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Methyl Jasmonate Induced Production of Taxol in Suspension Cultures of *Taxus cuspidata*: Ethylene Interaction and Induction Models

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Suspension cultures of *Taxus cuspidata* were challenged with various concentrations and combinations of methyl jasmonate and ethylene. Taxol productivity increased 19-fold when *T. cuspidata* suspension cultures were exposed to 5 ppm ethylene and 10 μ M methyl jasmonate. This increase was 15-fold when either 0 or 10 ppm ethylene was combined with 10 μ M methyl jasmonate. The induction of taxol occurred within 51 h after elicitation and would reduce fermentation times and costs. Ethylene concentration at 50 ppm had an inhibitory effect on taxol production but not on phosphate uptake rate, suggesting independent regulation of taxol and physiological functions of the cell. A simple induction model is proposed to explain the action and effects of both ethylene and methyl jasmonate with regard to receptor binding and regulatory systems in plants.

1. Introduction

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).

Elicitors stimulate synthesis of phytoalexins in cultured plant cells. Biotic elicitors include glucan polymers, glycoproteins, low molecular 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). Taxol, a natural diterpenoid with strong anticancer activity, can be obtained from various species of *Taxus*. Taxol production has previously been related to exogenous ethylene concentration (Mirjalili and Linden, 1995).

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 accumula-

tion, which suggested an integral role for this molecule in the signal transduction system of plants. Endogenous jasmonic acid and its methyl ester (MeJA) accumulate 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). The induced secondary metabolites were accompanied by an increase in PAL poly(A)+RNA that was followed by an increase in phenylalanine ammonia-lyase (PAL) activity to maximal values 25 and 33 h after elicitation, respectively. Gundlach *et al.* (1992) suggested that addition of methyl jasmonate initiates *de novo* transcription of PAL genes. 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-hydroxyphenyl)pyruvate 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. Particular signal combinations may synergistically hyperinduce plant defense genes and be more specifically related to gene function than 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).

The purpose of this study was to investigate the effects of methyl jasmonate and ethylene and their possible interaction on production of taxol. Since taxol (a phytoalexin) is presumed to be produced by plants as a response to pathogenic attack, understanding the effect of ethylene/MeJA on production of taxol may help elucidate the mechanism.

Table 1. Experimental Designs Used To Study Various Concentrations and Combinations of Ethylene and Methyl Jasmonate (MeJA) in *T. cuspidata* Suspension Cultures

no. of replicates	O ₂ % (v/v)	CO ₂ % (v/v)	C ₂ H ₄ (ppm)	MeJA (μ M)	growth rate (day ⁻¹)
2	10	0.5	0	0	0.16
2	10	0.5	0	10	0.19
2	10	0.5	0	100	0.19
2	10	0.5	5	0	0.14
2	10	0.5	5	10	0.17
2	10	0.5	5	100	0.29
2	10	0.5	10	0	0.16
2	10	0.5	10	10	0.22
2	10	0.5	10	100	0.19

2. Materials and Methods

2.1. Cell Line and Growth Conditions. The cell line used was *Taxus cuspidata* P991, kindly provided by Dr. Donna Gibson and Dr. Ray Ketchum (Plant, Soil and Nutrition Laboratory of the USDA Agricultural Research Service, Ithaca, NY). The growth medium was formulated by Gibson *et al.* (1993). The cultures were subcultured every 2 weeks in 125 mL Erlenmeyer flasks; 10 mL of inoculum was added to 40 mL of fresh medium. For maintenance, the culture flasks were capped with Bellco silicone closures (28 mm i.d.) and agitated at 125 rpm in a New Brunswick incubator shaker at 25 °C in the dark. This cell line is a clone of the one used in a previous study (Mirjalili and Linden, 1995).

2.2. Experimental Conditions. Cultures were subcultured into 250 mL Erlenmeyer flasks for the experiments (10 mL of inoculum into 40 mL of fresh medium). A rubber stopper equipped with two 0.2 μ m membrane filters was used as the closure for test flasks. The gases oxygen, carbon dioxide, and ethylene were fed into each flask through one of the filters at a total gas flow rate of 40 mL/min. The apparatus used for controlling the mixture of gases flowing to each flask is explained elsewhere (Mirjalili and Linden, 1995).

