ผลของการใช้ปัจจัยกระตุ้นต่อการสังเคราะห์สารอัลคาลอยด์โดยใบของแพงพวย

Catharanthus roseus

นางสาวจุนธนี วีรเจตบดีธัช

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ELICITATION EFFECTS ON BIOSYNTHESIS OF INDOLE ALKALOIDS BY *Catharanthus roseus* LEAVES.

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อ.ที่ปรึกษา : อ.ดร.เหมือนเดือน พิศาลพงศ์, อ.ที่ปรึกษาร่วม :
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การศึกษาการใช้ปัจจัยกระตุ้น 2 ชนิดคือ ไคโตแซน และ เมธิลจัสโมเนทที่มีต่อการสังเคราะห์สารอัลคาลอยด์ vindoline ในใบของแพงพวย โดยทำการฉีดพ่นสารละลายไคโตแซน (ระดับโพลิเมอร์ DP 7190, น้ำหนักโมเลกุล MW 1200 kDa, และ ระดับหมู่อะซีติล %DA 14) ความเข้มข้น 10 20 และ 30 ส่วนในล้านส่วน ลงบนลำต้นของต้นแพงพวย ทุกวัน วันละ 2ครั้งเป็นเวลานาน 7 วันในการทดลองกลุ่มที่หนึ่ง และทำการรมไอของสารละลายเมธิลจัสโมเนทความเข้มข้น 10 50 100 และ 1000 ส่วนในล้านส่วนต่อลำต้นของแพงพวยภายใต้ระบบปิด ทุกวัน วันละ 2 ชั่วโมงเป็นเวลานาน 7 และ 14วันในการทดลองกลุ่มที่สอง พบว่า เมธิลจัสโมเนทสามารถกระตุ้นการสังเคราะห์สาร vindoline จากใบของต้นแพงพวยได้อย่างมีนัยสำคัญ ในขณะที่ไคโตแซนไม่มีผลหรือมีผลน้อยมากในการสังเคราะห์สาร vindoline ความเข้มข้นที่เหมาะสมของเมธิลจัสโมเนทสำหรับการกระตุ้นการผลิตสาร vindoline คือ 100 ส่วนในล้านส่วน โดยหลังจากทำการกระตุ้นด้วยเมธิลจัสโมเนท 100 ส่วนในล้านส่วน เป็นเวลา 7 วัน และ 14 วัน พบว่าความเข้มข้นของ vindoline เพิ่มขึ้นเป็นประมาณ 2 มิลลิกรัมต่อกรัมของใบแห้ง และ 4 มิลลิกรัมต่อกรัมของใบแห้งตามลำดับ (2 – 4 เท่าของความเข้มข้นในกรณีที่ไม่มีการกระตุ้น) โดยในการใช้เมธิลจัสโมเนทความเข้มข้น 0 – 100 ส่วนในล้านส่วน รมไอเป็นเวลา 14 วัน จะมีปริมาณการสะสมของ vindoline ในใบเพิ่มขึ้นในลักษณะเป็นเส้นตรงเมื่อสัมพันธ์กับความเข้มข้นของเมธิลจัสโมเนท ข้อมูลของการเปลี่ยนแปลงการสะสมของ vindoline ในใบแพงพวย จากการกระตุ้นด้วยเมธิลจัสโมเนทที่ความเข้มข้นต่างๆสอดคล้องกับแบบจำลองของการจับตัวโดยตรงระหว่างสารกระตุ้นต่อรีเซพเตอร์เป็นอย่างดี

ภาควิชา.....วิศวกรรมเคมี.....ลายมือชื่อนิสิต.....
สาขาวิชา.....วิศวกรรมเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....
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KEYWORD: *Catharanthus roseus* / ALKALOID / ELICITATION / VINDOLINE / METHYL JASMONATE.

JUNTANEE VEERAJETBODITHAT ; ELICITATION EFFECTS ON BIOSYNTHESIS OF INDOLE ALKALOIDS BY *Catharanthus roseus* LEAVES. THESIS ADVISOR ; DR.MUENDUEN PHISALAPHONG, THESIS COADVISOR ; ASSOC.PROF. WICHAI CHERDSHEWASART, D.Sc., pp 70, ISBN 974-17-9814-8

Two elicitors, chitosan and methyl jasmonate, were used for the study of elicitation effects on *Catharanthus roseus* leaves. Chitosan (DP 7190, MW 1200 kDa, and %DA 14) concentrations 0 - 30 ppm were daily sprayed over the aerial parts of the plants twice a day for 7 days in the first experiment set. Methyl jasmonate at concentrations 0 - 1000 ppm were daily fumed under the closed system over the aerial parts of each plant for 2 hours every day for 7 and 14 days in the second experiment set. Methyl jasmonate fuming resulted in significant induction of the biosynthesis of vindoline in *C. roseus* leaves, while little, if any, induction of vindoline formation occurred when exposured to chitosan. The optimal concentration of methyl jasmonate for elicitation of vindoline production was 100 ppm. The vindoline concentration was 2 mg/g leaf DW and 4 mg/g leaf DW after elicitation with 100-ppm methyl jasmonate for 7 and 14 days respectively (2 – 4 fold over concentration for unelicited condition). With the treatments of 0 – 100 ppm methyl jasmonate fuming for 14 days, vindoline accumulation in leaves increased linearly with methyl jasmonate concentration. The direct binding model of elicitor to receptor was well fitted with the data of vindoline accumulation in *C. roseus* responded to methyl jasmonate concentration.

Department……Chemical Engineering…….Student's signature…….

Field of study….Chemical Engineering…….Advisor's signature…….

