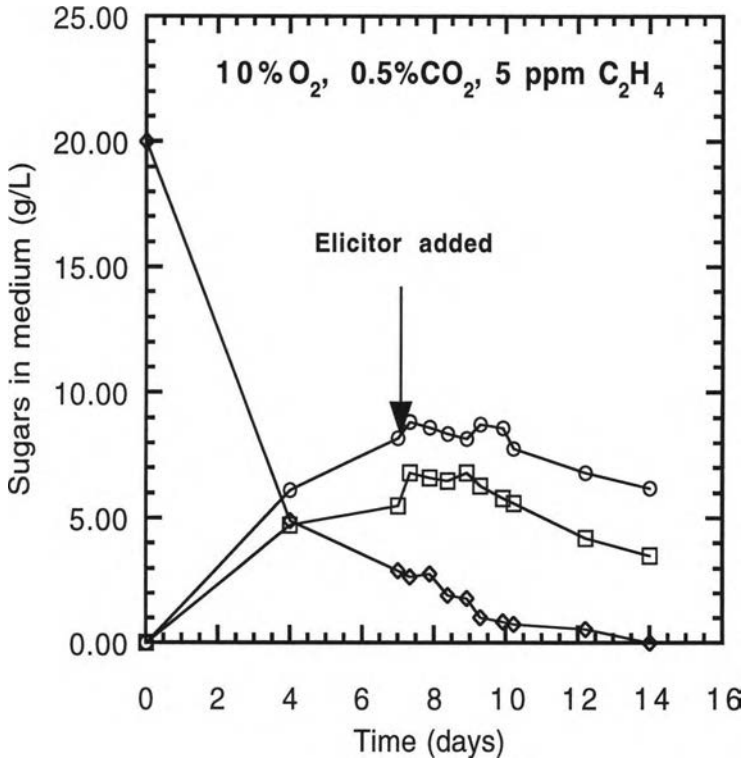

Figure 6.4: Kinetics of sugar utilization by the TC2 cells in Experiment H. $\diamond$ = sucrose; $\square$ = glucose; $\circ$ = fructose.

Patterns and kinetics of sugar utilization in Experiment H is shown in Figure 6.4. Again, sucrose was completely hydrolyzed in 10 days; and glucose and fructose were not completely utilizied by the cultures during the 14 days of the experiment. The kinetics of glucose and fructose utilization in cultures exposed to 10% (v/v) O_2, 0.5% CO_2, and 5 ppm C_2H_4 and 10 μM MeJA were similar in both experiments G and H.

6.3.3 Taxol analysis

Figures 6.5, 6.6 and 6.7 show the kinetics of taxol production at different concentrations and combinations of ethylene and methyl jasmonate. Independent of the ethylene concentration, cultures exposed to 0 μM MeJA, produced 0.5 mg/L taxol during the 14 days of the study (solid line in Figures 6.5, 6.6 and 6.7). When no ethylene was present (Figure 6.5), only the 10 μM MeJA treatment

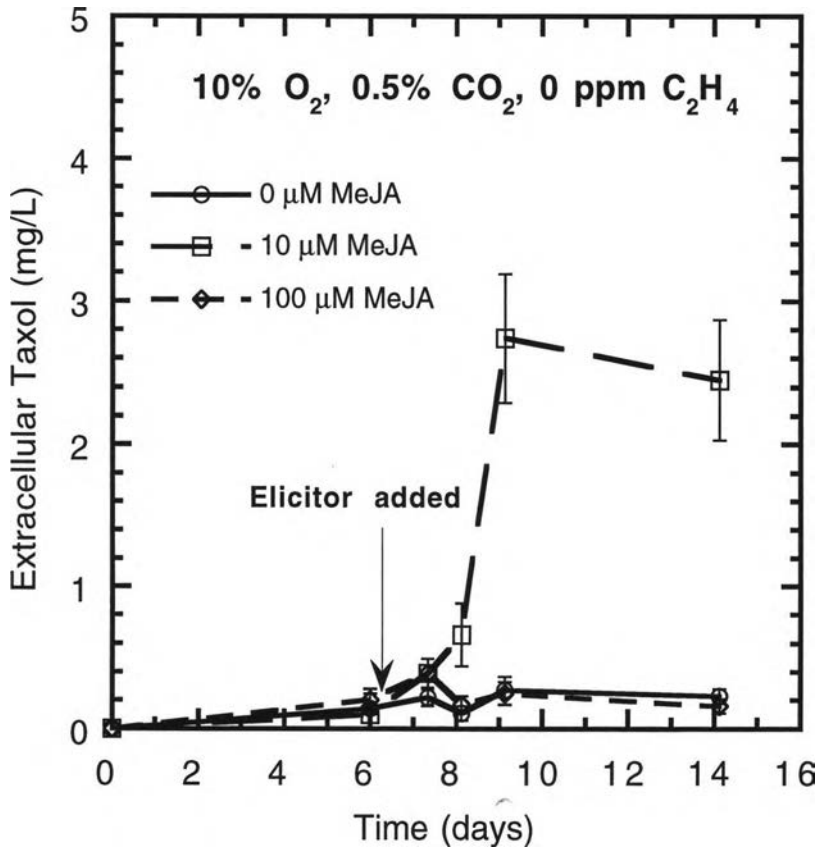

Figure 6.5: Kinetics of taxol production when the TC2 cell cultures were exposed to 0 ppm ethylene and various concentrations of methyl jasmonate.

resulted in an increase of taxol production to 2.7 mg/L. This maximum induction (5-fold increase) was observed 51 hours after elicitation. Cultures exposed 100 μM MeJA had comparable taxol production to cultures exposed to 0 μM MeJA.

When cultures were exposed to 5 ppm ethylene (Figure 6.6), 10 μM MeJA resulted in the highest taxol concentration (3.4 mg/L), again 51 hours after elicitation (7-fold increase); 100 μM MeJA resulted in an increase of taxol concentration from 0.5 to 1.1 mg/L but only after 171 hours (2-fold increase).

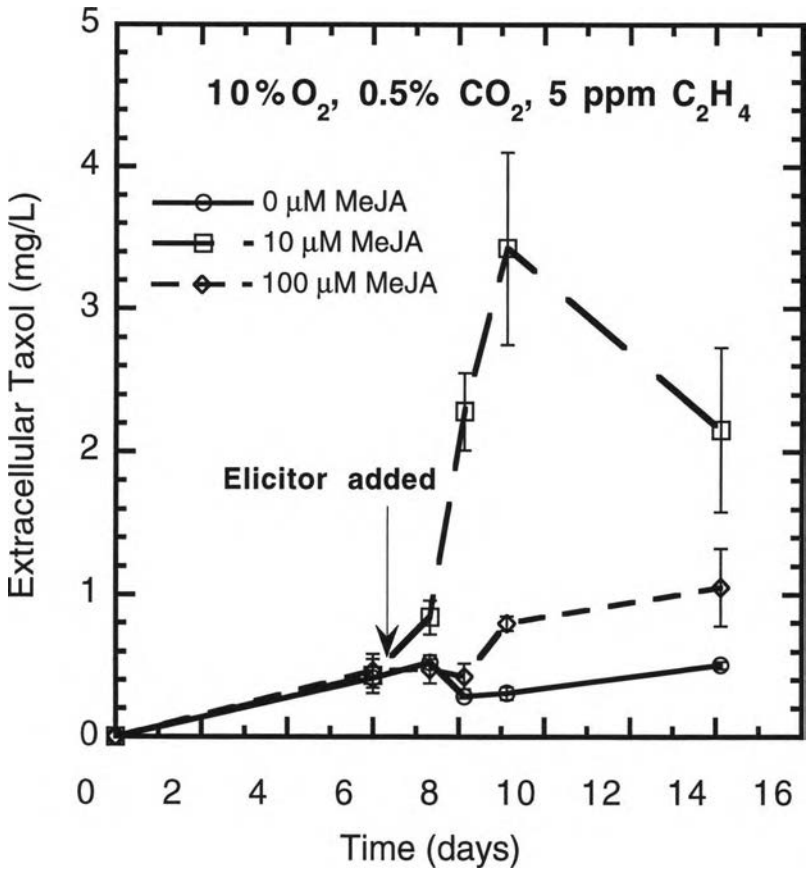

Figure 6.6: Kinetics of taxol production when the TC2 cell cultures were exposed to 5 ppm ethylene and various concentrations of methyl jasmonate.

However, at 10 ppm ethylene, taxol production in cultures exposed to both 10 and 100 μM MeJA increased dramatically from the basal level (Figure 6.7). Again, the highest taxol concentrations observed with 10 μM MeJA, were 51 hours after elicitation (2.8 mg/L). With 10 ppm ethylene and 100 μM MeJA, taxol concentration increased from 0.5 mg/L to 1.5 mg/L after 51 hours and to 2.3 mg/L 171 hours after elicitation (4-fold increase).

Statistical analysis of the taxol data showed that the interaction of methyl jasmonate and ethylene had a significant effect ($p \leq 0.05$) on production

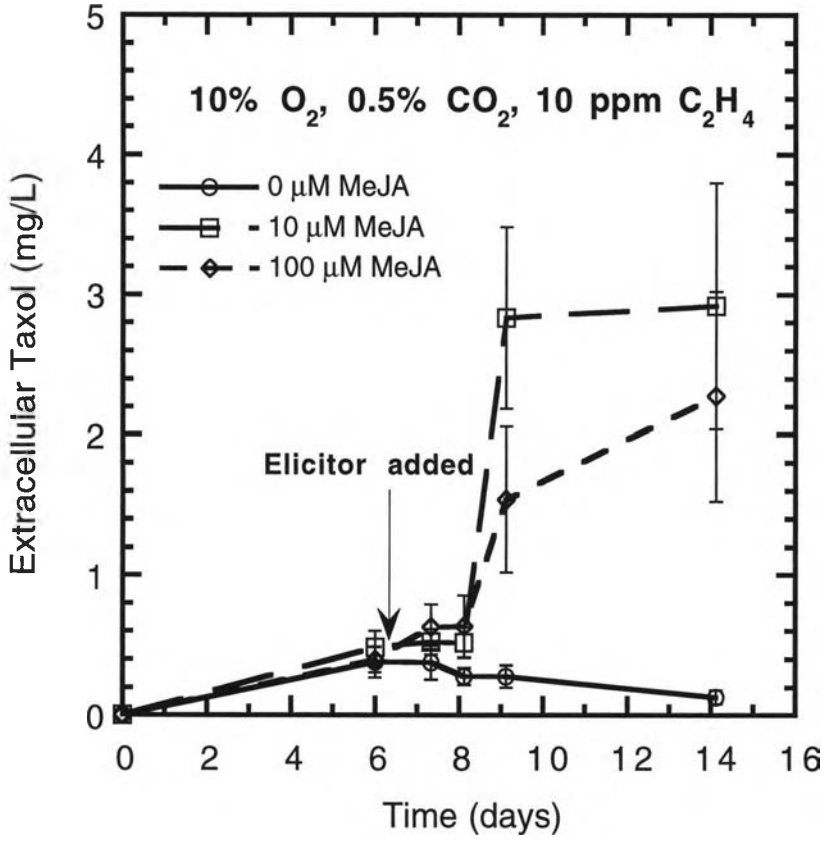

Figure 6.7: Kinetics of taxol production when the TC2 cell cultures were exposed to 10 ppm ethylene and various concentrations of methyl jasmonate.