Due to the small volume of the samples that could be taken from each culture, no cell dry weight data could be obtained from these experiments. Hence, glucose consumption rates were used as a way of relating to the growth of the cultures. The cell line used in the present study grew more slowly than the one used previously (Mirjalili and Linden, 1995). In order to be able to compare the two studies, taxol production and phosphate uptake rates per gram of glucose consumed were calculated for both studies. For the methyl jasmonate experiment, the final dry weight of the cultures was measured (after 14 days) and average growth rates were calculated.

2.3. Ethylene Experiments. The purpose of this study was to investigate the saturation kinetics of ethylene with respect to taxol production and phosphate uptake rate and to elucidate the ethylene action with regard to binding to putative receptors. Ethylene at concentrations of 5 and 50 ppm was mixed with 10% (v/v) oxygen and 0.5% (v/v) carbon dioxide using the above-mentioned gas apparatus from which it flowed into the head space of culture flasks.

2.4. Methyl Jasmonate Experiments. Table 1 shows the full factorial design used for one of these experiments. Elicitation was started 7 days after growth was initiated under 10% (v/v) oxygen and 0.5% (v/v) carbon dioxide and variable head space ethylene concentrations. These head space gas concentrations resulted in the highest taxol production in a previous study (Mirjalili and Linden, 1995). Methyl jasmonate (Beldoukian Research Inc., Danbury, CT) at concentrations of 0, 10, and 100 μ M was added to the cultures in 5 μ L of

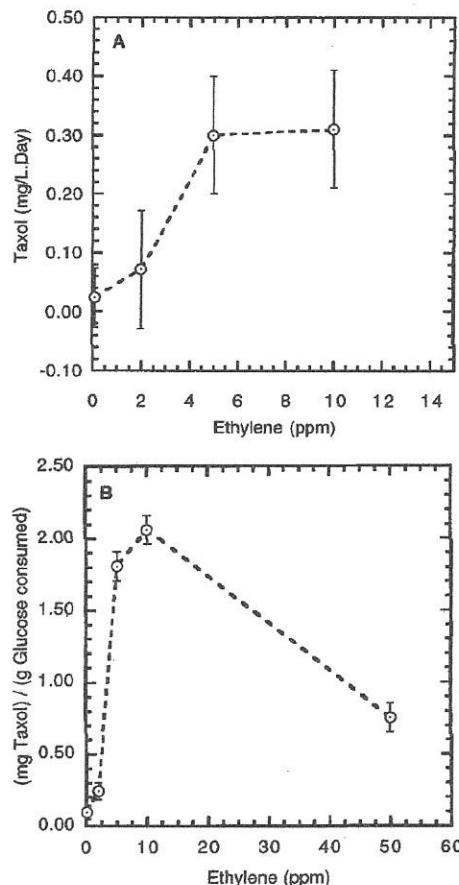


Figure 1. Taxol productivity at various gaseous concentrations of ethylene in the head space: (A) data from a previous study (Mirjalili and Linden, 1995); (B) data from both previous and present studies.

ethanol per milliliter of culture (Gundlach *et al.*, 1992). Equal volumes of ethanol (250 μ L) were added to all cultures. After elicitation, samples were taken every 8 h for the first day and then every 24 h until day 14. Samples were analyzed for extracellular glucose, phosphate, and taxol, as previously described by Mirjalili and Linden (1995). The taxol analysis was performed by high-pressure liquid chromatography using absorption at 228 nm with a Waters 486 UV detector. The taxol concentration in selected samples was confirmed using diode array analysis.

2.5. Statistical Analysis. The methyl jasmonate experiments were conducted using a three-level factorial design which provides information about the two main effects (ethylene and methyl jasmonate) and the two-factor interaction. The data from each gas experiment were 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).

3. Results

3.1. Ethylene Experiments. **3.1.1. Taxol Analysis.** In a previous study, the effect of head space ethylene at constant concentrations of 0, 2, 5, and 10 ppm was studied on production of taxol. Taxol concentration in the medium 21 days after inoculation increased as ethylene concentration was increased from 0 to 5 ppm; taxol concentration leveled out at 10 ppm (Figure 1A). In the present study ethylene at concentrations of 5 and 50 ppm was used.