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ABBREVIATIONS AND NOMENCLATURES

Ca^{2+}	:	Calcium ion
C.roseus	:	*Catharanthus roseus*
D4H	:	desacetoxyvindoline 4-hydroxylase
DA	:	Degree of acetylation
DAT	:	4-o-acetyltransferase
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxy ribonucleic acid
DP	:	Degree of polymerization
E	:	Elicitor
g	:	Gram
HPLC	:	High-performance liquid chromatography
JA	:	Jasmonic acid
k	:	Rate constant
K_m	:	Michaelis-Menten constant
MeJA	:	Methyl jasmonate
mg	:	Milligram
MIA	:	N-(methoxycarbonylethyl)-N-[2-(1*H*-indol-3yl)-ethyl]- β-methyl alaninate
MW	:	Molecular weight
ppm	:	Parts per million
R	:	Receptor
SSS	:	Strictosidine synthase
t	:	Time
TDC	:	Tryptophan decarboxylase
TIA	:	Terpenoid indole alkaloid
UV	:	Ultraviolet
V_m	:	Maximum forward velocity of the reaction
V/V	:	Volume by volume
XAD-7	:	Amberlite resin

CHAPTER 1

INTRODUCTION

1.1 General

The Madagascan periwinkle (*Catharanthus roseus*) is a source of pharmaceutically important indole alkaloids. Two of the various alkaloids are vinblastine and vincristine, which accumulate only in trace amount in plant. The only commercialized production of vinblastine and vincristine is the direct extraction from dried periwinkle plant. The precursors of the antitumour drugs are monomeric alkaloids; vindoline and catharanthine. Therefore, several efforts have been created to produce the precursors of the anticancer drugs instead because catharanthine and vindoline are found in relatively high concentrations in *C. roseus*. There are several researches about enhancing the indole alkaloid levels. Much of the work is focus on tissue-culture -level experiment such as cell suspension culture and hairy root culture. However, vindoline could not be synthesized in plant cell culture or hairy root culture. Moreover, in order to do such experiments, complicating techniques are needed. In other hands, carrying out the experiment with the intact field grown plants may give the alternative possibility to produce vindoline. In addition, it does not need many tedious techniques and might be easily applied to the nowadays-commercialized method because there is no need for skilled expertises.

Many secondary plant compounds protect plants against trivial environment. Their formation is greatly influenced by external factors. Therefore, we can take advantage of these ecological connections to produce *Catharanthus* alkaloids. By applying exogenously to plant, the selected elicitors may be able to stimulate the intact plant to produce the desired secondary products.

1.2 Objective of the study

To determine effects of elicitors, namely chitosan and methyl jasmonate, on the production of indole alkaloids by *C. roseus* leaves.

1.3 Scope of the study

1.3.1 Grow *C. roseus* plants in pots for the elicitation experiments approximately 50 plants every month for 4 months.

1.3.2 Determine effects of elicitors such as chitosan and methyl jasmonate on indole alkaloid biosynthesis of *C. roseus* leaves from the intact plants.

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

2.1 Background

2.1.1 Introduction to *Catharanthus roseus*

Madagascar periwinkle (*Catharanthus roseus* Don formerly *Vinca rosea*) belongs to Dogbane family (Apocynaceae). It is a tender erect perennial with oblong leaves, growing to 2 feet tall and everblooming. The flowers are rosy purple or white, 1.5 inches across. Its origin is unknown but now naturalized in the tropics of every continent. Madagascar periwinkle prefers a light sandy loam, and will not do well in a very heavy soil. When watering, the soil should be well soaked and then left alone for several days. It does well in semi-shady situations.

The Madagascar periwinkle produces an extremely diverse array of indole alkaloids of which the bisindole alkaloids vinblastine and vincristine are well known, due to their antimitotic and therapeutic applications. Their development is as anticancer drugs. They are now necessary components of combination chemotherapy regimens used to treat a number of cancers. While vinblastine is used in the treatment of Hodgkin's lymphoma, breast cancer, and testicular cancer, vincristine is commonly used to treat leukemia, Wilm's tumor, and embryonal rhabdosarcoma (Misawa and Goodbody,1996).

The alkaloids are a large family of nitrogen-containing secondary metabolites found in approximately 20% of the species of vascular plants. As a group, they are best known for their striking pharmacological effects on vertebrate animals. Alkaloids are usually synthesized from one of a few common amino acids – in particular, aspartic acid, lysine, tyrosine, and tryptophan. Most alkaloids are now believed to function as defenses against predators, especially mammals, because of their general toxicity and deterrence capability (Taiz and Zeiger, 1998).

2.1.2 Vindoline

Vindoline is a monoterpene indole alkaloid of *Aspidosperma* group. It exists predominantly in the green aerial parts particularly in the leaves. Only trace amount of vindoline is found in the cell and tissue cultures. Its biosynthetic enzyme is N-methyl transferase, which is located in chloroplasts. Vindoline is also the predominant alkaloid in hypocotyl and cotyledon. It can not be biosynthesized in hairy root culture and cell

Figure 2.1 *Catharanthus roseus*

suspension culture. Therefore, metabolic engineering may aid in the overproduction of this alkaloid (Shanks et al.,1998).

The biosynthesis of vindoline in *C. roseus* is a highly regulated process (Vazquez-Flota, 2000). The biosynthesis of vindoline involves the formation of strictosidine, which is a common intermediate for the formation of the more than 100 monoterpenoid indole alkaloids of *Catharanthus* (Scott, 1979). Two reactions involved in strictosidine biosynthesis (Figure 2.2) include tryptophan decarboxylase, which converts tryptophan into tryptamine and strictosidine synthase (SSS), which couples tryptamine with iridoid-glycoside secologanin. Different intramolecular rearrangements of the terpenoid moiety of strictosidine result in the formation of the corynanthe-, iboga and aspidosperma- type alkaloids. Strictosidine can be converted into tabersonine by an essentially uncharacterised pathway and tabersonine is converted into vindoline by a sequence of six enzymatic reactions. The first four reactions transform tabersonine into

desacetoxyvindoline, and the last two reactions involve 4-hydroxylation of desacetoxyvindoline to yield deacetylvindoline, followed by 4-o-acetylation to yield vindoline. These reactions are catalysed by desacetoxyvindoline 4-hydroxylase (D4H) and by deacetylvindoline 4-o-acetyltransferase (DAT), respectively (Vazquez-Flota, 2000).

Vindoline is isolated from the plant basically with solvent extraction by partitioning the alkaloid bases and their salts between organic and aqueous phases. The final isolation is done by thin-layer chromatography or HPLC (Stockigt and Soll., 1980; Rahman et al., 1983). As vindoline is polar and not very volatile, high-performance liquid chromatography (HPLC) on a reversed-phase column is commonly used for indole alkaloid analysis of *C. roseus* using gradient or isocratic systems. UV detection at the wavelength 254 nanometre is widely used in the analysis (Naaranlahti et al., 1987).