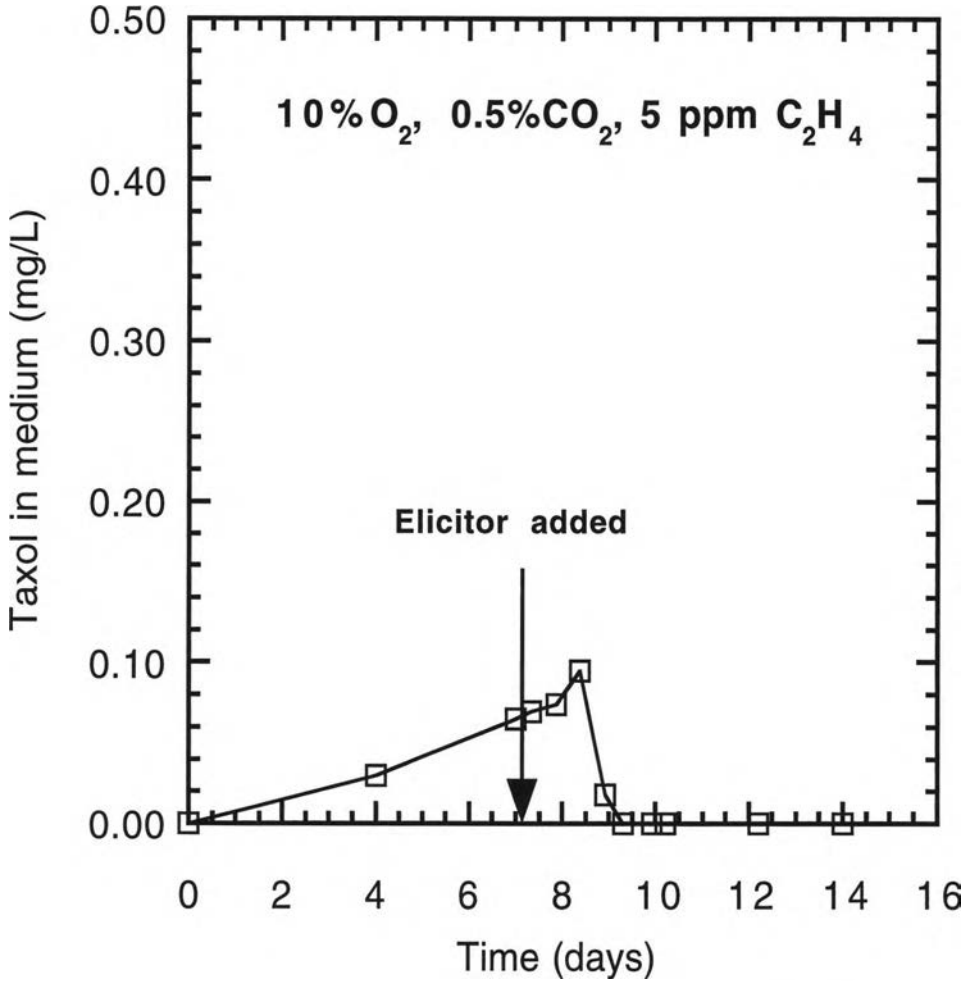

Figure 6.8: Kinetics of taxol production when the TC2 cells were exposed to 5 ppm ethylene and 10 μM methyl jasmonate in Experiment H.

of taxol 51 hours after elicitation in Experiment G (Equation 6.2).

$$Taxol(51hrs) = 1.4 + 1.6[MeJA] + 0.3[MeJA \times C_2H_4] \qquad (6.2)$$

Taxol concentrations were much lower in Experiment H compared to Experiment G. However, the interaction of 5 ppm ethylene and 10 μM MeJA resulted in a 5-fold increase of taxol production; this increase was observed 33 hours after elicitation. In this experiment, taxol levels declined 46 hours after elicitation (Figure 6.8).

6.3.4 Phosphate uptake

The patterns of phosphate uptake by the TC2 cells are shown in Figure 6.9. There was an increase in the medium phosphate concentration 8 hours after elicitation that appeared to be in response to addition of alkaloid solution of methyl jasmonate, and again later 75 hours after elicitation. An increase in medium phosphate equates to a release of phosphate from the intracellular pools. This behavior was independent of ethylene and methyl jasmonate concentrations. This increase in medium phosphate concentration was repeated in Experiment H (Figure 6.10). Since the release of phosphate was observed even at 0 ppm ethylene and 0 μM MeJA, it may be attributed to the concentration of ethanol in the cultures. To all of these cultures, even the controls, 250 μl of ethanol was added at the time of elicitation. Methyl jasmonate was first dissolved in ethanol and then added to the cultures. The concentration of ethanol was 81 mM.

6.3.5 Calcium uptake

In contrast with phosphate patterns, in Experiment G, the concentration of calcium in the medium decreased temporarily 8 hours after elicitation, then increased to pre-elicitation concentration before declining to new lows in 51 hours. A decrease in medium calcium means there was an uptake of calcium by the cells from the medium. Figure 6.11 shows the patterns and kinetics of calcium uptake in this experiment. Even controls receiving the ethanol placebo exhibited the same patterns. The decrease in the medium calcium concentration was not observed in Experiment H (Figure 6.10). Statistical analysis showed that neither methyl jasmonate nor ethylene had significant effects on the uptake of calcium from the medium.

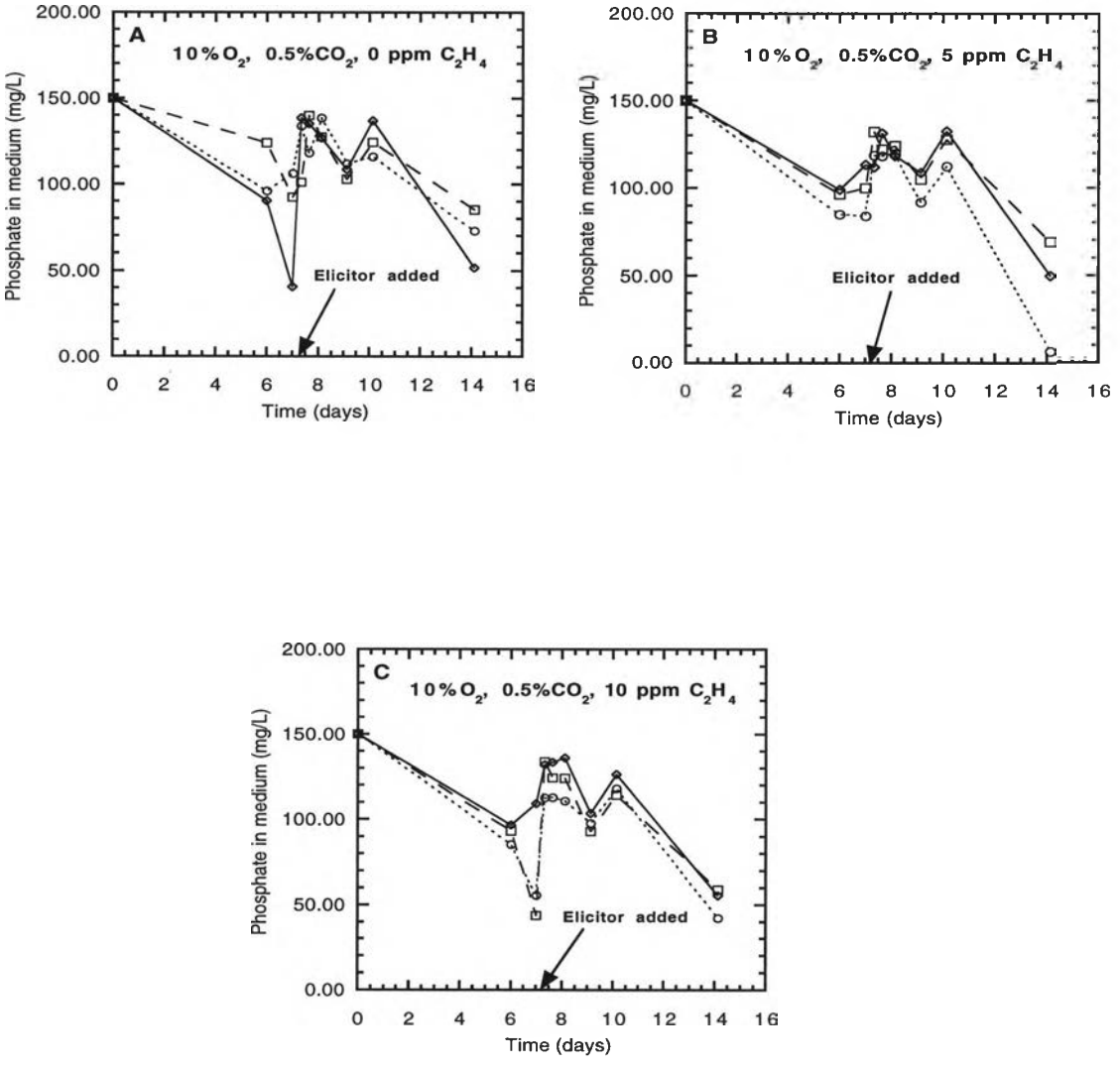

Figure 6.9: Kinetics of phosphate uptake when the TC2 cell cultures were exposed to various concentrations and combinations of ethylene and methyl jasmonate in Experiment G. $\diamond$ = 0 μM MeJA; $\square$ = 10 μM MeJA; $\circ$ = 100 μM MeJA.

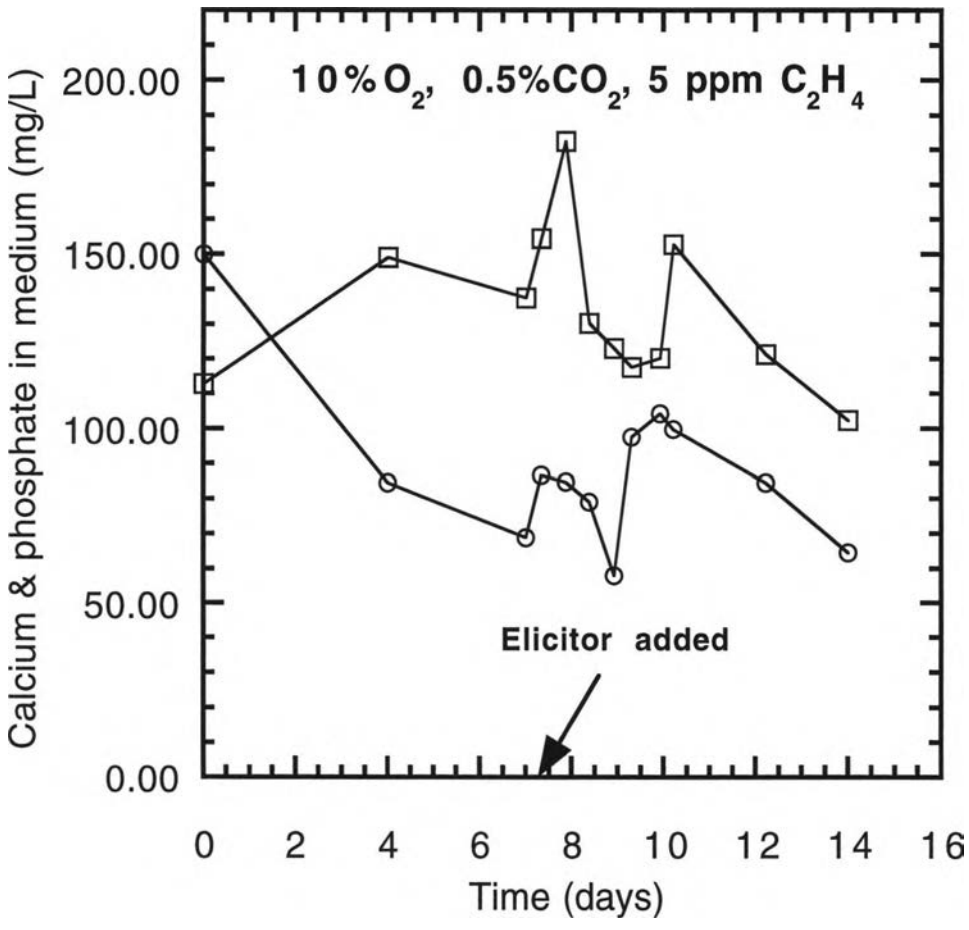

Figure 6.10: Phosphate and calcium uptake patterns in TC2 cultures in Experiment H. □ = calcium; ◇ = phosphate.