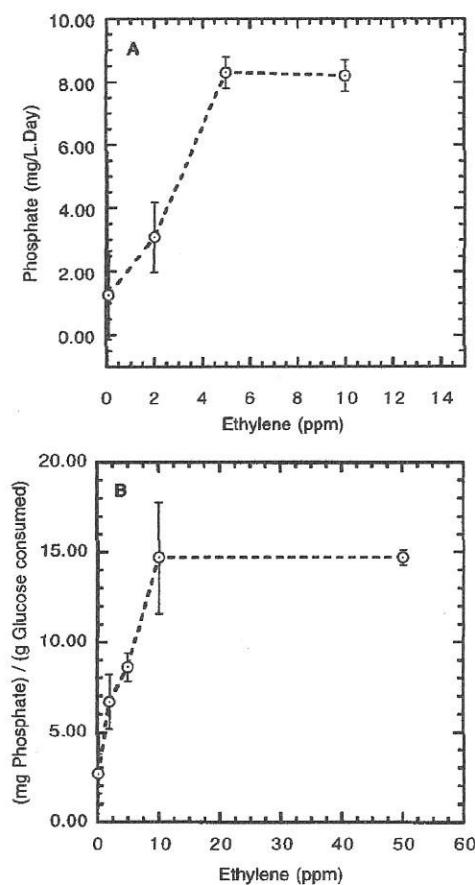


Figure 2. Phosphate uptake rate at various gaseous concentrations of ethylene in the head space: (A) data from a previous study (Mirjalili and Linden, 1995); (B) data from both previous and present studies.

Figure 1 shows taxol productivity at different levels of gaseous ethylene. Taxol productivity with 5 ppm in this study ($0.20 \text{ mg/(L}\cdot\text{day)}$) was lower than the productivity in the previous one ($0.31 \text{ mg/(L}\cdot\text{day)}$) (Mirjalili and Linden, 1995). Taxol productivity of cells exposed to 50 ppm ethylene was $0.17 \text{ mg/(L}\cdot\text{day)}$. Even when taxol concentrations were normalized using the glucose consumption rates, taxol productivity in the 50 ppm ethylene treatments was less than the maximum. 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 an optimum concentration. For example, optimal cell division in wild-type tobacco protoplasts occurred at a defined auxin (NAA) concentration (Walden *et al.*, 1994).

3.1.2. Phosphate Uptake Rate. The importance of phosphate esters and anhydrides to cellular metabolism is presented in detail by Westheimer (1987); no other compound appears to 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).

Using phosphate uptake rate per gram of glucose consumed, a saturation curve could be constructed as a function of ethylene concentration (Figure 2B). In the previous study (Mirjalili and Linden, 1995), phosphate uptake rate ($\text{mg/(L}\cdot\text{day)}$) increased as the exogenous ethylene concentration increased from 0 to 5 ppm and

reached saturation limits at 10 ppm (Figure 2A). Due to the differences in the cell lines, the amount of (mg) phosphate taken up per gram of glucose consumed by the cells for both studies was calculated. Figure 2B shows the effect of ethylene on phosphate uptake and indicates saturation kinetics over the 0–50 ppm range. 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.

3.2. Methyl Jasmonate Experiments. 3.2.1.

Growth Analysis. Table 1 shows the dry weight data when the cultures were exposed to various concentrations of ethylene and MeJA. Statistical analysis showed that MeJA had a significant effect ($p \leq 0.05$) on growth of the cultures. Growth rate of the cultures increased as the concentration of MeJA increased; from 0.15 day^{-1} to 0.19 day^{-1} to 0.22 day^{-1} at 0, 10, and $100 \mu\text{M}$ 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 (data not shown). The highest growth rate was observed with 5 ppm ethylene and $100 \mu\text{M}$ MeJA (0.29 day^{-1}). The average growth rate with this cell line (0.19 day^{-1}) was 67% of that of the cell line used in the previous study (Mirjalili and Linden, 1995).