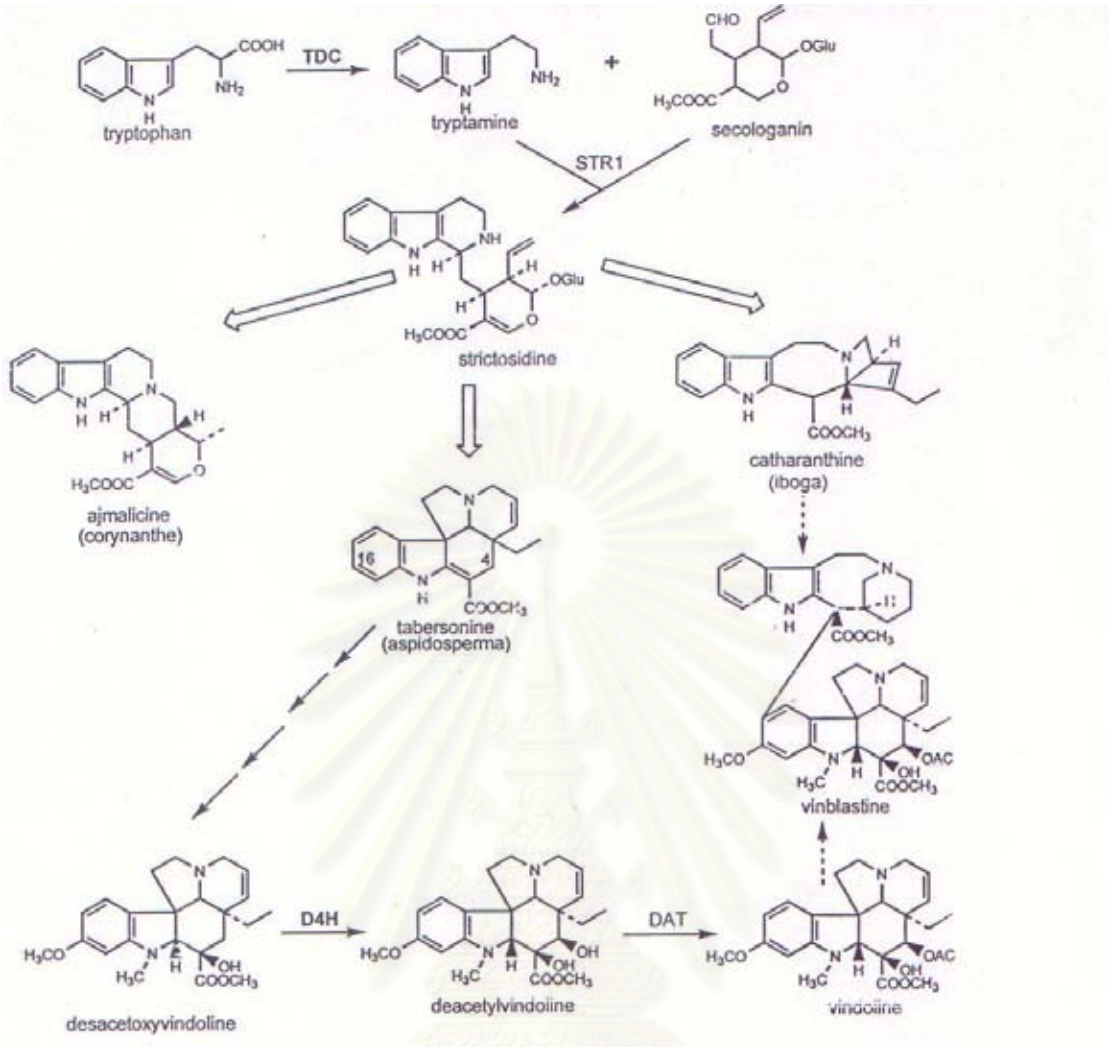

Figure 2.2 Scheme of monoterpenoid indole alkaloid biosynthesis leading to the corynanthe, aspidosperma and iboga types, which are derived from the central intermediate strictosidine. Tabersonine (Aspidosperma) is converted to vindoline by 6 enzymatic steps. Vindoline is coupled enzymatically to catharanthine (iboga) to yield dimeric indole alkaloids like vinblastine. Solid lines represent single reactions, dashed lines represent multiple reactions.(From Vazquez-Flota et al.,2000)

2.1.3 Elicitors

The accumulation of secondary products by plant cell cultures can be enhanced by stress factors such as osmotic shock, addition of inorganic salts, heavy metal ions, fungal homogenates and UV irradiation . As a response to the stress, the enzymes of biosynthetic pathways other than the primary metabolism are induced, resulting in an accumulation of secondary products. These stress agents are normally referred as elicitors. Several *C.roseus* cell lines respond to elicitation with an induction of the indole alkaloid biosynthetic pathway. The response to elicitation is highly dependent on the cell line (Moreno et al., 1995).

2.1.3.1 Chitosan

Chitosan, the deacetylated form of chitin, is a polysaccharide formed primarily of repeating units of β-(1$\rightarrow$4)-2-amino-2-deoxy-D-glucose (or D-glucosamine). Chitosan is insoluble in most organic solvents and in water at neutral pH. However, it dissolves in acidic solutions. Chitosan has been shown to suppress several plant diseases, to act as an anti-fungal agent against various fungi, and as an elicitor of various plant defense responses (Ben-Shalom et al., 2000).

2.1.3.2 Methyl jasmonate

It has been demonstated in numerous plants and cell cultures that jasmonic acid (JA) and methyl jasmonate (MeJA), a plant hormone, trigger the accumulation of the secondary metabolites. MeJA is produced in the "lipoxygenase pathway" (Aerts et al., 1996). JA has been suggested to act as a signal mediator of various types of stress to physiological responses including the accumulation of secondary metabolites (Ishihara et al., 2002). MeJA can also induce the production of terpenoid indole alkaloid secondary metabolites (Menke et al., 1999).