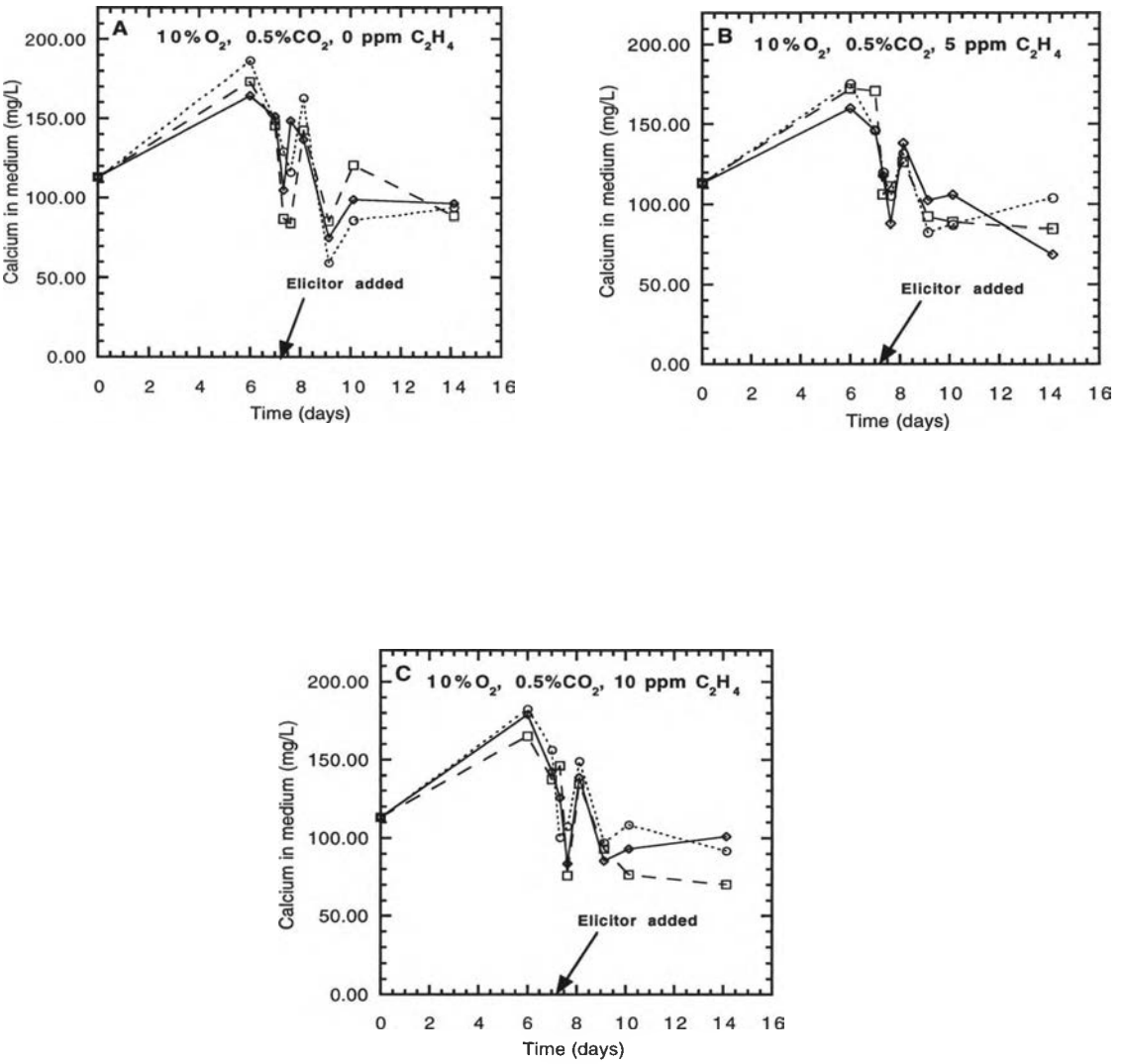

Figure 6.11: Kinetics of calcium uptake when the TC2 cell cultures were exposed to various concentrations and combinations of ethylene and methyl jasmonate in Experiment G. $\diamond$ = 0 μM MeJA; $\square$ = 10 μM MeJA; $\circ$ = 100 μM MeJA.

6.4 Discussion

Our results here are comparable with those of other investigators. For example, a 10-fold increase in rosmarinic acid (RA) content in cultured cells of *L. erythrorhizon* was observed after their exposure to 100 μM MeJA (Mizukami *et al.*, 1993). The 100 μM MeJA also caused a maximal induction of alkaloids in *E. californica* cultures; this maximum was detected within 6 hrs after elicitation; alkaloids continued to accumulate at a linear rate for 136 hours. In the studies by Gundlach *et al.* (1992), of 36 plant cell cultures, methyl jasmonate (0.1–500 μM) induced the secondary metabolites by a factor of 9–30 over the control values. Induction by MeJA was not specific to any one type of secondary metabolite but rather general to a wide spectrum of low molecular weight substances ranging from flavonoids, guaianolides and anthraquinones to various classes of alkaloids. The induced secondary metabolites were accompanied by an increase in PAL poly(A)+ RNA that was followed by an increase in PAL enzyme activity to maximal values 25 and 33 hours after elicitation, respectively (Gundlach *et al.*, 1992).

In another study by Kutchan (1991), methyl jasmonate, yeast cell wall preparation and 12-oxo-phytodienoic acid produced similar patterns of transcript accumulation of the berberine bridge enzyme; maximal levels of transcript occurred 6–8 hours after elicitation with methyl jasmonate concentration of 50 μM.

The combination of ethylene and MeJA (5ppm and 45 μM) induced both osmotin mRNA and protein accumulation to levels nearly as high as those found in salt adapted tobacco cells (Xu *et al.*, 1994). This accumulation of protein occurred within 3 days of the treatment, similar to the time required for protein to accumulate in response to fungal infection and salt adaptation. Bressan's coworkers have found evidence that the combination of ethylene/MeJA has effects

both on osmotin promoter induction and on stabilization of osmotin mRNA (Xu *et al.*, 1994). Although MeJA in combination with ethylene or salicylic acid (SA) both hyperinduced osmotin and PR-1b mRNA accumulation, osmotin protein accumulated to very high levels only when MeJA was combined with ethylene; PR-1b protein did so only when MeJA was combined with SA (Xu *et al.*, 1994). Xu *et al.* (1994) suggested that SA and ethylene exert translational or protein stability effects on PR-1b and osmotin, and the maximal accumulation of more than one defense protein requires multiple signals.

Both simulation and inhibition of ethylene biosynthesis by MeJA have been reported (Saniewski *et al.*, 1987). The ability of MeJA and ethylene to co-regulate the osmotin gene was not a result of any effect of MeJA on ethylene production (Xu *et al.*, 1994), since the ability of MeJA to increase the induction of the osmotin promoter occurs at ethylene concentrations above saturation level of ethylene (5 ppm).

In our study, MeJA induction occurred at all ethylene concentrations including 0 ppm ethylene. The gas mixing apparatus was designed in such a way that the cells were exposed to constant concentrations of oxygen, carbon dioxide and ethylene throughout the experiments. Ethylene synthesized as a result of MeJA addition was flushed out of the cultures. Also if the effects were because of ethylene synthesis as a result of MeJA addition, an effect at 0 ppm and 100 μM MeJA would be expected.

The volumetric phosphate uptake rates by the TC2 cell cultures at various concentrations and combinations of ethylene and methyl jasmonate are presented in Figure 6.12. Phosphate uptake rate decreased as the concentration of MeJA was increased from 0 to 100 μM (Figure 6.12). The highest volumetric phosphate uptake rate was observed when cultures were exposed to 0 ppm ethylene and 0 μM MeJA (13.9mg/L·Day) and the lowest was observed with 5

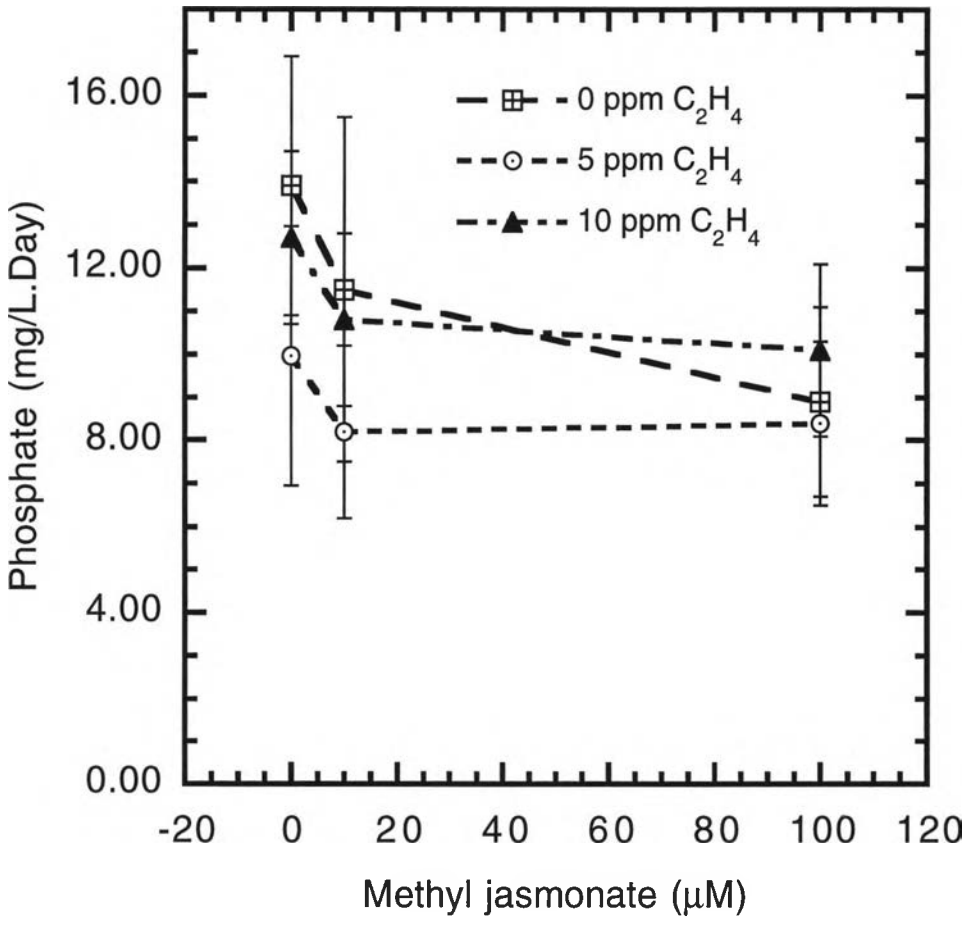

Figure 6.12: Volumetric phosphate uptake rates in Experiments G and H using TC2 cell cultures.

ppm ethylene and 10 μM MeJA (8.2 mg/L·Day). Volumetric taxol production had a maximum at 5 ppm ethylene and 10 μM MeJA (Figure 6.13). The highest phosphate uptake rate correlated with the lowest taxol productivity (compare Figures 6.12 and 6.13), whereas, with TC1 cell cultures, the opposite was true with ethylene alone as the independent variable (Chapter 3). On the basis of such differences, one may conclude that phosphate uptake and taxol productivity are not linked.

Xu *et al.* (1994) suggest the binding of ethylene to its receptors on the plasma membrane might sensitize MeJA receptors on the membrane. Their work with tobacco seedlings shows that continuous presence of ethylene is required

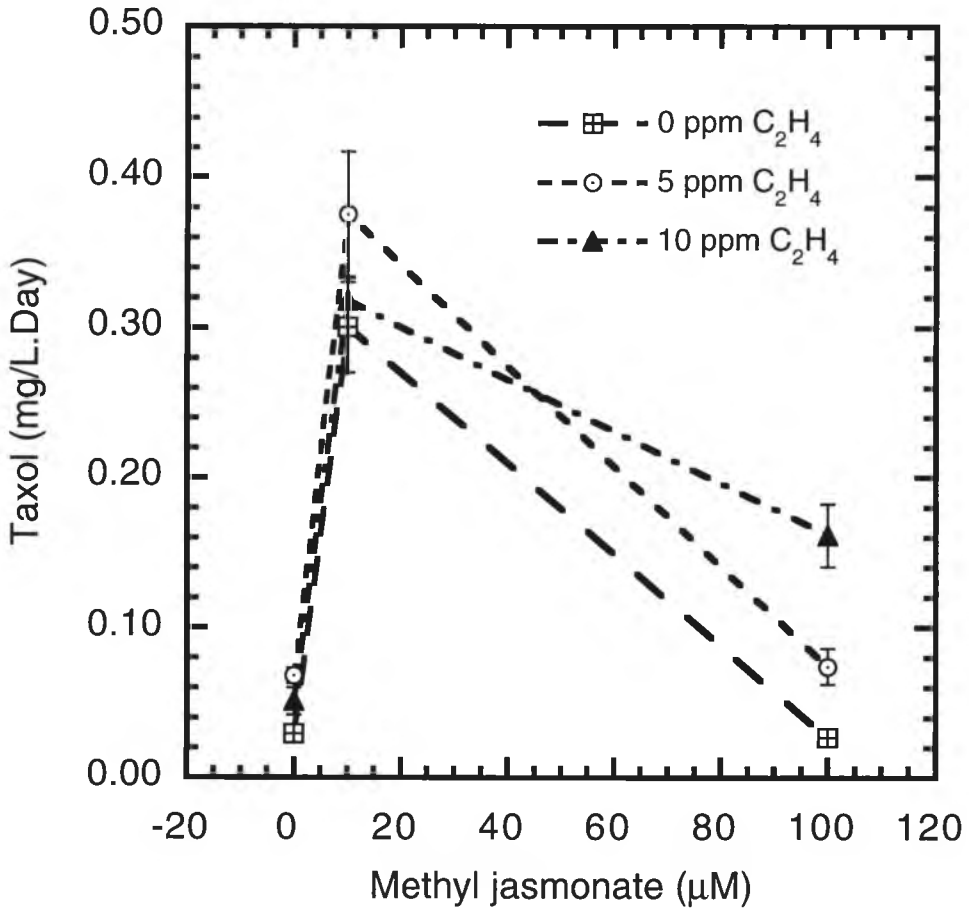

Figure 6.13: Volumetric taxol productivity in in Experiments G and H using TC2 cell cultures.

for methyl jasmonate binding for the synergistic effect of MeJA and ethylene on inducing the osmotin gene, i.e. the continuous binding of ethylene with its receptors might be required or that a short-lived participant in signal transduction is induced which results in inducing the osmotin gene. The observation that the MeJA conditioning causes the parsley cells to become more sensitive to low concentrations of the fungal elicitor suggests an improved cellular signal perception/transduction system (Kauss *et al.*, 1992). The view expressed by Enyedi *et al.* (1992) says that at least two separate systems of induction of resistance exist, one involving pathogens through salicylic acid and another involving wounding through ethylene.