3.2.2. Taxol Analysis. Figure 3 shows the kinetics of taxol production at different concentrations and combinations of ethylene and methyl jasmonate. When no ethylene was present (Figure 3A), only $10 \mu\text{M}$ MeJA resulted in an increase of taxol production to 2.7 mg/L . This maximum induction was observed 51 h after elicitation. When cultures were exposed to 5 ppm ethylene (Figure 3B), $10 \mu\text{M}$ MeJA resulted in the highest taxol concentration (3.4 mg/L), again 51 h after elicitation; $100 \mu\text{M}$ MeJA resulted in an increase of taxol concentration from 0.5 to 1.1 mg/L but only after 171 h. However, at 10 ppm ethylene, taxol production in cultures exposed to both 10 and $100 \mu\text{M}$ MeJA increased dramatically from the basal level (Figure 3C). Again, the highest taxol concentrations observed with $10 \mu\text{M}$ MeJA, were 51 h after elicitation (2.8 mg/L). With 10 ppm ethylene and $100 \mu\text{M}$ MeJA, taxol concentration increased to 1.5 mg/L after 51 h and to 2.3 mg/L 171 h after elicitation.

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 of taxol 51 h after elicitation. 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 exposure to $100 \mu\text{M}$ MeJA (Mizukami *et al.*, 1993). MeJA ($100 \mu\text{M}$) also caused a maximal induction of alkaloids in *E. californica* cultures; this maximum was detected within 6 h after elicitation; alkaloids continued to accumulate at a linear rate for 136 h. In the studies by Gundlach *et al.* (1992), of 36 plant cell cultures, methyl jasmonate (0.1 – $500 \mu\text{M}$) 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. In another study by Kutchan (1991), methyl jasmonate, a yeast cell wall preparation, and 12-oxophytodienoic acid produced similar patterns of transcript accumulation of the berberine bridge enzyme; maximal levels of transcript occurred 6–8 h after elicitation with a methyl jasmonate concentration of $50 \mu\text{M}$.

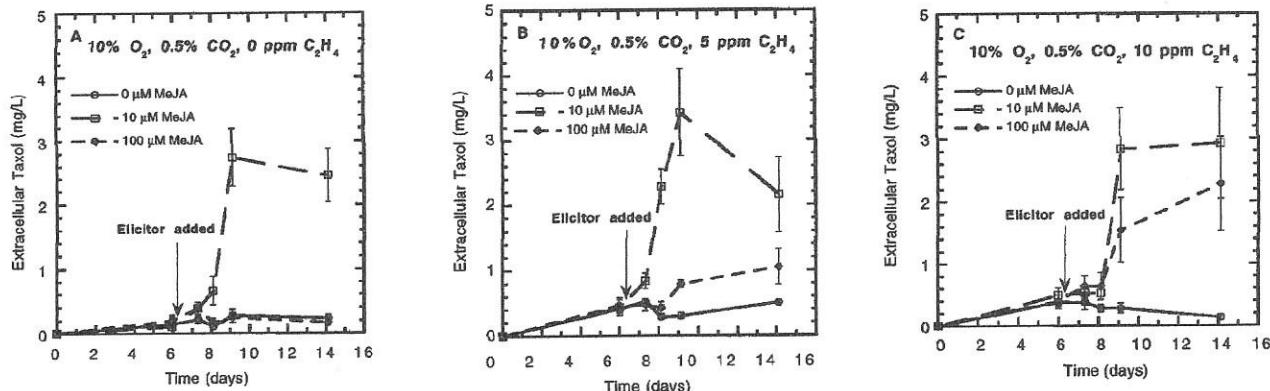


Figure 3. Kinetics of taxol production at different concentrations and combinations of ethylene and methyl jasmonate (MeJA). All cultures were exposed to 10% (v/v) O₂ and 0.5% (v/v) CO₂: ○, 0 µM MeJA; □, 10 µM MeJA; △, 100 µM MeJA.

The combination of ethylene and MeJA (5 ppm and 45 µM) induced both osmotin mRNA and protein accumulation to levels nearly as high as those found in salt-adapted 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 co-workers (Xu *et al.*, 1994) have found evidence that combination of ethylene/MeJA has effects both on osmotin promoter induction and on stabilization of osmotin mRNA. Although MeJA in combination with ethylene or salicyclic 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 demonstrate translational or protein stability effects on PR-1b and osmotin, and the maximal accumulation of more than one defense protein requires multiple signals.