2.1.4 Developmental process

The process that cells commit to differentiate into specialized structures is called developmental process. The developmental processes result in biochemical specialization of cells for the biosynthesis or accumulation of secondary metabolites. Studies with germinating seedlings have suggested that alkaloid biosynthesis and accumulation are associated with seedling development (St-Pierre et al., 1999). Studies with mature plants also reveal this type of developmental control (Westekemper et al., 1980; Frischknecht et al.,1986). Vindoline biosynthesis in *C. roseus* also appears to be under this type of developmental control (St-Pierre et al., 1999). In the leaves of *C.roseus*, vindoline is enzymatically coupled with catharanthine to produce the powerful cytotoxic dimeric alkaloids vinblastine and vincristine. Vindoline as well as the dimeric alkaloids are restricted to leaves and stems, whereas catharanthine is distributed equally throughout the aboveground and underground tissues (Westkemper et al., 1980). The biosynthesis of catharanthine and vindoline are differentially regulated. Vindoline biosynthesis is under more rigid tissue-, development-, and environment-specific control than that of catharanthine (St-Pierre et al., 1999).

2.1.5 Subcellular compartmentation

Another important factor in the regulation of alkaloid metabolism is the subcellular compartmentation (Moreno et al.,1995). This mechanism allows the plant cell to separate the enzymes from their substrates and end products. Transport of enzymes, substrates and products is thus an important regulatory factor. So far only a limited number of enzymes in the alkaloid pathway have been clearly localized. A hypothetical compartmentation of alkaloid biosynthesis can be seen in Figure 2.3 The involvement of three cellular compartments has been demonstrated in alkaloid biosynthesis, namely vacuole, cytosol, and plastids (Meijer et al., 1993d).

The conversion of tryptophan into tryptamine was shown to take place in cytosol, whereas SSS has been localised inside the vacuoles. The strictosidine formed inside the vacuoles has to be transported to the cytoplasm where the glucose moiety will be

removed leading to the biosynthesis of different alkaloids. As *C.roseus* cell suspension cultures accumulate large amounts of strictosidine, alkaloid biosynthesis can also be regulated by the transport of strictosidine to the cytoplasm. These findings point to an important regulatory role of the mechanisms involved in the transport of the several intermediates into the different compartments.

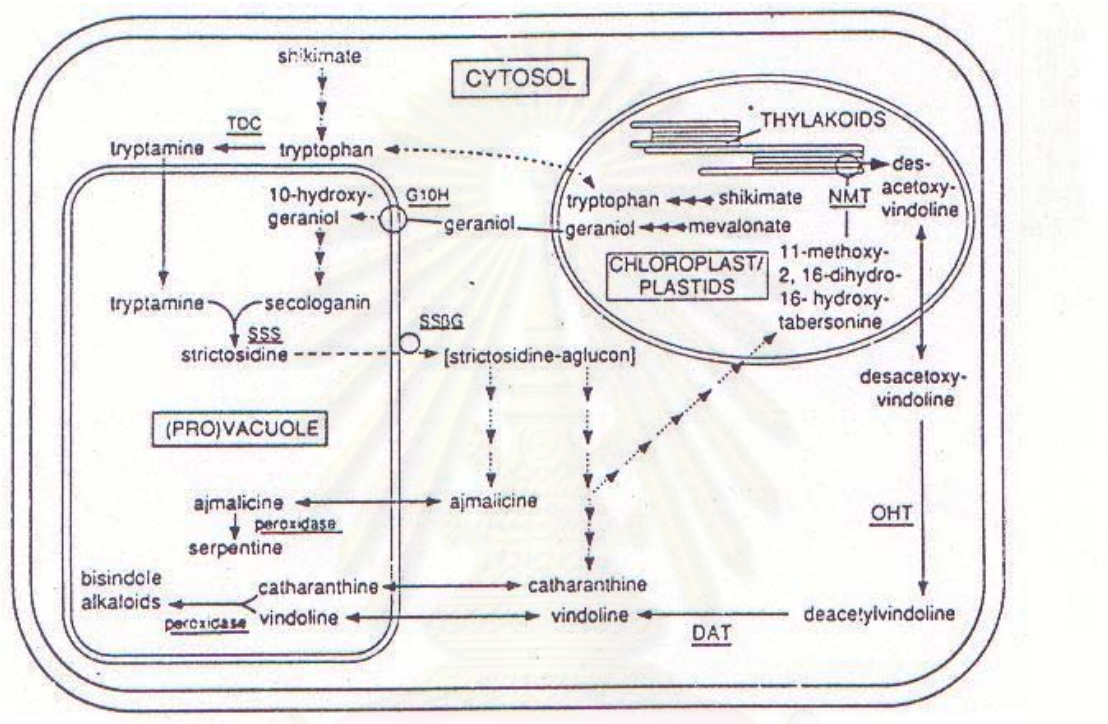

Figure 2.3 Compartmentalisation of terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. The localisation of enzymatic steps indicated with dased arrows is hypothetical. Circles indicate membrane-associated enzymes (From Moreno et al.,1995).

2.1.6 Environmental effects

Biosynthesis of natural products is also strongly affected by environmental influences. One of the most important external factors is water. Plants living under environmental stress conditions could increase levels of chemical defense compounds. In addition, in cell culture systems, the application of environmental stress factors may promote secondary product formation (Frischknecht et al., 1987).

2.2 Literature review

A complete analysis of a 14-week-old vegetative plant was performed to determine the distribution of vindoline in *Catharanthus* plants . It was found that the very youngest leaves in the upmost apical region showed about a 10-fold lower concentration than the more developed leaves (Figure 2.4). The average contents of the alkaloid increased with age of the leaf, however, a drastic and consistent drop in vindoline concentration occurred in older leaves. The highest concentration of vindoline was found in just fully matured, non growing-old leaves. It was suggested that alkaloid was translocated out of the growing-old tissue or further metabolized within the leaf to material with little or no immuno reactivity. In addition, the average vindoline content is dependent on the species (Westkemper et al., 1980). The relative distribution of the vindoline concentration in different *Catharanthus* species is shown in Table 2.1. The localization and storage of alkaloids in a cell suspension culture of *C. roseus* was investigated. Fluorescence microscopy and electron microscopy indicated alkaloid accumulation to occur inside the vacuoles of particular cells (Neumann et al.,1983).

Table 2.1 Average vindoline concentration in leaf tissue of different *Catharanthus* species.