The amount of phytoalexin produced by the cell culture as a result of elicitation often follows saturation behavior (Yoshikawa *et al.*, 1983; Basse *et al.*, 1992). This production is strongly dependent at low elicitor addition and independent of elicitor concentration at high dosages. In our studies, the amount of taxol produced does not follow saturation behavior with respect to elicitor concentration. The response could saturate as a result of three factors: saturation of the enzymatic capacity; saturation of the capacity for resource allocation to the pathway; or saturation of the tissue's ability to perceive the elicitor (Singh *et al.*, 1994). The addition of methyl jasmonate reduced the time in taxol production, hence increasing the productivity. Similar phenomenon was observed by Eilert *et al.* (1987), where accumulation of alkaloids in *Papaver somniferum* cell cultures treated by fungal elicitors did not result in a higher yield of the product, but the time required for alkaloid production was reduced. Therefore, elicitation techniques can be used to reduce the cost of secondary metabolite production from cultured plant cells.

Chapter 7

MODULATION (INDUCTION) MODEL

Removal of regulatory repressors, genetic manipulation of metabolic pathways or the addition of specific metabolic inducers can increase secondary metabolism. It is suggested that the secondary biosynthetic capabilities of the plant cells are repressed in cell culture systems and need a stimulus for expression (DiCosmo and Misawa, 1985). Providing that stimulus to the culture is the basis of a set of techniques which could be used to exploit the biotechnological potential; as such elicitors have been used as a tool to understand the regulation of phenylpropanoid secondary metabolites in plants (Chappell *et al.*, 1984; Hahlbrock and Grisebach, 1979; Ryder *et al.*, 1984; Cramer *et al.*, 1985). Chapter 6 showed the effect of methyl jasmonate and ethylene elicitation on production of taxol. This chapter develops a simple induction/repression model with analogy to the regulation of bacterial metabolism.

7.1 Regulation of developmental processes in plants

The activity of the enzymes present in the cell are under control. Enzyme catalyzed reactions are in accordance with the demands of the cell for energy and for cellular constituents. This control is accomplished by the ability of the cell to increase or to decrease the activity of certain enzymes of the metabolic pathway (Prescott *et al.*, 1993). Compartmentation is one method of

regulation in eukaryotic microorganisms. Compartmentation makes possible the simultaneous, but separate operation and regulation of similar pathways (Fosket, 1994). Pathway activities can be coordinated through regulation of the transport of metabolites and coenzymes between cell compartments.

Expression of the regulatory and effector genes control the developmental processes in plants (Fosket, 1994). Regulatory genes encode for proteins that regulate the expression of the other genes. Effector genes, on other hand, encode for proteins that modify the structure or metabolism of the cell. Many effector genes encode enzymes that are required for a particular biochemical pathway. The regulatory genes are switches that control developmental and biochemical pathways by regulating the expression of genes encoding proteins that make these possible. For example, anthocyanins (members of a class of compounds known as flavonoids) are pigments whose synthesis is regulated during plant development (Fosket, 1994). Anthocyanins are synthesized in response to stresses such as cold, exposure to ultraviolet light, mechanical damage and attack by pathogens (Fosket, 1994). Anthocyanin synthesis is brought about by several enzymes, including phenylalanine ammonia lyase (PAL) and chalcone synthase. Tissues in which the regulatory genes are expressed will synthesize anthocyanin pigments; those that do not express the regulatory genes do not synthesize anthocyanins.

The phenylpropanoids are the most thoroughly studied group of secondary metabolites. Within this group are the lignins and the flavonoids (of which anthocyanin is a member). Hahlbrock and Grisebach (1979) have reported the roles of flavonoids to be protection of the plant from UV light and infection. The timing and appearance of the key enzymes and their mRNAs in the phenylpropanoid pathway, suggest that these pathway enzymes are coordinately induced (Chappell *et al.*, 1984; Ryder *et al.*, 1984; Cramer *et al.*, 1985). Also, induction of transcription has been reported to be very rapid and likely to

be an early event in a defense response (Dixon *et al.*, 1990; Bolwell *et al.*, 1985; Robbins *et al.*, 1985). Robbins *et al.* (1985) observed that the elicitor treatments resulted in the selective induction of enzymes involved in phytoalexin synthesis. Activities of the other enzymes not involved in phenylpropanoid metabolism were not induced by elicitor treatment.

Chappell *et al.* (1984) reported that transcriptional regulation governs the timing and appearance of phenylpropanoid metabolites and enzyme levels may in some cases be controlling the pathway flux. There is evidence in the literature that primary and secondary metabolism compete for a common pool of precursors and that the rate limitation to phenylpropanoid production is the precursor supply (Chappell *et al.* (1984) and the references therein). Intracellular physiological signals such as adenylates and/or energy charge may also be regulating the phenylpropanoid metabolites (Chappell *et al.*, 1984). Cinnamic acid has been reported to act as a feedback regulator by reducing the induced synthesis of PAL and by enhancing PAL inactivation reactions (Bolwell *et al.*, 1985).

Due to the limited understanding of the metabolic pathways in secondary production in plants, optimum elicitor types and dosage have been found by conducting dose response experiments. In these experiments, plant cells are exposed to various types and concentrations of elicitors. These types of experiments are both time consuming and costly.

7.2 Example: The *lac* operon system in bacteria

Many important aspects of the regulation of gene expression are different in eukaryotes and prokaryotes. Nevertheless, it is instructive to consider how this process is regulated in bacteria. One of the most studied examples of the negative control of gene expression in bacteria is the regulation of the transcription

of genes involved in lactose metabolism in *E. coli.* Most of the genes that must be expressed for lactose utilization are part of the *lac* operon (Prescott *et al.*, 1993). In the presence of lactose and the absence of glucose, the *lac* operon is expressed, leading to the synthesis of the enzyme β-galactosidase. This enzyme breaks the bond between glucose and galactose in the lactose molecule, enabling the bacterium to use these sugars for its metabolism. The enzyme β-galactosidase is an inducible enzyme; that is, its level rises in the presence of molecules called inducers. Enzymes whose amount is reduced by the presence of an end product are repressible enzymes. Repressible enzymes are necessary for synthesis and are always present unless the end product of their pathway is available. In contrast to repressible enzymes, inducible enzymes are required only when their substrate is available; i.e., they are missing in the absence of the inducer (Prescott *et al.*, 1993). Induction and repression results from changes in the rate of transcription, and control of gene expression complements the regulation of enzyme activity.

Transcription of the *lac* operon is regulated by two DNA binding proteins, the *lac* repressor and the catabolite activator protein. The *lac* repressor is a negative regulator of transcription. It turns off transcription by binding to a specific DNA sequence, known as the operator, blocking the access of RNA polymerase to the operon (Prescott *et al.*, 1993). The binding of the *lac* repressor protein is regulated by inducers. The inducers initiate gene expression by binding directly to the *lac* repressor. The binding of the inducer to the *lac* repressor brings about a conformational change in its structure so that its DNA binding site is not exposed to the surface of the protein. The *lac* operon is also regulated by the catabolite activator protein (CAP). If CAP is bound to a site near the RNA polymerase binding site, it strengthens polymerase binding to the *lac* promoter and ensures *lac* operon transcription (Prescott *et al.*, 1993). CAP is a positive regulator of *lac* operon transcription, and can bind to the CAP binding

site in the absence of glucose. CAP has influence on transcription when cyclic AMP has bound to it; it then aquires the ability to bind to very specific sites on DNA. Cyclic AMP (cAMP) is required for the transcription of all the operons that are inhibited by glucose which is also known as the catabolite repression (Prescott *et al.*, 1993).

7.3 An induction model for the taxol system

Adjustment of the activity of regulatory enzymes controls the functioning of many metabolic pathways. The activity of regulatory enzymes can be changed by a small molecule called an effector or modulator. The effector binds reversibly by non-covalent forces to a regulatory site and causes a change in the shape or conformation of the enzyme (Prescott *et al.*, 1993). A positive effector increases the activity of the enzyme, whereas a negative effector decreases or inhibits enzyme activity. The changes in activity result from alterations in the affinity of the enzyme for its substrate (Prescott *et al.*, 1993).

As an analogy to the derivation of an expression for the regulation of the *lac* operon by Yagil and Yagil (1971), the relationships between the rate of taxol production, the rate of phosphate uptake and the effector concentration, F, were evaluated. The following expression was derived from equilibrium analysis of binding repressor molecules to the operator of the *lac* operon. In our analysis, the repressor is called a modulator and the basal level of taxol productivity (or phosphate uptake) is denoted as β_b, and β is the response at any given concentration of F.

The two equations for inducible systems are (Yagil and Yagil, 1971):

$$nF + R \overset{K_1}{\rightleftharpoons} RF_n \tag{7.1}$$

$$O + R \overset{K_2}{\rightleftharpoons} OR \tag{7.2}$$

Where F is an effector molecule (inducer or repressor); R is an unbound modulator molecule in the cell; RF_n is a modulator molecule which binds n effector molecules; O is an operator free to be transcribed and OR is an operator which is binding a modulator. $[O_T]$ is the total number of operators in a cell. If the fraction of modulators partially binding the effector is small, then $[R_T]$ is the total concentration of the modulator:

$$[O_T] = [O] + [OR] \tag{7.3}$$

$$[R_T] = [R] + [RF_n] \tag{7.4}$$

The two equilibrium constants are:

$$K_1 = \frac{[R][F]^n}{[RF_n]}$$

and

$$K_2 = \frac{[O][R]}{[OR]}$$

Re-arranging the above equations with the assumptions that the fraction of free operators in a population is $\beta = [O]/[O_T]$ and the fact that when the concentration of effector [F] is zero (repression is maximal), then the enzyme is synthesized at the basal rate, the following equation can be derived (Yagil and Yagil, 1971).

$$log(\frac{\beta}{1-\beta} - \beta_b) = \pm n log[F] + log\beta \pm logK_1 \tag{7.5}$$

This equation allows linear plotting of experimental data and the evaluation of two quantities: n, the number of effector molecules combining with a modulator molecule and K_1, the dissociation constant of this interaction. A positive slope indicates that the binding of effector molecules with modulator molecules induces the observed response; whereas a negative slope suggests that binding of effector molecules interacts with modulator molecules to cause repression of the response (Yagil and Yagil, 1971).