Both stimulation and inhibition of ethylene biosynthesis by MeJA have been reported (Saniewski *et al.*, 1987); the ability of MeJA and ethylene to coregulate 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 occurred at ethylene concentrations above the saturation level of ethylene. In our study, 10 µM MeJA induction occurred at all ethylene concentrations including 0 ppm ethylene (Figure 4B). 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 experiment. Ethylene synthesized as a result of MeJA addition was flushed out of the cultures. Also, if the effects were because of ethylene synthesis that resulted from MeJA addition, an effect at 100 µM MeJA would be expected.

3.2.3. Phosphate Uptake Rate. The volumetric phosphate uptake rates by the cultures decreased as the concentration of MeJA was increased from 0 to 100 µM (Figure 4A). The highest volumetric phosphate uptake rate was observed when cultures were exposed to 0 ppm ethylene and 0 µM MeJA (13.9 mg/(L·day)), and the lowest was observed with 5 ppm ethylene and 10 µM MeJA (8.2 mg/(L·day)). The highest phosphate uptake rate correlated with the lowest taxol productivity, whereas in the previous study, the opposite was true with ethylene alone as the independent variable. These differences are not understood except to conclude that phosphate uptake and taxol productivity appear not to be linked (Figure 4). In our studies phosphate uptake may be con-

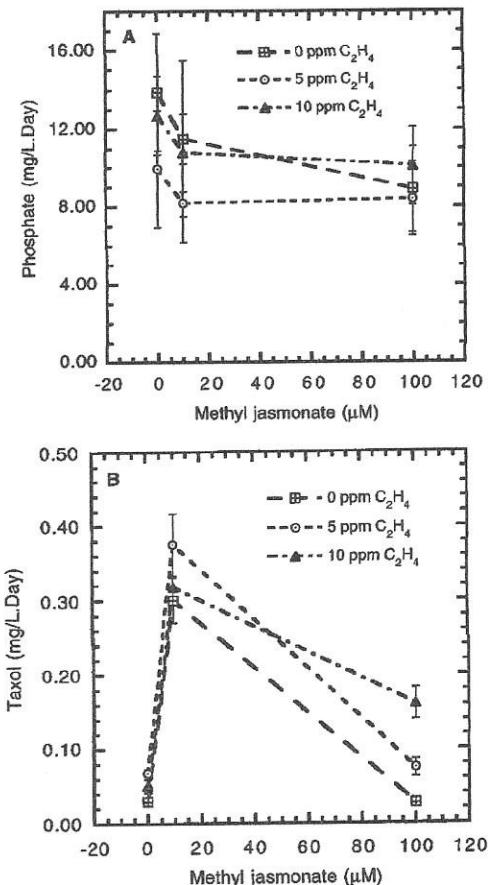


Figure 4. Volumetric phosphate uptake rates by the cultures (A) and volumetric taxol productivity (B) at different concentrations and combinations of ethylene and methyl jasmonate (MeJA).

sidered as a physiological handle, i.e. an indicator of culture activity.

3.3. Modeling. 3.3.1. Direct/Indirect Model for Ethylene Binding. 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 cofactor in some reaction, (2) by being oxidized to some component and being incorporated into tissue, or (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

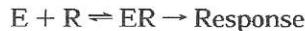
are unlikely (see Sisler (1991) and references therein). The third one is under extensive research (Kieber *et al.*, 1993; Hall *et al.*, 1990; Chang *et al.*, 1993).

Two types of ethylene binding sites (receptors) have been characterized on the basis of high and low values of association/dissociation constants. The fast-action receptor had 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). Sisler (1991) reported that the responses from exposing plants to ethylene ceased when the ethylene was removed.

Chang *et al.* (1993) and Kieber *et al.* (1993) have identified the genes for several proteins involved in ethylene signal transduction. The gene products may bind to specific DNA sequences located within the promoter of ethylene-induced genes. Alternatively, they may perceive ethylene and transduce the signal through phosphate transfer reactions to proteins that act indirectly as 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 product 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 two-component regulatory 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 hypothetically a negative regulator of ethylene response (Kieber *et al.*, 1993). In bacteria, the two protein components of the signal transducer 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 represents a plausible two-component system homologue in the signal transduction pathway in plants (Chang *et al.*, 1993). Whether ethylene is binding to ETR1 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 complexed with 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 follows:

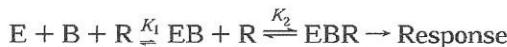


$$\text{Response} = \frac{V_m [E]}{K_m + [E]} \quad (1)$$

For phosphate uptake as the response, $K_m = 4 \times 10^{-8}$ M and $V_{max} = 15$ (mg of phosphate)/(g of glucose consumed). For taxol productivity, $K_m = 4 \times 10^{-8}$ M and $V_{max} = 0.35$ mg/(L·day). 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

the head space gas phase and the liquid throughout the experiments. Hence, the dissolved concentration of ethylene (M) in the culture medium was calculated using Henry's law.