Species	Vindoline (%dwt)
Catharanthus lanceus	0.33
Catharanthus longifolius	0.50
Catharanthus ovalis	0.17
Catharanthus roseus	0.48
Catharanthus trichophyllus	0.39

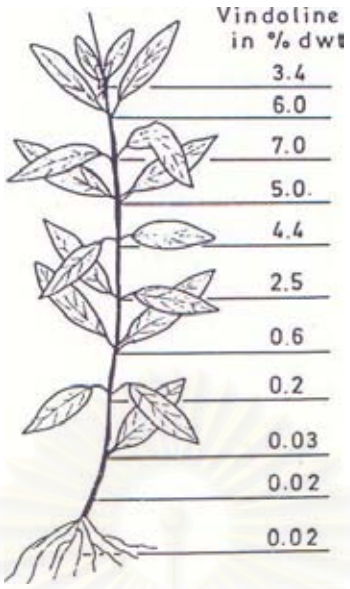

Figure 2.4 Distribution of vindoline (%dwt) within a 14 week old plant of *C. roseus* (From Westekemper et al., 1980)

Indole alkaloid formation in *C.roseus* under drought and wounding stress caused by cutting off plant parts was monitored. Drought stress effected a decrease of the relative alkaloid content. In growing tissue, wounding stress led to an increase up to 100% in alkaloid accumulation. Moreover it was found that variation of alkaloid accumulation in leaves during stress reduced, degree of chemical defense in growing leaves at the moment of stress increased, but degree in fully developed leaves unchanged (Frischknecht et al.,1987). In germinating seedlings of *C. roseus*, the expression of indole alkaloid biosynthetic capability was shown to be under developmental control. The activities of enzymes involved in indole alkaloid biosynthesis to the final product vindoline were under strict developmental control. It was demonstrated that while the majority of genes controlling indole alkaloid biosynthesis were expressed early in germination and in all plant tissues, those involved in the late stages of vindoline biosynthesis were only expressed in the aerial parts of the plant. The results demonstrated that the biosynthetic pathway was separated developmentally. Moreover expression of the late stages of vindoline biosynthesis might not simply correlate with the presence or absence of chloroplasts (DE Luca et al., 1988). The biotransformation of a synthetic product, N-(methoxycarbonylethyl)-N-[2-(1*H*-indol-3yl)-

ethyl]- β- methyl alaninate (MIA), in order to stimulate the biosynthesis of biologically active indole alkaloids in the roots and aerial parts of *C.roseus* cultivated *in vitro* was investigated. The results showed a large increase (90%) of ajmalicine, transformed from MIA, in the roots. In the aerial parts, vindoline levels were age dependent and increased by about 57% from MIA transformation in 35 days (Bonzom et al.,1997).

Various plant signalling molecules were tested for their ability to enhance alkaloid synthesis in *Catharanthus* seedling. The compound tested included plant hormones, fatty acid-derived messengers and agents that could induce systemic-acquired resistance in plants. Only MeJA enhanced the synthesis of monomeric alkaloids. In addition, all the lipoxygenase pathway inhibitors tested did not block alkaloid accumulation in the seedlings. It was concluded that although MeJA was able to enhance alkaloid synthesis when supplied exogenously, the lipoxygenase pathway probably did not play a role in the regulation of alkaloid synthesis during normal germination of *Catharanthus* (Aerts et al.,1995). The elicitation on *Taxus cuspidata* suspension cultures with various concentrations and combinations of methyl jasmonate was carried out. Taxol productivity increased 19-fold when *T. cuspidata* suspension cultures were exposed to 5-ppm ethylene and 10μM methyl jasmonate. This increase was 15-fold when either 0 or 10-ppm ethylene was combined with 10-μM MeJA (Mirjalili and Linden, 1996). JA was found to induce the production of phytoalexin in suspension-culture rice (*Oryza sativa* L) (Nojiri et al.1996). The elicitation on *Taxus canadensis* suspension cultures with MeJA was examined. A dramatic increase of pacilitaxel was obtained when the cultures were elicited with 100-μM MeJA (Phisalaphong and Linden,1999). The induction of paclitaxel biosynthesis was enhanced when adding MeJA with chitin and chitosan derived elicitors in the plant cell suspension cultures of *Taxus canadensis*. The N-acetylchitohexaose 0.6 mg/l with the 100-μM MeJA made the paclitaxel concentrations greater than 100-μM MeJA treatment-controls about 3-fold and nearly 15-fold greater than MJ-minus controls. Co-mediation of chitosan hydrolysate and 100-μM MeJA revealed the dose-of-chitosan dependence of the paclitaxel production. Using 75 mg/l of chitosan hydrolysate together with MeJA also showed a function of MeJA concentrations in paclitaxel production (Linden and Phisalaphong,

2000). The effects of JA on secondary metabolism in barley (*Hordeum vulgare* L.) were investigated. The amount of a particular compound increased in excised barley leaf segments that had been treated with JA (Ishihara et al., 2002).

Late exponential phase hairy root cultures of *C.roseus* were elicited with pectinase and JA. An increase of 150% in tabersonine specific yield was observed upon addition of 72 units of pectinase. JA was found to be a unique elicitor leading to an enhancement in flux to several branches in the indole alkaloid pathway. JA addition caused an increase in the specific yields of ajmalicine(80%), serpentine (60%), lochnericine (150%), and horhammericine (500%) in dosage studies by adding 0.25mg to 2.5mg of JA per flask, harvested at the time range 14 hours to 100 hours depending on the alkaloid optimum time period (Rijhwani and Shanks, 1998). The effect of elicitor dosage and exposure time on biosynthesis of indole alkaloids by *C.roseus* hairy root cultures, using MeJA as elicitor was studied. It was found that 100-μM MeJA gave the maximum secretion of ajmalicine and catharanthine. At 10-μM MeJA, ajmalicine content was four-fold with respect to the untreated roots (Vazquez-Flota et al.,1994). The elicitation by chitin and JA in *C.roseus* hairy root cultures was studied. It was found that chitin could enhance ajmalicine level by 50%. JA could increase the specific yields of ajmalicine by 80% and serpentine by 60%. Other alkaloid levels (lochnericine and horhammericine) increased with increasing JA concentration (Shanks et al., 1998).