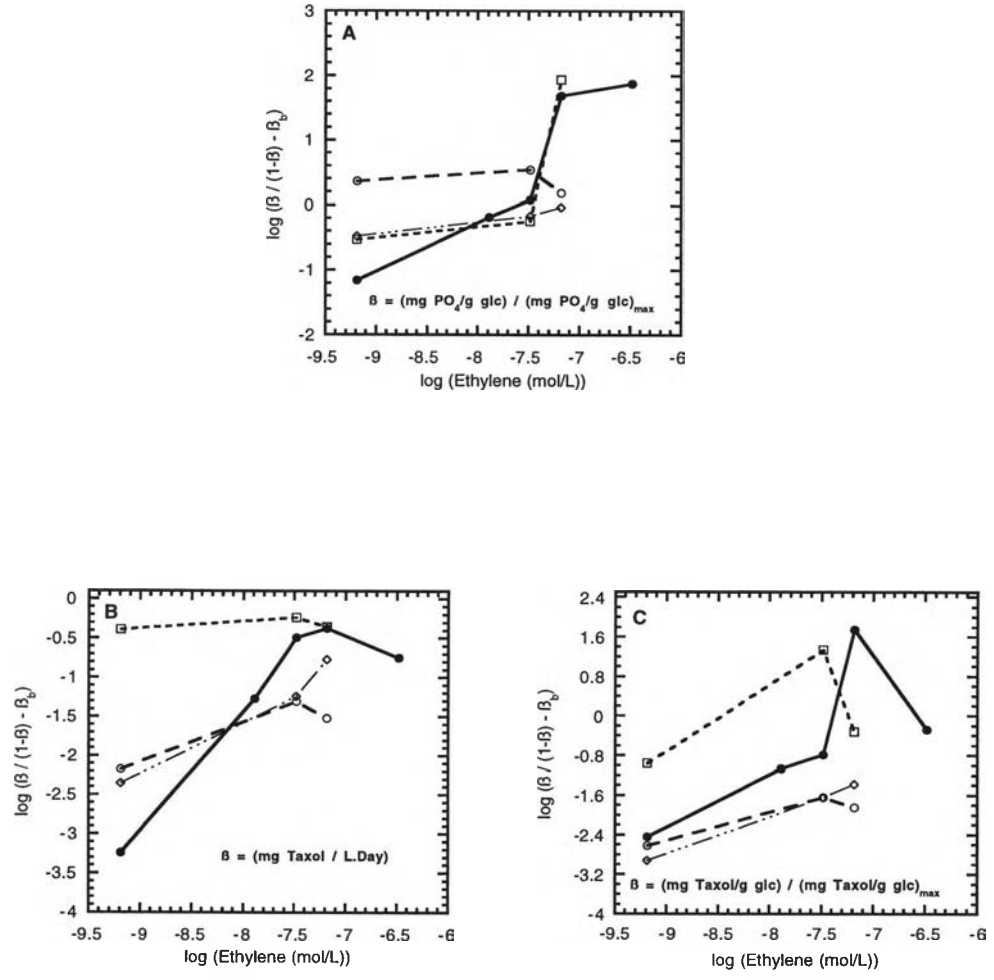

Figure 7.1: The effect of ethylene and methyl jasmonate on taxol productivity and phosphate uptake rates based on an expression from equilibrium analysis of binding repressor molecules to the operator derived by Yagil and Yagil (1971). • = 0 μ M MeJA, 0 ethanol; ○ = 0 μ M MeJA, 81 mM ethanol; □ = 10 μ M MeJA, 81 mM ethanol; ◇ = 100 M MeJA, 81 mM ethanol.

The effect of ethylene and methyl jasmonate on taxol productivity and phosphate uptake rates are shown in Figure 7.1. As mentioned in Chapter 2, MeJA was dissolved in ethanol and 250 μl ethanol was added to all cultures. This volume of 95% ethanol in 50 ml of cultured cells corresponded to 81 mM ethanol. This concentration of ethanol had an effect on specific phosphate uptake at 5 and 50 ppm ethylene (Figure 7.1A, compare • and o); whereas, there is no effect of ethanol on the volumetric and specific productivities of taxol (Figure 7.1, B & C). Methyl jasmonate at concentrations of 0 and 100 μM had positive effects on specific taxol at all the ethylene concentrations; however, 10 μM methyl jasmonate had a positive effect at 0 and 5 ppm ethylene and a negative effect between 5 and 10 ppm ethylene. In comparison, 0 and 100 μM methyl jasmonate did not have significant effects on uptake of phosphate, but 10 μM had a positive effect at the higher concentration of ethylene (10 ppm). Hence, it appears that the effector, MeJA, may act both as inducer and repressor, or according to the definitions above, as a modulator of gene product expression. This phenomenon has also been observed with other elicitors. For example, specific fungal molecules can either direct the plant cell metabolism to either synthesize or cause suppression of secondary metabolite production (DiCosmo and Misawa, 1985). Similar observation has been made with cellular expression in a bacterial system (Linden and Shiang, 1991). Not all receptor binding events will elicit a cellular secondary metabolic response.

7.4 Discussion

Methyl jasmonate, a linoleic acid-derived molecule, can act as a volatile signal that induces the accumulation of proteinase inhibitor proteins to even higher levels than can be induced by wounding (Farmer and Ryan, 1990). The concentration of jasmonic acid and/or methyl jasmonate in different tissues and

species are typical of plant hormones, ranging from 10 ng to 3 μg/g fresh weight (Staswick, 1992).

The mechanism by which oligosaccharide elicitors (chitosan, β-glucan, homogalacturonan) activate the proteinase inhibitor genes is not known, but oligosaccharide elicitors have been found associated with membrane receptors and changes in phosphorylation patterns of membrane proteins (Hahn, 1995). There is evidence which shows that the mechanism of elicitation can, in some cases, operate at the level of gene expression in the cell (Gundlach *et al.*, 1992; Mizukami *et al.*, 1993; Kutchan, 1991). The results presented in Chapter 6 indicate that treatment of cultured plant cells with elicitors rapidly altered gene expression for secondary metabolite synthesis. Therefore, production of taxol in cell suspension cultures of *T. cuspidata* may be regulated to some extent by exogenous methyl jasmonate and ethylene levels.

Dixon *et al.* (1990) proposed a model for the induction of plant defense responses. This model assumes that the elicitor binds to a specific receptor, probably located in the plant plasma membrane, and that this binding indirectly leads to changes in the transcriptional activity of genes involved in the production of antimicrobial agents by the host. The evidence for the presence of specific high affinity elicitor binding sites in the soybean plasma membrane has been shown by Cosio *et al.* (1990) and Schmidt and Ebel (1987). Researchers are studying the signal chain between the elicitor-receptor complex and the gene activation process (Dittrich *et al.*, 1992; Cosio *et al.*, 1990; Chappell and Hahlbrock, 1984; Brooks and Watson, 1991). It is suggested that protein phosphorylation of cultured plant cells is affected by fungal elicitor treatments (Dietrich *et al.*, 1990). Farmer and Ryan (1990) suggest that these signals are either transported locally by diffusion through intercellular and extracellular fluids or infection sites or systemically through the vascular system of the plants.

Gundlach *et al.* (1992) have proposed a hypothetical mechanism of action for MeJA: An elicitor-receptor complex activates a lipase releasing α-linolenic acid, which is then transformed by constitutive enzymes to jasmonic acid and methyl jasmonate and activates, in different plant systems, "jasmonate-induced" proteins. A multitude of species-specific genes involved in the formation of high and low molecular weight compounds are expressed in response to these signal transducer molecules (Mueller-Uri *et al.*, 1988). Creelman *et al.* (1992) suggested that cell wall fragments are involved in the rapid induction of the defense genes, whereas MeJA acts later in this process. Other evidence that MeJA acts at the level of transcription is reported by Tamari *et al.* (1995).

It is possible that different elicitor signals induce different parts of the defense response (Grosskope *et al.*, 1991). Particular combinations of signal molecules produce a specific "signature" set of inducers that initiate a specific type of environmental protection; the occurrence of "cross-talk" between signaling molecules can be explained by an interactive signal transduction system (Xu *et al.*, 1994). Ethylene and MeJA may represent "cross-talk" signaling in plants.

Chapter 8

CONCLUSIONS

8.1 Recapitulation

Products of plant origin include an impressive list of pharmaceuticals, food colors and flavors, and agricultural chemicals. Despite their importance, there are often difficulties in securing adequate supplies of many plant derived products. Plant cell culture offers advantages of assured and expandable supply, in comparison with reliance on plants that are rare, and produce the desired compound on a seasonal basis. Taxol, an extractive of *Taxus brevifolia* has shown strong anticancer activity. Although the mechanism of action of taxol is known (Horwitz *et al.*, 1981), there is a lack of understanding about taxol biosynthesis and plant physiology affecting taxol production. In an effort to produce taxol from cell cultures of *Taxus cuspidata*, the effect of gas phase compositions and elicitation on growth, sugar utilization, calcium and phosphate uptake and production of taxol were studied. The success of our system was embedded in the ability of controlling the concentration of the gases of interest in an independent manner, and without altering other parameters important in plant cell culture systems.

The gas phase composition influences the timing and rate of taxol production in *Taxus cuspidata* cultures as well as patterns of nutrient utilization. Below-ambient oxygen and above-ambient carbon dioxide and ethylene concentrations are favorable for taxol production. The most effective combination of

gas phase compositions tested were 10% (v/v) oxygen, 0.5% (v/v) carbon dioxide and 5 ppm ethylene. These cultures produced 6.5 mg/L of taxol in 21 days. The addition of fructose (10 g/L) on day 11 improved taxol concentration to 12.2 mg/L. Maximum taxol concentrations in the culture medium correlated with low rates of calcium uptake, high rates of phosphate uptake and adequate fructose for energy production.

The effect of elicitation on taxol production was studied by exposing suspension cultures of *Taxus cuspidata* to various concentrations and combinations of methyl jasmonate and ethylene. A dose dependent response was observed for methyl jasmonate and ethylene. Taxol productivity increased 15–19 fold when *Taxus cuspidata* suspension cultures were exposed to 10 μM methyl jasmonate and all ethylene concentrations. Responses using 100 μM methyl jasmonate were highly dependent on ethylene concentration; these changes occurred within 2 days after elicitation. The growth rate of the cultures increased as methyl jasmonate concentration increased and was independent of ethylene concentration. Ethylene and methyl jasmonate had synergistic effects on reducing the time of taxol production and hence, increasing taxol productivity.

Table 8.1 shows the maximum taxol concentration, volumetric taxol productivity and specific taxol productivity obtained using the different treatments studied in this dissertation. The highest taxol production (12.2 mg/L) and the highest volumetric productivity (0.49 mg/L day) of taxol were obtained in cultures to which 10 g/L fructose was added (head space concentrations of 10% O_2, 0.5% CO_2 and 5 ppm C_2H_4). However, the highest specific taxol productivity (6.7 mg/g glucose consumed) was obtained in cultures grown under 5 ppm ethylene and induced with 10 μM methyl jasmonate (head space concentrations of 10% O_2, 0.5% CO_2). This was an evidence of inducing the biosynthetic pathway of taxol in the cells.

Table 8.1: Taxol concentration and productivity at the various treatments.

Treatment $O_2/CO_2/C_2H_4$	Taxol (mg/L)	Taxol (mg/L·day)	Taxol (mg/g Glc_c)
10/0.5/2	0.5	0.02	0.09
10/0.5/2	3.5	0.07	0.25
10/0.5/5	6.5	0.31	2.06
10/0.5/5 + F	12.2	0.49	1.2
10/0.5/10	6.5	0.31	2.06
10/0.5/50	0.5	0.17	0.76
10/0.5/0 + 0 μM MeJA	0.27	0.03	0.06
10/0.5/0 + 10 μM MeJA	2.74	0.30	0.72
10/0.5/0 + 100 μM MeJA	0.25	0.03	0.05
10/0.5/5 + 0 μM MeJA	0.5	0.07	0.19
10/0.5/5 + 10 μM MeJA	3.4	0.38	6.7
10/0.5/5 + 100 μM MeJA	1.05	0.07	0.19
10/0.5/10 + 0 μM MeJA	0.38	0.05	0.14
10/0.5/10 + 10 μM MeJA	2.9	0.32	2.27
10/0.5/10 + 100 μM MeJA	2.3	0.16	0.32

Ethylene ($H_2C{=}CH_2$) is a plant hormone that can affect various physiological process in plants. It has been known for a long time that ethylene gas can modify the growth of plants. The mechanism of action of ethylene is not well known. In an effort to better understand the role of ethylene involving the signal transduction in plants and secondary metabolite production, indirect and direct binding theories were reviewed. Ethylene does not have an inhibitory effect on specific phosphate uptake rate; phosphate uptake rate demonstrates saturation kinetics. Whereas high concentrations of ethylene have inhibitory effect on production of taxol, for which the reasons are not understood. In both cases (taxol productivity and phosphate uptake rate), the indirect binding of ethylene to a putative receptor, fits the experimental data better than the direct binding model. Ethylene action is closely related to the two-component regulatory systems in bacteria.