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:



$$\text{Response} = pF_o = p \frac{K_1 K_2 [B][E]}{1 + K_1 [E](1 + K_2 [B])} \quad (2)$$

where $1/K_m = K_1(1 + K_2[B])$. The ethylene binding site concentration [B] for various plant vegetative tissue is reported by Sisler (1991). Using the average value reported for all binding site concentrations ($[B] = 4 \times 10^{-9}$ M), $K_1 = 4 \times 10^3$ ($M^{-1} s^{-1}$). Therefore, with $K_m = 4 \times 10^{-8}$ M, $K_2 = 6.3 \times 10^{12}$ M $^{-1}$. The high and low values of [B] were used to evaluate the sensitivity of this parameter. The value for p was calculated using the maximal value of the response and F_o at saturation (100%), i.e. V_{max} from the Michaelis–Menten analysis.

Figure 5 shows eqs 1 and 2 applied to our system. The specific phosphate uptake rate and the specific and volumetric taxol productivities have been used as responses. The limits of sensitivity of the indirect model are shown by the dotted lines on Figure 5. 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. The dashed lines represent the direct binding model. Figure 5A 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, which is in equilibrium with 3.4×10^{-7} M dissolved ethylene). The curves are drawn using parameters calculated by disregarding the 50 ppm ethylene data. Again, the indirect binding model fits the data better than the direct binding model at the lower physiological concentrations of ethylene. Because taxol productivity (Figure 5B,C) relationships are different than that of phosphate uptake (Figure 5A), taxol biosynthesis may be unrelated to physiological functions of the cell, but under the regulation of ethylene effects on gene transcription.

3.3.2. Modulation (Induction) Model. Removal of regulatory repressors, genetic manipulation of enzyme 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 exploiting the biotechnological potential of plant cells. Elicitors have been used as a tool to understand the regulation of phenylpropanoid secondary metabolites in plants (DiCosmo and Misawa, 1985).

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 concentrations of jasmonic acid and/or methyl jasmonate in different tissues and species are typical of

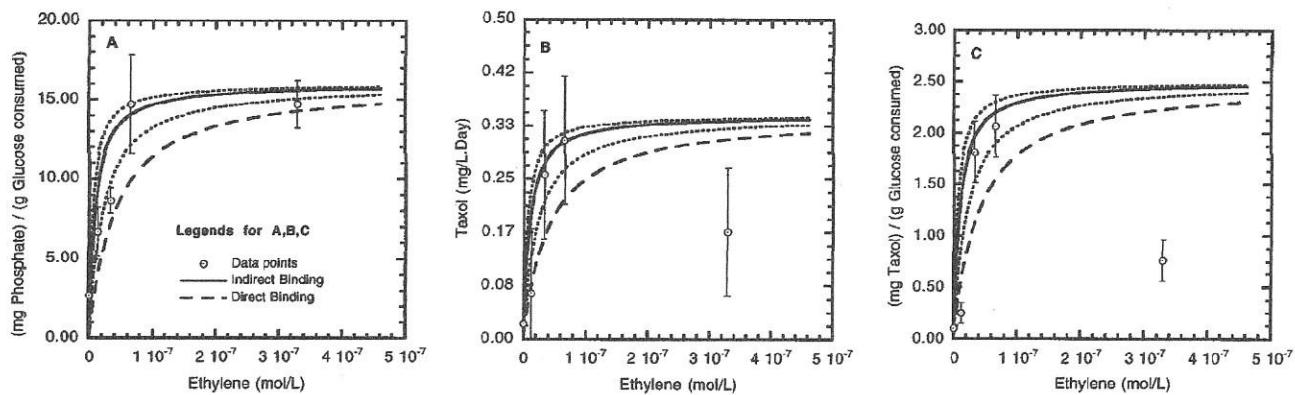


Figure 5. Mathematical models of direct (eq 1) and indirect (eq 2) binding of ethylene to receptor applied to our data.