The interactions between cytokinins and ethylene on alkaloid accumulation in a periwinkle cell line were examined. Exogenously applied either cytokinins or ethylene enhanced the ajmalicine accumulation in cell suspension culture of *C.roseus*. Moreover, cytokinin-enhancing effect was not the result of endogenous ethylene enhancement. In addition, the responses to exogenous cytokinin and ethylene were additive (Yahia et al., 1998). The effects of exogenous cytokinin on *C.roseus* cell culture were investigated. Cytokinins were found to stimulate the alkaloid synthesis induced by removing auxin from the medium (Decendit et al., 1992). The elicitation of *C.roseus* cell suspension culture by cell wall of *Phytophthora megasperma* f.sp glycinea (Pmg elicitor) was studied. The cells were pretreated with 2,6-dichloroisonicontinic (DCIA) or 5-chlorosalicylic (5CSA) acid before elicitor addition 1 day. It was indicated that these compounds improved the elicitor reception system (Frankmann and Kauss, 1994).

The combined actions of *in situ* adsorption, permeabilization, and fungal elicitation by *Penicillium* sp. on the production and secretion of catharanthine and ajmalicine were investigated. The release ratio of catharanthine and ajmalicine by XAD-7 and DMSO was enhanced by 20 and 70 %, respectively, which was 3.4 and 2 times higher than in the culture with *in situ* adsorption by XAD-7 alone. Over a 27-day culture period, the total production of catharanthine and ajmalicine were 67 and 30.2 mg/l, respectively, which was about 3 and 13 times higher than in the culture with the addition of XAD-7 alone (Sim et al., 1994).

The effects of the degrees of polymerization (DP)and N-acetylation (%DA) of chitosan in elicitation of callose formation in *C.roseus* cell suspension were studied. It was found that increasing the DP caused an increase in callose formation at low concentrations of chitosan, and also raised the maximum values attained. For fully deacetylated chitosan (0%DA), the chitosan of DP 2500, MW 552 kDa gave better callose formation than DP 30 about 14 fold. For 23%DA chitosan, the chitosan of DP 7567, MW 1666 kDa produced higher callose than chitosan of DP 82, MW 18 about 9 fold (Kauss et al., 1989). The effect of chitosan treatment in eliciting tomato leaves by spraying the leaves of the whole plant was studied. It was found that the higher percentage of DA, the better enzyme induction. The 30% DA chitosan polymer of molecular weight about 500 kDa enhanced greater activity than the 5% DA chitosan polymer of the same molecular weight. The 5% DA chitosan gave the induced activity of three enzymes; bound chitosanase, bound chitinase, and bound β-1,3-glucanase by 5.6, 9.3, and 6.7 fold, respectively. The 30% DA chitosan induced the activity of bound chitosanase, bound chitinase, and bound β-1,3-glucanase by 8.2, 10.0, and 9.7 fold respectively. In addition, chitosan polymer induced elicitation whereas chitosan oligomers did not. It was suggested that 30% DA chitosan controlled the elicitation through a combination of the degree of acetylation and molecular size (Ben-Shalom et al., 2000).

Plant cell suspensions of grape cells (*Vitis vinifera* L.cv Gamay Freaux) were grown in shake flasks operated both in the batch and semicontinuous mode. A mathematical model described grape cell growth, sucrose uptake and secondary metabolite (anthocyanin) production was developed. Viable biomass along the process

and non-growth-associated production of secondary metabolite were assumed. The equation of secondary metabolite included the rate of secondary metabolite catabolism. The model was able to predict the results for semicontinuous experiments (Guardiola et al., 1995). A simple induction model to explain the action and effects of both ethylene and MeJA on Taxol production was proposed. The model regarded to receptor binding and regulatory systems in plants (Mirjalili and Linden, 1996). A mathematical model for plant cell culture of *C.roseus* in an airlift fermentor was proposed. It included viability into biomass equation and regarded non-growth associated production as well as growth-associated production of secondary metabolite in product equation (Werning et al., 1992). The stoichiometry of maintenance and carbohydrate storage as well as ajmalicine production kinetics of non-dividing *C.roseus* cells in the second stage of a two-stage batch process was investigated. In the mathematical model, the biomass was divided in two compartments: active biomass and storage carbohydrates. In addition, the model coupled the production of a secondary metabolite with several essential environmental conditions. The results showed that the model predicted maintenance, biomass formation and ajmalicine production with a reasonable accuracy under various process conditions, despite the fact that the model parameters were obtained from batch cultures (Schlatmann et al., 1999). Kinetics of paclitaxel production *by Taxus canadensis* cultures in batch and semicontinuous with total cell recycle was studied. Paclitaxel accumulation in semicontinuous culture with total cell recycle increased by a factor of 4.0 relative to that in the batch culture during 35 days of cultivation. Production yield and total sugar uptake in the semicontinuous culture with cell recycle increased about 3-fold relative to those in the batch culture (Phisalaphong and Linden, 1999a). Ethylene and MeJA acted as co-mediators in the taxane production of *Taxus canadensis*. Inhibition of paclitaxel production by MeJA was observed at the MeJA concentrations greater than 200 μM. A mathematical equation was shown for the inhibition. A Hill plot was performed to calculate several allosteric parameters relating paclitaxel productivity to MeJA concentration. (Phisalaphong and Linden, 1999b),

A method of vindoline isolation by a combination of selective precipitation and preparative high pressure liquid chromatography was reported (Atta-ur-Rahman et al.,1983). A radioimmunoassay for the quantification of vindoline was developed. The

amount of 0.2-45 nanogram of vindoline could be measured. Several hundred-quantitation assays could be performed in one day per person. Crude extracts could also be used for the test (Westkemper et al., 1980).

CHAPTER 3

THEORY

A number of physical and chemical factors could influence plant secondary metabolism. Elicitors, compounds triggering the formation of secondary metabolites, are considered to be one of the effective tools for increasing biosynthesis pathways in plants.

3.1 Elicitation

Plants exhibits a variety of defense mechanisms against potential pathogens, including phytoalexin production, reinforcement of plant cell walls through the deposition of callose, the synthesis of lignin-like materials, and the enhancement of the activities of certain hydrolytic enzymes such as chitinase or glucanase (Chang and Sim,1994)(Figure 3.1).