The pathways regulating plant defense mechanisms are closely related to those that induce secondary metabolism. Elicitors often simultaneously induce pathogenesis related proteins as well as enzymes of secondary metabolism (Seifert *et al.*, 1994; Lindsay *et al.*, 1993; Xu *et al.*, 1994). The synergistic effect of methyl jasmonate and ethylene was observed with regard to taxol production and phosphate uptake rates. However, the two compounds have at least partially separate signal transduction pathways. Both methyl jasmonate and ethylene can act as inducers and inhibitors of taxol production in *Taxus cuspidata* cell cultures.

The feasibility of producing taxol in cultured plant cells was demonstrated here. The gas mixing apparatus used in this study is applicable to large scale production systems. Plant cell culture systems can also be important in understanding the biosynthesis pathways for the novel plant metabolites, leading to the discovery of new compounds.

8.2 Shortcomings

The limitations of this dissertation are numerous. The simple approach taken in this study allowed a clear interpretation and understanding, and was thus preferable to a more complex, but potentially uninterpretable one (for instance, investigating taxol production in bioreactors). The characterization of head space gases, and their interactions, while revealing a number of important issues, fell short of being complete.

Within the task studied, a number of limitations can be put forward. The factor oxygen was studied in the simplest form: Only two experiments were presented studying various levels of oxygen on taxol production and its physiological relations in the cell culture system. A more thorough study with oxygen at four levels of 25, 15, 10, and 5% (v/v) will reveal detailed effect(s) of oxygen on growth and taxol production.

The concentration of carbon dioxide resulting in the highest taxol production in this study was 0.5% (v/v). While 10% (v/v) carbon dioxide was inhibitory to taxol production, this factor was not optimized. Concentrations of carbon dioxide, in the range of 0.5% (v/v) to 2% (v/v), are to be studied to fully understand the effect of this gas and its interaction with ethylene on growth and taxol production. The results of gas studies can be used to model the system; on-line measurement and dynamic control of the head space gases, can result in higher taxol productivities.

8.3 Impact of this dissertation on some related issues

Plant cell cultures have the potential of producing valuable secondary metabolites. Currently a methodology for rapid development of plant cell cultures for secondary metabolite production does not exist. The experience and knowledge gained in this project on taxol production is valuable for development of a general methodology. The production of secondary metabolites from plant cell culture, requires an interdiciplinary team as was demonstrated in this study. The importance of combining engineering basics with the knowledge of metabolic behavior of the cells is the key to large scale production of secondary metabolites. The successful production of taxol, an important anticancer agent, from plant cells is encouraging for production of other plant metabolites and will motivate other researchers to this task.

8.4 Further directions

The supply problems of taxol demonstrate the need for large scale processes. Although the crisis in the supply of taxol has eased and it is commercially available, controllable alternative sources are still needed (Ellis, 1995). Due to the differences of plant cells with bacterial systems, special care and attention is

needed for large scale production of novel compounds using this method. Cell line selection and stability is one of the most important factors. More research should be toward environmental effects on plant cell growth and production. One of the biggest problems encountered in this study was the inherent instability in production capacity of the cell lines used. This is a problem that has been encountered in other cell lines as well (Dougall, 1987). This instability can be due to genetic (loss of gene expression), epigenetic (a stable but reversible change in phenotype), or environmental factors (temperature, light, etc.) (Dougall, 1987). The cycling phenomena of high to low to high taxol production has been noted in our work (Hirasuna *et al.*, 1994; Mirjalili and Linden, 1995b). The environmental changes or metabolic conditions causing the observed changes in taxol production need to be defined more extensively. The relationship between environmental factors and genetic and epigenetic factors on taxol production should be studied.

In order to increase production of secondary metabolites, a knowledge of the biosynthetic pathway of the product is desirable. It is especially important to understand the inhibitory or induction effects of various compounds on the product formation. Increasing fermentation yields requires redirecting the cellular metabolism in favor of the desired product as opposed to biomass or other side-products. It is very useful to have some knowledge of the biochemical reactions of critical metabolic pathways. The factors causing the shift from one pathway to another could be influenced by the availability and demand of ATP and reduction energies, the rate of substrate uptake, intracellular pH and oxygen supply, to name a few.

Using bioreactors will enable determination of several fermentation parameters, such as effects of shear stress and analysis of the respiratory quotient. Controlled feeding of a culture with carbon substrates and other nutrients such

as calcium and phosphate is facilitated in bioreactors. Fermentation exhaust gas analysis, using process mass spectroscopy, can be used as an on-line method for following cellular metabolism. By analyzing the composition of the inlet and outlet gas, carbon dioxide evolution rate, oxygen uptake rate, and hence the respiratory quotient (RQ) can be determined. The respiratory quotient can then be correlated to sugar consumption rate, protein synthesis, and nitrate assimilation, indicating the existence of shifts in metabolism.

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Appendix A

EXPERIMENTAL DATA

The data for Experiments A–C for which the cell line TC1 was used, are presented in this appendix. The data for Experiments D–H are presented in Appendix B. The statistical regression models based on the data are presented in Appendix C. The headings in the data set tables are in accord with the roman letters assigned to each treatment. Hence, the repeated treatments in various experiments are assigned one unique roman letter.

A.1 Experiment A

The experimental design for Experiment A is shown in Table A.1.

Table A.1: Experimental design for Experiment A.

Treatments	O_2%(v/v)	CO_2%(v/v)	C_2H_4(ppm)	Comments
i	10	10	2	–
ii	10	10	0	–
iii	10	0.5	2	–
iv	10	0.5	0	–
v	21	0.03	0	humidified air

Table A.2: Taxol data for Experiment A.

	day	iii	SD	iv	SD	i	SD	ii	SD	v	SD
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	6.00	3.58	0.330	3.12	0.680	2.84	0.350	0.680	0.150	0.00	0.00
2	13.0	1.33	0.220	1.48	0.450	1.80	0.450	1.55	0.320	1.10	0.320
3	20.0	1.65	0.350	0.630	0.220	1.35	0.360	0.530	0.110	1.74	0.350
4	28.0	1.29	0.550	0.530	0.210	1.33	0.340	0.210	0.100		

Table A.3: Glucose data for Experiment A.

	days	iii	SD	iv	SD	i	SD	ii	SD	v	SD
0	0	0	0	0	0	0	0	0	0	0	0
1	6	5.99	0.9	5.5	0.6	6	0.5	4.87	0.6	4.9	0.8
2	13	4.63	0.5	6.18	1.3	5.45	0.7	6.25	0.8	3.87	0.5
3	20	4.56	1.2	5.6	0.8	3.88	0.8	1.37	0.5	0.14	0
4	28	2.3	0.5	6.09	1.2	2.06	0.65	0	0	0.15	0

Table A.4: Fructose data for Experiment A.

	days	iii	SD	iv	SD	i	SD	ii	SD	v	SD
0	0	0	0	0	0	0	0	0	0	0	0
1	6	7.68	0.5	7.1	0.9	7.6	0.88	6.3	0.69	6.3	0.69
2	13	8.4	0.9	7.8	0.8	8.3	0.59	9.1	0.55	7.2	1.3
3	20	8.76	0.8	7.3	1.3	6.5	0.87	4.1	0.85	4.4	0.89
4	28	7	1.2	5.1	1.2	4.7	0.56	3.5	1.1	2.3	0.56

Table A.5: Calcium data for Experiment A.

	days	iii	SD	iv	SD	i	SD	ii	SD	v	SD
0	0	113	0	113	0	113	0	113	0	113	0
1	6	90.2	5.1	75.8	12.4	75.4	4.4	91.5	2.8	46.6	1.5
2	13	66.7	13.1	65.3	11.6	91.5	6.3	65.3	5.2	97.5	12.9
3	20	46.4	3.6	60.3	15.7	57.3	4.4	38.2	5.9	53.3	11.2
4	28	37.6	6.3	41.8	15.2	50.6	9.1	33.4	11.8	28.3	5.5

Table A.6: Phosphate data for Experiment A.

	days	iii	SD	iv	SD	i	SD	ii	SD	v	SD
0	0	150	0	150	0	150	0	150	0	150	0
1	6	101	9	105	11.2	115	8.2	95.4	19.1	99.7	8.2
2	13	93.5	6.5	112	10.1	95.2	11.7	107	19.7	85.6	4.4
3	20	82.5	4.5	118	18.7	101	1.8	98.9	12.8	62.8	11.2
4	28	80.8	15.7	112	10.2	99.9	3.2	103	18.4	61.9	7.1

Table A.7: DNA data for Experiment A.

	days	iii	SD	iv	SD	i	SD	ii	SD	v	SD
0	0	4.55	1	3.98	0.8	4.325	0.88	4.3	0.91	4.2	0.65
1	6	14.29	3.1	14.7	1.5	14.13	0.9	12.3	1.1	16.2	1.5
2	13	18.7	2.2	14.29	1.1	15.1	1.2	13.1	0.7	17.1	2.3
3	20	8.74	0.68	4.94	0.6	9.2	0.81	5.2	0.78	10.3	0.91
4	28	4.31	0.55	2.24	0.3	4.3	0.45	3.9	0.36	4.23	0.15

A.2 Experiment B

The experimental design for Experiment B is shown in Table A.8.

Table A.8: Experimental design for Experiment B.

Treatments	O_2%(v/v)	CO_2%(v/v)	C_2H_4(ppm)	Comments
vi	10	0.5	5	–
i	10	0.5	2	–
vii	10	0.1	5	–
viii	10	0.1	2	–
v	21	0.03	0	humidified air
ix	21	0.03	0	250 ml Bellco capped
x	21	0.03	0	125 ml Bellco capped

Table A.9: Taxol data for Experiment B.

	Days	vi	SD	i	SD	vii	SD	viii	SD	x	SD	ix	SD	v	SD
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	7	2.88	0.25	3.73	0.23	1.57	0.11	1.56	0.15	0.815	0.15	0.55	0.16	0.56	0.22
3	11	3.16	0.21	1.69	0.15	1.07	0.18	0.89	0.07	1.54	0.25	1.5	0.34	1.07	0.32
4	14	3.42	0.21	1.5	0.15	1.25	0.15	0.97	0.08	2.26	0.31	2.17	0.41	1.42	0.35
5	18	4.96	0.33	1.36	0.13	1.31	0.11	0.58	0.05	2.2	0.33	2.3	0.35	1.94	0.41
6	21	6.57	0.33	1.73	0.15	1.35	0.11	0.59	0.05	2.55	0.41	2.59	0.25	1.74	0.32
7	25	5.93	0.36	1.58	0.14	1.21	0.14	0.22	0.05	3.25	0.36			1.62	0.25
8	28	1.67	0.15	1.24	0.17	1.13	0.12	0.18	0.05	2	0.33				

Table A.10: Glucose data for Experiment B.

	vi	SD	i	SD	vii	SD	viii	SD	x	SD	ix	SD	v	SD
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	7.09	0.350	7.54	0.450	6.78	0.360	6.62	0.330	7.34	0.440	7.24	0.420	6.92	0.360
2	3.24	0.220	2.58	0.330	1.56	0.330	2.52	0.410	7.85	0.410	8.43	0.410	7.92	0.450
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.99	0.350	5.56	0.330	3.92	0.330
4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.25	0.220	1.62	0.220	1.12	0.120
5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table A.11: Fructose data for Experiment B.

	vi	SD	i	SD	vii	SD	viii	SD	x	SD	ix	SD	v	SD
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	11.4	0.610	12.2	0.610	11.5	0.550	11.2	0.660	12.3	0.560	10.4	0.580	10.1	0.620
2	10.1	0.550	11.4	0.660	10.5	0.620	10.2	0.620	11.4	0.620	10.5	0.540	9.93	0.440
3	7.29	0.410	3.03	0.510	1.64	0.410	4.79	0.510	11.2	0.510	9.81	0.360	9.03	0.520
4	5.68	0.360	1.22	0.330	0.212	0.150	2.12	0.330	3.56	0.410	5.24	0.220	4.26	0.360
5	2.34	0.330	0.00	0.00	0.00	0.00	0.560	0.150	1.21	0.220	2.24	0.00	2.21	0.380
6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.612	0.150
7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			0.00	0.00
8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			0.00	0.00

Table A.12: Calcium data for Experiment B.