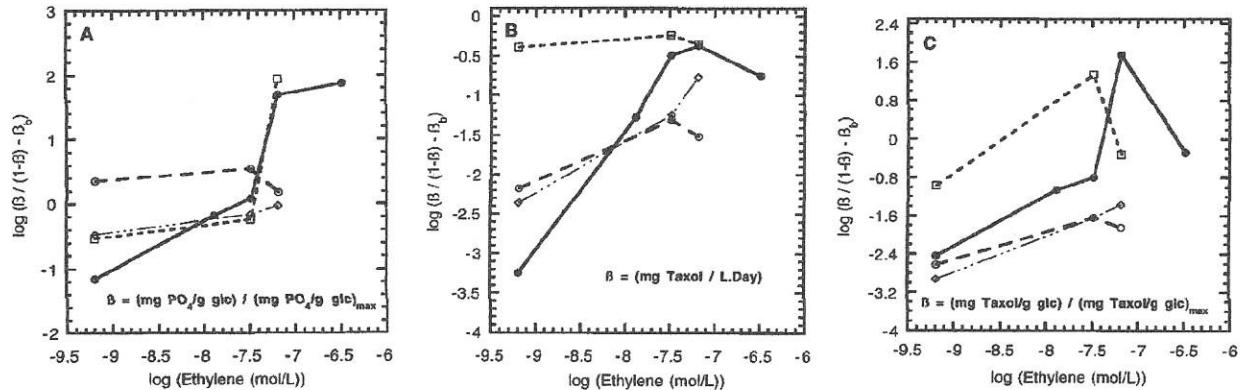


Figure 6. Effect of ethylene and methyl jasmonate on taxol productivity and phosphate uptake rates based on an expression from equilibrium analysis of binding repressor molecules to the operator derived by Yagil and Yagil (1971): ●, 0 μ M MeJA, 0 ethanol; ○, 0 μ M MeJA, 81 mM ethanol; □, 10 μ M MeJA, 81 mM ethanol.

plant hormones, which range from 0.01 to 3 μ g/gram of 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; changes in protein phosphorylation patterns of membrane and cellular proteins have been associated with the induction process (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 here indicate that treatment of cultured plant cells with elicitors rapidly altered gene expression for secondary metabolite synthesis (Figures 3 and 4). Therefore, production of taxol in cell suspension cultures of *T. cuspidata* may be regulated to some extent by exogenous methyl jasmonate and ethylene levels. 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, 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 .

$$\log \left(\frac{\beta}{1-\beta} - \beta_b \right) = \pm n \log [F] + \log \beta \pm \log K_1 \quad (3)$$

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 binding of effector molecules with modulator molecules to induce 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).

The effects of ethylene and methyl jasmonate on taxol productivity and phosphate uptake rates are shown in Figure 6. As mentioned in section 2.4, MeJA was dissolved in ethanol and 250 μ L of 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 5–50 ppm ethylene (Figure 6A, compare ● and ○), whereas there is no effect of ethanol on the volumetric and specific productivities of taxol (Figure 6B,C). Methyl jasmonate at concentrations of 0 and 100 μ M had positive effects on specific taxol at all the ethylene concentrations; however, 10 μ M 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 μ M methyl jasmonate did not have significant effects on uptake of phosphate, but 10 μ M 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 as a moderator 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 synthesize antibiotics or may cause suppression of secondary metabolite production (DiCosmo and Mis-