The initiation of a plant defense requires elicitors which may be the perception of pathogen-derived (exogenous) or plant derived (endogenous) signal molecules. They are classified depending on their origin as biotic or abiotic elicitors. Biotic elicitors include polysaccharides derived from plant cell walls (e.g., pectin or cellulose) and microorganisms (e.g., chitin, chitosan, or glucans). The primary reactions upon elicitation with a biotic elicitor are thought to be composed of recognition of the elicitor and its binding to a specific receptor protein on the plasma membrane. The next step in elicitation is thought to be inhibition of plasma membrane ATPase, which reduces the proton electrochemical gradient across this membrane (Dornenberg and Knorr, 1995). Some of the changes in protein phosphorylation are among the earliest known events following elicitation (Chang and Sim,1994).

A model for the induction of plant defense responses was proposed with the assumption 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 (Dixon et al.,1990). It was suggested that the transduced signals between the elicitor-receptor complex and the gene activation process were either transported locally by diffusion through intercellular and extracellular fluids or infection sites or systemically through the vascular system of the plants (Farmer and Ryan, 1990). The response capacity to elicitors could be limited by three factors: enzymatic capacity, resource allocating capacity to the pathway, or elicitor perception capacity of the tissue (Singh et al., 1994). However, molecular basis of the specific perception and transduction of these signals largely remain unsolved.

3.1.1 Chitosan

Oligosaccharide elicitors derived from the cell surface of pathogenic microbes, as well as host plants, are found to be an effective inducer for various defense responses in plants. Addition of oligosaccharides to plant cell cultures mimics the effects of elicitation from some pathogenic microbes.

The degree of acetylation of chitosan and its molecular size are important in inducing defense metabolisms in the plant cell cultures (Dornenberg and Knorr, 1995; Ben-Shalom et al., 2000). Qualitative (time of elicitation) and quantitative (extent of elicitation) of chitosan are factors affecting elicitation (Ben-Shalom et al., 2000).

The susceptibility of the plant material is also an important factor for chitosan to elicit the plant cells. In 4 * 10^5 *C.roseus* protoplasts in 2 ml of buffer, 1μg of chitosan elicites a considerable response, while in 300 mg of cells at least 20μg of chitosan is required (Kauss et al., 1989).

When the polycations of chitosan bind to the polyanions of phospholipids at plasma membrane, chitosan, with the presence of Ca^{2+} (15 μM outside the plasma membrane and 5μM in cytoplasm) is able to stimulate the DNA transcription of enzymes or proteins (Figure 3.2). Callose formation occurs, only when there is a balance between inflowed Ca^{2+} and outflowed substrates, followed by chitinase or proteinase inhibitor synthesis (Kohle et al., 1985). Treatments inducing callose deposition also leads to other changes in cell metabolism such as the formation of a phytoalexin in soybean cell suspension (Kohle et al.,1984; Kauss 1987a).

3.1.2 Methyl jasmonate

The induction of defense response can be mediated by elicitation to the plant or wounding the plant. The defense responses include the biosynthesis of secondary metabolites and proteinase inhibitors. The signal transduction pathway of defense response involves a lipid based octadecanoid pathway leading to jasmonic acid (JA). Upon wounding, the octadecanoid pathway is activated by polypeptides and oligouronoids, resulting in the elevated levels of JA. Then JA activates the synthesis of wounding-inducible proteinase inhibitors. In other words, the octadecanoid pathway is involved in signal transduction, leading to transcriptional activation of proteinase inhibitor genes. In the same way, JA also involves as an intermediate signal in elicitor-induced secondary metabolite accumulation. Therefore, in elicitor-induced secondary metabolism, JA plays a similar role to its role in the accumulation of wounding-induced proteinase inhibitors. Some biotic elicitors and abiotic elicitors include wounding and UV radiation can trigger a transient increase in endogenous JA in plants (Menke et al., 1999).

JA exerts 2 possible roles on alkaloid accumulation. Firstly, enlargement of the alkaloid precursor pool and secondly, enhancement of several enzyme activities in alkaloid biosynthesis (Aerts et al.,1995).

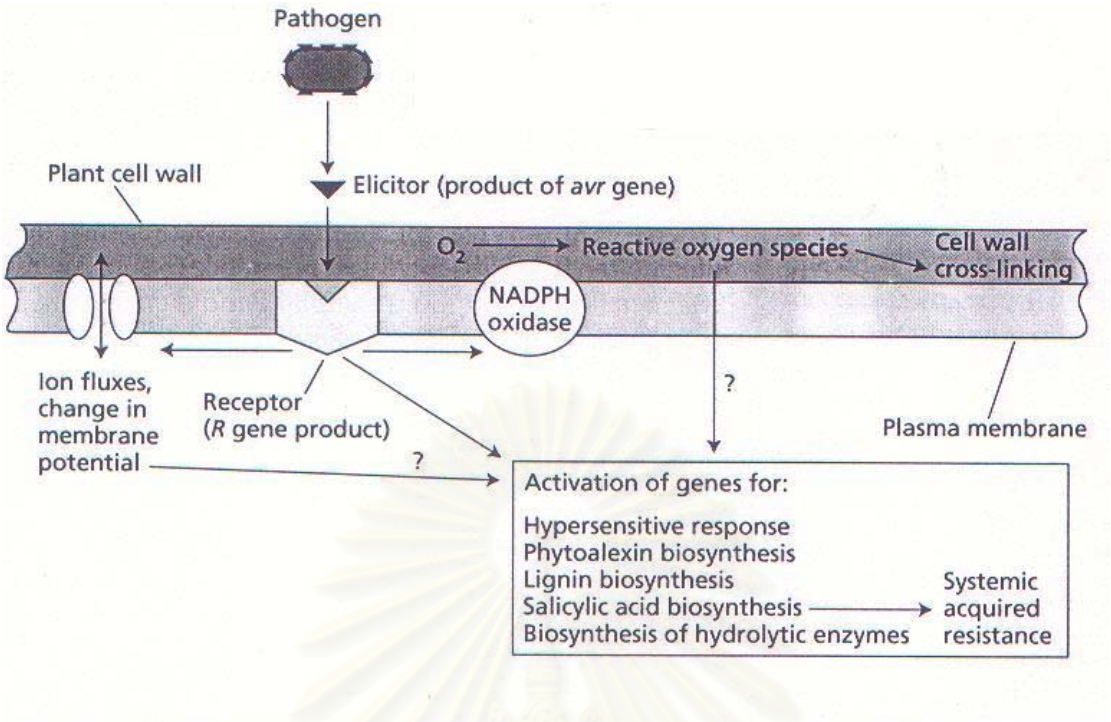

Figure 3.1 Many modes of antipathogen defense are induced by infection. Fragments of pathogen molecules called elicitors initiate a complex signaling pathway leading to the activation of defensive responses (From Taiz and Zieger, 1988).