	days	i	SD	vi	SD	viii	SD	vii	SD	ix	SD	x	SD	v	SD
0	0	113		113		113		113		113		113		113	
1	3	97.8	5.05	112	11.2	90.2	4.38	102	2.83	106	2.23	105	7.97	93.2	2.60
2	7	95.5	5.12	108	11.6	85.0	5.68	87.7	5.24	92.5	9.57	88.0	3.19	60.9	4.20
3	11	78.9	8.20	98.5	10.6	67.7	6.31	85.7	5.99	105	1.59	106	6.38	94.8	5.60
4	14	74.4	3.64	85.0	5.63	63.2	5.63	61.7	5.63	99.3	3.19	103	5.68	60.2	2.25
5	18	67.7	6.20	54.1	5.80	48.1	4.45	57.2	4.23	85.7	3.19	95.9	4.78	42.0	3.44
6	21	57.9	5.06	52.6	8.31	40.2	5.23	51.1	3.36	82.3	1.59	97.0	3.19	35.2	4.69
7	25	37.6	3.21	37.3	6.30	39.3	5.14	24.3	2.25	60.2		56.0	5.65	30.2	6.51
8	28	27.3	3.30	32.2	5.60	26.4	6.32	21.2	3.46	41.5		38.0	2.82	20.2	1.15

Table A.13: Phosphate data for Experiment B.

	vi	SD	i	SD	vii	SD	viii	SD	x	SD	ix	SD	v	SD
0	150	0.00	150	0.00	150	0.00	150	0.00	150	0.00	150	0.00	150	0.00
1	103	10.7	110	8.20	54.3	0.630	53.0	0.760	80.9	0.330	74.5	0.330	66.2	1.30
2	93.8	3.11	95.8	4.40	9.86	0.850	0.447	0.130	112	7.05	120	4.36	112	2.50
3	76.1	4.95	81.2	2.80	2.38	0.110	0.724	0.110	59.7	0.330	66.2	0.672	75.3	0.380
4	49.8	9.54	75.6	3.50	0.724	0.110	0.328	0.180	1.44	0.00	1.67	0.168	9.79	0.470
5	29.3	1.65	49.5	3.30	2.55	0.0600	3.93	0.110	2.39	0.330	3.16	0.250	0.526	0.0600
6	0.00	0.00	32.2	2.30	1.67	0.230	2.78	0.180	0.784	0.0800	0.843	0.167	0.447	0.0600
7	0.00	0.00	10.2	1.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table A.14: DNA data for Experiment B.

	days	vi	SD	i	SD	viii	SD	vii	SD	v	SD	x	SD	ix	SD
0	0	4.28	0.550	4.25	0.550	4.28	0.5	4.28	0.35	4.28	0.46	4.28	1.1	4.28	0.62
1	3	5.49	0.350	4.32	1.30	4.41	0.6	5.87	0.85	7.45	0.11	4	0.55	5.06	0.89
2	7	11.3	1.20	12.2	1.23	5.36	0.7	17.4	2.2	13.5	1.1	7.13	3.2	9.52	1.1
3	11	30.7	2.50	16.4	1.16	18.3	0.3	14.3	1.2	29.5	2.1	45.7	3.5	9.15	1.1
4	14	21.1	1.60	16.5	1.15	27.2	2.5	12.7	0.55	23.9	2.5	21.9	2.2	15.8	2.3
5	18	15.2	0.690	9.05	1.10	11	1.1	8.25	0.36	13.3	0.69	13.2	1.1	6.51	0.36
6	21	12.1	0.550	6.72	1.00	10.1	0.8	8.1	0.63	10.1	0.58	14.6	1.1		
7	25	11.9	0.450	6.63	0.250	7.75	0.5	4.77	0.55	6.32	0.66	6.23	0.36		
8	28	8.25	0.520												

A.3 Experiment C

The experimental design for Experiment C is shown in Table A.15.

Table A.15: Experimental design for Experiment C.

Treatments	O_2%(v/v)	CO_2%(v/v)	C_2H_4(ppm)	Comments
vi	10	0.5	5	–
xi	10	0.5	10	–
xii	10	0.5	5	1% fructose on day 11
ix	21	0.03	0	250 ml Bellco capped
x	21	0.03	0	125 ml Bellco capped

Table A.16: Taxol data for Experiment C.

	days	vi	SD	xii	SD	xi	SD	x	SD	ix	SD
0	0	0	0	0	0	0	0	0	0	0	0
1	3	0.0098		0.112	0.001	0.529	0.05	0	0	0	0
2	7	2.98	0.15	2.85	0.15	3.17	0.23	0.88	0.05	0.534	0.1
3	11	3.23	0.21	3.16	0.18	3.64	0.25	1.7	0.11	1.89	0.35
4	14	4.27	0.21	4.72	0.25	5.33	0.25	2.33	0.23	2.18	0.22
5	18	4.94	0.23	6.35	0.31	5.78	0.33	2.74	0.35	2.53	0.25
6	21	6.62	0.31	8.35	0.33	6.64	0.25	4.28	0.41	2.2	0.25
7	25	5.69	0.26	12.2	0.44	6.61	0.31	5.15	0.38	1.84	0.26
8	28	2.16	0.15	6.84	0.32	3.33	0.27	4.04	0.33	1.55	0.2

Table A.17: Glucose data for Experiment C.

	days	vi	SD	xi	SD	xi	SD	x	SD	ix	SD
0	0	0	0	0	0	0	0	0	0	0	0
1	3	6.47	0.55	6.61	0.32	6.39	0.45	6.9	0.56	6.83	0.52
2	7	8.55	0.52	8.65	0.64	8.61	0.55	8.62	0.45	8.53	0.42
3	11	6.82	0.46	7.37	0.44	7.29	0.62	7.59	0.44	8.15	0.44
4	14	3.88	0.32	5.69	0.33	5.86	0.41	6.98	0.33	6.85	0.45
5	18	0	0	2.37	0.31	2.23	0.25	3.39	0.22	4.6	0.31
6	21	0	0	0	0	0.759	0.15	1.01	0.15		
7	25	0	0	0	0	0	0	0	0		
8	28	0	0	0	0	0	0	0	0		

Table A.18: Fructose data for Experiment C.

	days	vi	SD	xii	SD	xi	SD	x	SD	ix	SD
0	0	0	0	0	0	0	0	0	0	0	0
1	3	8.42	0.44	8.5	0.53	8.32	0.64	8.83	0.42	8.67	0.55
2	7	11.5	0.62	11.6	0.62	11.6	0.52	11.4	0.36	11.3	0.42
3	11	10.6	0.51	12	0.51	11.8	0.44	11.6	0.51	12.2	0.56
4	14	10	0.41	20.1	0.42	11.7	0.35	12.3	0.55	11.9	0.36
5	18	6.22	0.35	18	0.41	10.5	0.36	11	0.46	11.6	0.35
6	21	0.801	0.15	8.56	0.32	8.92	0.28	5.64	0.41		
7	25	0	0	3.86	0.25	5.32	0.35	1.75	0.22		
8	28	0	0	1.21	0.15	2.24	0.22	0.325	0.1		

Table A.19: Calcium and phosphate data for Experiment C.

	days	xi	SD	vi	SD	xii	SD	x	SD	ix	SD
0	0	113	0	113		113		113		113	
1	3	102	5.6	106	1.7	106	7.2	111	4.9	114	1.3
2	7	114	2.7	106	1	106	2	121	8.9	112	8.6
3	11	93.2	4.4	102	2	102	1	108	5.6	119	11.2
4	14	84.5	3.6	93.2	9.6	81.6	3	123	7.4	85.1	7.2
5	18	73.5	4.6	57.1	6.4	65.6	8.9	63.2	2.9	83.2	5.2
6	21	51.6	5.5	51.2	1.7	54.7	5.5	54.1	5.2		
7	25	39.3	9	44.7	5	45.3	4.2	18	2.8		
8	28	47.3	6.1	43.7	5	55.3	2.3	32	2.8		

	days	xi	SD	vi	SD	xii	SD	x	SD	ix	SD
0	0	150		150		150		150		150	
1	3	121	10.9	103	7.7	121	5.8	110	4.9	102	3.2
2	7	96	2.4	93.8	3.1	101	4.4	96.7	5.9	99.5	2
3	11	67.2	3.2	76.1	4.9	70.9	8.7	81.7	3.9	91.1	2.9
4	14	53.7	2.9	49.8	9.5	47.3	0.5	79.6	1.4	73.7	7.4
5	18	46.2	5.6	29.3	1.7	0.73	0.3	3.9	0.6	25.2	3.9
6	21	4.6	0.3	0		0		0			
7	25	0		0		0		0			
8	28					0		0			

Appendix B

EXPERIMENTAL DATA

The data for Experiments D–H are presented in this appendix. The cell line TC2 was used in these experiments. As mentioned in Appendix A, the headings in the data set tables are in accord with the roman letters assigned to each treatment for each experiment.

B.1 Experiment D

The experimental design for Experiment D is shown in Table B.1.

Table B.1: Experimental design for Experiment D for which the cell line TC2 was used.

Treatments	O_2%(v/v)	CO_2%(v/v)	C_2H_4(ppm)	Comments
vi	10	0.5	5	–
xii	10	0.5	50	–
x	21	0.03	0	125 ml Bellco capped

Table B.2: Sucrose data for Experiment D.

	days	vi	SD	xiii	SD
0	0	20		20	0
1	3	9.89	0.38	9.28	0.34
2	7	6.81	0.64	3.78	0.39
3	10	7.04	0.15	3.67	0.32
4	14	6.45	0.17	0.599	0.26
5	21	0.25	0.05	0	
6	24	0		0	
7	28	0		0	

Table B.3: Glucose data for Experiment D.

	days	vi	SD	xiii	SD
0	0	0	0	0	
1	3	4.45	0.16	4.54	0.22
2	7	5.79	0.47	6.97	0.74
3	10	6.48	0	7.26	0.46
4	14	7.28	0.49	8.52	0.04
5	21	4.12	0.41	7.75	0.35
6	24	2.32	0.35	6.12	0.26
7	28	1.65	0.51	5.25	0.32

Table B.4: Fructose data for Experiment D.

	days	vi	SD	xiii	SD
0	0	0		0	
1	3	7.03	0.33	7.66	0.5
2	7	9.39	0.94	11.3	1.03
3	10	10.5	0.11	11.4	0.16
4	14	12.7	1.3	14.4	0.17
5	21	10.9	0.62	12.1	0.66
6	24	7.85	0.65	9.22	0.53
7	28	3.95	0.45	8.65	0.54

Table B.5: Calcium data for Experiment D.

	days	vi	SD	xii	SD
0	0	113		113	
1	3	136	8.6	133	13.6
2	7	131	13.5	115	12.2
3	10	111	10.6	114	9.6
4	14	120	11.3	129	7.4
5	17	95.3	9.2	110	7.2
6	23	89.6	5.8	95.6	8.5
7	28	81.2	6.4	85.4	6.6

Table B.6: Phosphate data for Experiment D.

	days	vi	SD	xii	SD
0	0	150		150	
1	3	120	7.4	121	8.8
2	7	120	4.7	119	12.2
3	10	111	3.3	117	9.4
4	14	79.6	8.5	117	11.3
5	17	83.2	4.1	69.7	8.8
6	23	49.2	5.6	59.8	10.5
7	28	35.6	2.5	43.5	6.7

B.2 Experiment E

The cultures in Experiment E became contaminated after Day 7 of the experiment, therefore, no nutrient data are available for this experiment.

B.3 Experiment F

Table B.7: Data for cultures exposed to 50 ppm ethylene in Experiment F.

	days	suc	gluc	fruc	cal	po4	taxol
0	0	20	0	0	113	150	0
1	3	0.38	4.7	8.23	141.1	66.1	0.02
2	7	0.29	3.1	7.91	154.6	66.9	0.017
3	10	0	1.5	7.75	127.6	64.5	0.058
4	14	0	0.71	4.6	99.8	61.2	0.078
5	17	0	0.34	1.22	89.1	33.1	0
6	23	0	0.3	0.54	69.3	36.1	0
7	28	0	0	0			

Table B.8: Data for the reference cultures in Experiment F.

	days	suc	gluc	fruc	cal	po4	taxol
0	0	20	0	0	113	150	0
1	3	0.514	4.39	7.56	154.6	106.8	0
2	7	0.456	4.2	8.4	157.3	73.9	0.016
3	10	0	2.9	8.2	121.4	68	0.03
4	14	0	0.71	5.3	98.9	56.4	0
5	17	0	0.24	1.69	90.9	32.9	0
6	23	0	0.12	0.33	106.9	38.4	0
7	28	0	0	0	92.5	21.3	0

B.4 Elicitation experiments, G and H

The experimental design for Experiments G and H are presented in Table B.9.

Table B.9: Experimental designs used in Experiments G and H (TC2 cell line).