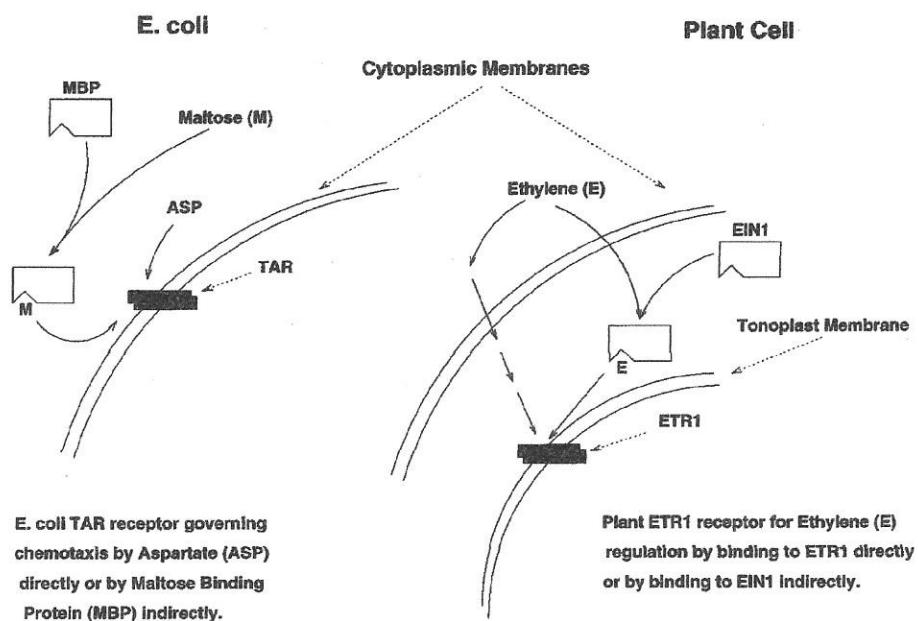


Figure 7. Hypothetical mechanism of action of ethylene, compared to the *E. coli* chemotaxis system.

awa, 1985). Similar observations have been made with enzyme production in bacterial systems (Linden and Shiang, 1991).

4. Discussion

A model for the induction of plant defense responses is proposed by Dixon *et al.* (1990). 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. 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 transduction 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). 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 was reported by Tamari *et al.* (1995).

Mild mechanical stress to plant tissues may lead to an enhanced synthesis of ethylene, which thereby can be called a possible second messenger (Blowers and Trewavas, 1989). The enzymes involved in ethylene synthesis are located in the tonoplast membrane (Guy and Kend, 1984). Increased production of ethylene, from

the stimulation of the methionine pathway, starts 15–30 min after elicitation (Boller, 1990).

Xu *et al.* (1994) suggest that the binding of ethylene to its receptor on the plasma membrane might sensitize methyl jasmonate receptors on the membrane. Their work with seedlings shows that continuous presence of ethylene is required for the synergistic effect of MeJA and ethylene on inducing the osmotin gene. The continuous binding of ethylene with its receptors might be required or a short-lived participant in signal transduction may be induced. The observation that the MeJA conditioning causes parsley cells to become more sensitive to low concentrations of fungal elicitor suggests an improved cellular signal perception/transduction system (Kauss *et al.*, 1992). The view expressed by Enyedi *et al.* (1992) suggests the existence of two separate systems of induction, one involving pathogens through SA and another involving wounding.

In our studies, the amount of taxol produced does not follow saturation behavior with respect to ethylene concentration. The physiological basis of saturation in hormone response is not known. 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 tissue ability to perceive the elicitor (Singh *et al.*, 1994).

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 (Xu *et al.*, 1994). 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 this plant culture system.

5. Conclusion

The results and modeling presented here could be summarized as follows: The effect of ethylene on the activity of the cell, as indicated by the phosphate uptake "handle", is independent of the effect on secondary metabolism. Taxol productivity appears to be under ethylene control by regulation of transcription of biosyn-

thetic enzymes. While these conclusions give no physical evidence of the mechanism of ethylene action, it is frequently useful when the available information is collected into potential models. Such a model is presented in Figure 7 in the form of a drawing. The ETR1 receptors are indicated as integral of the tonoplast membranes only because Hall *et al.* (1990) isolated receptors on internal membrane systems rather than the plasma membrane. Ethylene is synthesized intracellularly; exogenous ethylene diffuses readily. Precedent for intracellular receptors of the two-component regulatory system comes from phosphate regulation of bacteria (Stock and Surette, 1992). As mentioned above, indirect binding of maltose in the chemotaxis model serves as a representation of EIN1 involvement. The data presented here allow 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 CTR1, which has been shown to be genetically linked downstream to ETR1, may be responsible for promoter interaction (Kieber *et al.*, 1993).

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