Treatments	O_2% (v/v)	CO_2% (v/v)	C_2H_4 (ppm)	MeJA (μM)
Expt. G				
VII	10	0.5	0	0
VIII	10	0.5	0	10
IX	10	0.5	0	100
I	10	0.5	5	0
II	10	0.5	5	10
III	10	0.5	5	100
IV	10	0.5	10	0
V	10	0.5	10	10
VI	10	0.5	10	100
Expt. H				
2	10	0.5	5	10

Table B.10: Taxol data for Experiment G.

	days	elic(h)	VII	SD	VIII	SD	IX	SD
0	0		0		0		0	
1	6	0	0.14	0.04	0.1	0.15	0.2	0.08
2	7.33	8	0.22	0.06	0.39	0.1	0.39	0.1
3	8.13	27	0.11	0.05	0.66	0.22	0.17	0.06
4	9.13	51	0.27	0.1	2.74	0.45	0.25	0.08
5	14.1	171	0.23	0.05	2.45	0.42	0.16	0.05

	days	elic(h)	I	SD	II	SD	III	SD
0	0		0		0		0	
1	6	0	0.415	0.005	0.43	0.12	0.465	0.12
2	7.33	8	0.525	0.025	0.84	0.12	0.475	0.1
3	8.13	27	0.285	0.015	2.28	0.27	0.425	0.09
4	9.13	51	0.305	0.045	3.42	0.375	0.795	0.05
5	14.1	171	0.505	0.025	2.15	0.175	1.05	0.17

	days	elic(h)	IV	SD	V	SD	VI	SD
0	0		0		0		0	
1	6	0	0.38	0.11	0.485	0.065	0.395	0.09
2	7.33	8	0.375	0.12	0.525	0.055	0.63	0.11
3	8.13	27	0.28	0.06	0.52	0.11	0.635	0.12
4	9.13	51	0.28	0.08	2.84	0.135	1.54	0.11
5	14.1	171	0.13	0.05	2.92	0	2.28	0.3

Table B.11: Sucrose data for Experiment G.

	days	I	II	III	IV	V	VI	VII	VIII	IX
0	0	20	20	20	20	20	20	20	20	20
1	6	5.5	4.92	4.16	5.65	4.88	4.29	7.53	6.16	5.89
2	7	5.1	4.71	3.61	4.63	4.55	4.22	8.82	6.93	6.39
3	7.33	5.59	4.66	3.37	5.88	4.46	3.69	8.4	6.64	6.03
4	7.62	5.16	4.2	3.03	5.36	4.14	3.29	8.05	6.17	6
5	8.13	4.33	3.49	2.17	4.99	3.1	2.16	7.57	5.42	4.89
6	9.13	3.35	2.52	0.939	4.16	1.83	0.849	7.05	4.65	3.67
7	10.1	2.14	1.37	0.449	3.36	0.35	0.351	6.09	3.74	1.73
8	14.1	0.11	0.01	0	0	0	0	0	0	0

Table B.12: Glucose data for Experiment G.

	days	I	II	III	IV	V	VI	VII	VIII	IX
0	0	0	0	0	0	0	0	0	0	0
1	6	4.6	4.75	4.96	4.46	4.56	4.83	3.46	4.32	4.57
2	7	5.28	5.2	5.94	4.39	5.87	6.71	4.43	5.62	5.83
3	7.33	6.25	6.46	6.48	6.01	6.21	4.46	4.43	5.78	5.86
4	7.62	6.22	6.5	6.54	5.83	6.27	6.65	4.36	6.38	5.45
5	8.13	6.44	6.67	6.7	6.14	6.43	6.78	4.54	5.68	6.12
6	9.13	7.24	7.6	7.41	6.99	7.06	7.47	4.89	6.14	6.97
7	10.1	7.2	7.96	8.3	7.65	8.56	8.07	5.31	7.11	7.15
8	14.1	6.53	8.26	5.33	6.12	7.26	4.68	5.05	6.21	5.01

Table B.13: Fructose data for Experiment G.

	days	I	II	III	IV	V	VI	VII	VIII	IX
0	0	0	0	0	0	0	0	0	0	0
1	6	5.65	6.24	6.42	5.44	5.47	5.87	4.34	5.34	5.41
2	7	5.86	6.53	8.43	5.95	8.62	10.1	6.78	8.33	8.7
3	7.33	8.27	8.77	9.38	8.18	8.95	9.57	6.24	7.15	7.4
4	7.62	7.17	8.13	8.54	7.03	8.08	7.29	5.91	8.36	6.82
5	8.13	7.53	8.01	8.39	7.24	7.92	8.65	5.57	6.2	7.3
6	9.13	10.5	11.3	11.2	9.89	8.65	8.87	7.84	8.46	11
7	10.1	11.6	9.51	10.7	10.2	10.8	10.1	8.91	10.1	10.7
8	14.1	8.36	8.41	9.61	7.58	11.4	9.56	10.3	8.52	8.33

Table B.14: Calcium data for Experiment G.

	days	I	II	III	IV	V	VI	VII	VIII	IX
0	0	113	113	113	113	113	113	113	113	113
1	6	160	172	175	179	165	183	186	173	164
2	7	146	171	146	142	137	156	148	145	151
3	7.33	118	106	120	126	146	100	129	87	104
4	7.62	87.7	111	105	83.4	76.1	107	116	84.1	148
5	8.13	138	126	131	139	134	149	163	142	137
6	9.13	102	92.1	82.6	85.5	92.8	97.2	59.3	85.5	75.3
7	10.1	106	88.7	87.4	93.1	76.6	108	86.1	120	98.8
8	14.1	68.9	84.8	104	101	70.2	91.8	93.7	88.7	96.3

Table B.15: Phosphate data for Experiment G.

	days	I	II	III	IV	V	VI	VII	VIII	IX
0	0	150	150	150	150	150	150	150	150	150
1	6	98.8	96.5	84.8	96.5	93.2	85.2	90.4	124	96.2
2	7	113	99.7	84.1	109	43.6	55.6	40.4	92.3	106
3	7.33	112	132	118	132	134	113	138	101	134
4	7.62	131	122	118	133	124	113	135	140	118
5	8.13	118	124	120	136	124	111	127	127	138
6	9.13	109	105	91.9	103	92.9	97.8	109	103	112
7	10.1	132	127	112	126	114	118	137	124	116
8	14.1	50	69.3	6.62	55.3	58.7	42.3	51.6	85.3	72.9

Table B.16: Data for Experiment H.

	days	suc	gluc	fruc	po4	cal	taxol	unkn1	unkn2
0	0	20	0	0	150	113	0	0	0
1	4	4.91	4.74	6.12	84.5	149	0.0295	0	0
2	7	2.91	5.5	8.2	68.9	138	0.065	0	0
3	7.33	2.67	6.8	8.84	86.8	155	0.0695	0	0
4	7.87	2.78	6.6	8.61	84.8	182	0.074	0	0
5	8.38	1.91	6.5	8.38	79.2	130	0.095	0	0
6	8.92	1.81	6.8	8.18	58	123	0.018	0	0
7	9.29	1.02	6.3	8.74	97.6	118	0	0.531	0.444
8	9.92	0.86	5.8	8.6	104	120	0	0.407	0.101
9	10.2	0.75	5.6	7.77	99.9	153	0	0.576	0.148
10	12.2	0.55	4.2	6.82	84.5	121	0	0.362	0
11	14	0	3.5	6.19	64.5	102	0	0.06	0

Appendix C

STATISTICAL REGRESSION EQUATIONS

The data from each experiment was analyzed using a statistical software, Minitab. Linear regression equations were then constructed from the results of the statistical analysis. An explaination of the statistical terms is presented in Chapter 2 (Section 2.4). Only coefficients that are valid at 95% significance level ($p \leq 0.05$) were used to develop the regression equations. The regression equations for taxol, calcium and phosphate uptake, sugar utilization, and DNA content for each experiment are listed in this chapter. The lack of a regression equation for a nutrient siginifies the fact that the treatments in that experiment did not result in a significant change of that nutrient uptake/utilization. The regression equations for taxol are also listed in the corresponding sections of Chapter 3.

C.1 Experiment A

$$Taxol(6days) = 2.56 - 0.42[CO_2] + 0.55[C_2H_4] - 0.79[CO_2 * C_2H_4] \quad \text{(C.1)}$$

$$DNA(day7) = 13.1 + 1.0(CO_2) + 0.5(C_2H_4) - 0.7(CO_2 * C_2H_4) \quad \text{(C.2)}$$

$$DNA(day14) = 15.0 - 0.9(CO_2) + 1.4(C_2H_4) - 0.3(CO_2 * C_2H_4) \quad \text{(C.3)}$$

$$DNA(day21) = 6.0 - 0.9(CO_2) - 0.9(CO_2 * C_2H_4) \quad \text{(C.4)}$$

$$Calcium(uptake, wk2) = 72.2 - 6.3(CO_2) - 6.2(C_2H_4) + 6.9(CO_2 * C_2H_4) \quad (C.5)$$

$$Phosphate(uptake, wk2) = 102.6 - 8.5(CO_2) + 10.9(C_2H_4) - 11.8(CO_2 * C_2H_4) \quad (C.6)$$

C.2 Experiment B

$$Taxol(day7) = 2.43 + 0.87[CO_2] - 0.21[C_2H_4] + 0.21[CO_2 * C_2H_4] \quad (C.7)$$

$$Taxol(day11+) = 1.70 + 0.72[CO_2] + 0.41[C_2H_4] + 0.32[CO_2 * C_2H_4] \quad (C.8)$$

$$Glucose(utilization, wk1) = 2.5 - 0.49(CO_2) + 0.46(CO_2 * C_2H_4) \quad (C.9)$$

$$Fructose(utilization, wk2) = 4.2 - 0.97(CO_2) - 1.86(CO_2 * C_2H_4) \quad (C.10)$$

$$DNA(day7) = 7.6 - 3.3(CO_2) + 3.8(C_2H_4) - 2.6(CO_2 * C_2H_4) \quad (C.11)$$

$$DNA(day14) = 19.9 - 3.6(CO_2) + 2.5(C_2H_4) - 4.5(CO_2 * C_2H_4) \quad (C.12)$$

$$Calcium(uptake, wk2) = 2.1 - 12.4(CO_2) - 9.4(C_2H_4) + 10.5(CO_2 * C_2H_4) \quad (C.13)$$

$$Phosphate(uptake, wk1) = 6.5 - 1.4(CO_2) - 5.8(C_2H_4) + 1.1(CO_2 * C_2H_4) \quad (C.14)$$

$$Phosphate(uptake, wk2) = 0.32 + 0.21(CO_2) - 0.11(C_2H_4) - 0.09(CO_2 * C_2H_4) \quad (C.15)$$

$$Phosphate(uptake, wk3) = 1.5 + 0.76(CO_2) + 0.23(C_2H_4) + 0.33(CO_2 * C_2H_4) \quad (C.16)$$

C.3 Experiment D

$$Taxol(3d) = 0.04 - 0.032(C_2H_4) \quad (C.17)$$

$$Taxol(7d) = 0.05 - 0.035(C_2H_4) \quad (C.18)$$

C.4 Elicitation experiment: G

$$Taxol(8h) = 0.49 + 0.13(C_2H_4) + 0.10(MeJA) + 0.13(C_2H_4 * MeJA) \quad \text{(C.19)}$$

$$Taxol(27h) = 0.59 + 0.40(C_2H_4) + 0.55(MeJA) + 0.73(C_2H_4 * MeJA) \quad \text{(C.20)}$$

$$Taxol(51h) = 1.4 + 1.6(MeJA) + 0.29(C_2H_4 * MeJA) \quad \text{(C.21)}$$

$$Taxol(171h) = 1.3 - 0.37(C_2H_4) + 1.2(MeJA) - 0.27(C_2H_4 * MeJA) \quad \text{(C.22)}$$

$$DWT(14d) = 1.6 + 0.32(MeJA) \quad \text{(C.23)}$$

$$Glucose(utilization, 15h) = 1.3 - 0.35(C_2H_4) - 0.24(MeJA) \quad \text{(C.24)}$$

$$Fructose(utilization, 27h) = 1.1 - 0.25(C_2H_4) - 0.46(MeJA) \quad \text{(C.25)}$$

$$Calcium(uptake, 15h) = 1.6 - 0.58(MeJA) \quad \text{(C.26)}$$

$$Phosphate(uptake, 15h) = 2.1 + 0.73(MeJA) \quad \text{(C.27)}$$