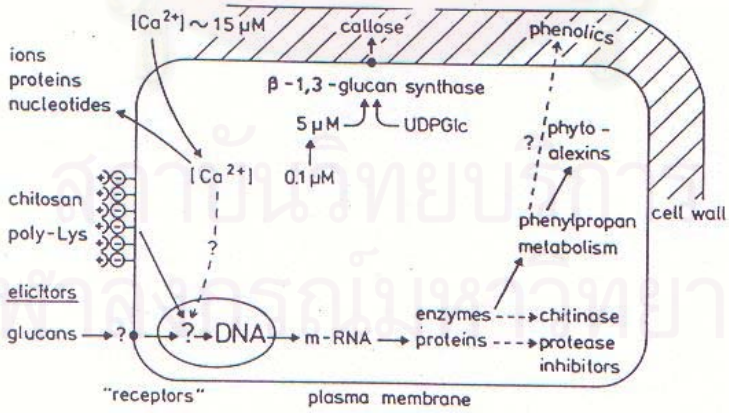

Figure 3.2 Proposed events leading to chitosan-elicited callose synthesis followed by induction of some other metabolic changes regarded to be of importance in resistance against pathogens (From Kohle et al., 1985).

A hypothetical mechanism of action for MeJA was proposed (Gundlach et al., 1992). Exogenously applied JA may be recognized by a plasma membrane receptor. An elicitor-receptor complex activates a lipase releasing α-linolenic acid, which is then transformed by constitutive enzymes to JA and MeJA, 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. Figure 3.3 shows a hypothetical model for elicitor transduction by Menke at al.(1999). Elicitation of terpenoid indole alkaloid (TIA) biosynthetic gene expression is mediated through protein phosphorylation and the octadecanoid pathway leading to jasmonate.

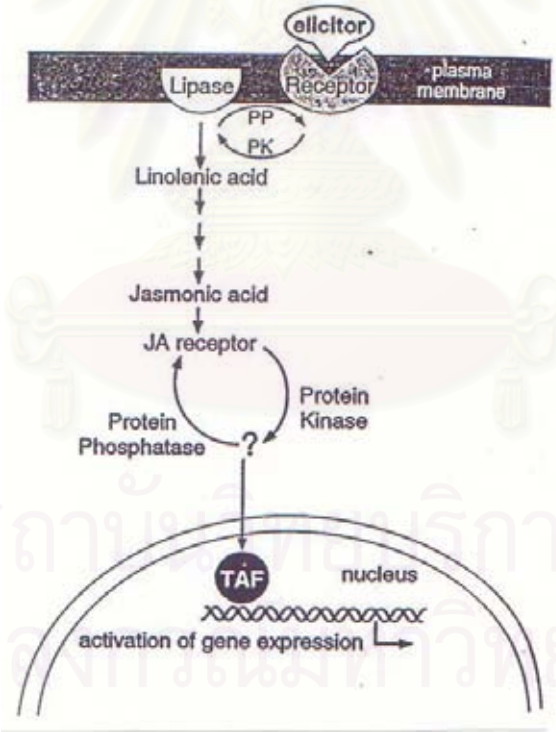

Figure 3.3 Model for elicitor signal transduction leading to TIA biosynthetic gene expression. The model shows the involvement of protein phosphorylation and the octadecanoid pathway in elicitor induced TIA biosynthetic gene expression. (from Menke et al.,1999) PK, Protein kinase; PP, protein phosphatase; TAF, transcription-activating factor.

3.2 Biosynthesis and regulation

Several studies have been made to explain the biosynthetic pathway leading to monoterpenoid indole alkaloids and the enzymatic steps involved. The information available on the biosynthesis of indole alkaloids was mostly obtained from experiments with *C.roseus*. At present, several enzymes involved in the indole alkaloid biosynthesis have been isolated and characterised. This will enable more detailed studies on the regulation of alkaloid biosynthesis. Such studies may eventually lead to an improvement of alkaloid production by genetic modification of the plant or cell cultures (Moreno et al.,1995).

The biosynthesis of *Catharanthus* alkaloids arises from the precursors tryptamine and secologanin, derived from two different pathways. Tryptamine is formed by the decarboxylation of tryptophan by enzyme tryptophan decarboxylase (TDC). Secologanin is formed through several enzymatic steps initiated after the hydroxylation of geraniol by the enzyme geraniol-10-hydroxylase (G10H). Strictosidine synthase (SSS) couples these two products to form strictosidine, the universal precursor of all monoterpenoid indole alkaloids. TDC and G10H link primary and secondary metabolism, and SSS is the first enzymatic step in terpenoid indole alkaloid biosynthesis. The first step in the alkaloid biosynthesis after the formation of strictosidine is the removal of the sugar moiety from strictosidine to form an unstable aglycone.

Several steps in the biosynthesis of terpenoid indole alkaloids in *C. roseus* are under developmental regulation. Alkaloid metabolism seems to be restricted to certain tissues and is modulated by different developmental and environmental mechanisms.

Various environmental factors influence the secondary metabolism in cell and tissue cultures of *C.roseus*. These regulatory mechanisms in cell cultures might differ from those of the intact plant, and some of the enzymatic steps of the biosynthetic pathway might be blocked in undifferentiated cultured cells.

Vindoline and bisindole alkaloids accumulate only in green tissues, and are not found in root or cell suspension cultures. In seedlings of *C.roseus*, the expression of TDC and SSS was not only under strong developmental control but also these enzyme

activities were further modulated by tissue-specific and/or light-depentdent factors. In *C.roseus* leaves, the concentrations of vindoline is age dependent (Moreno et al., 1995).

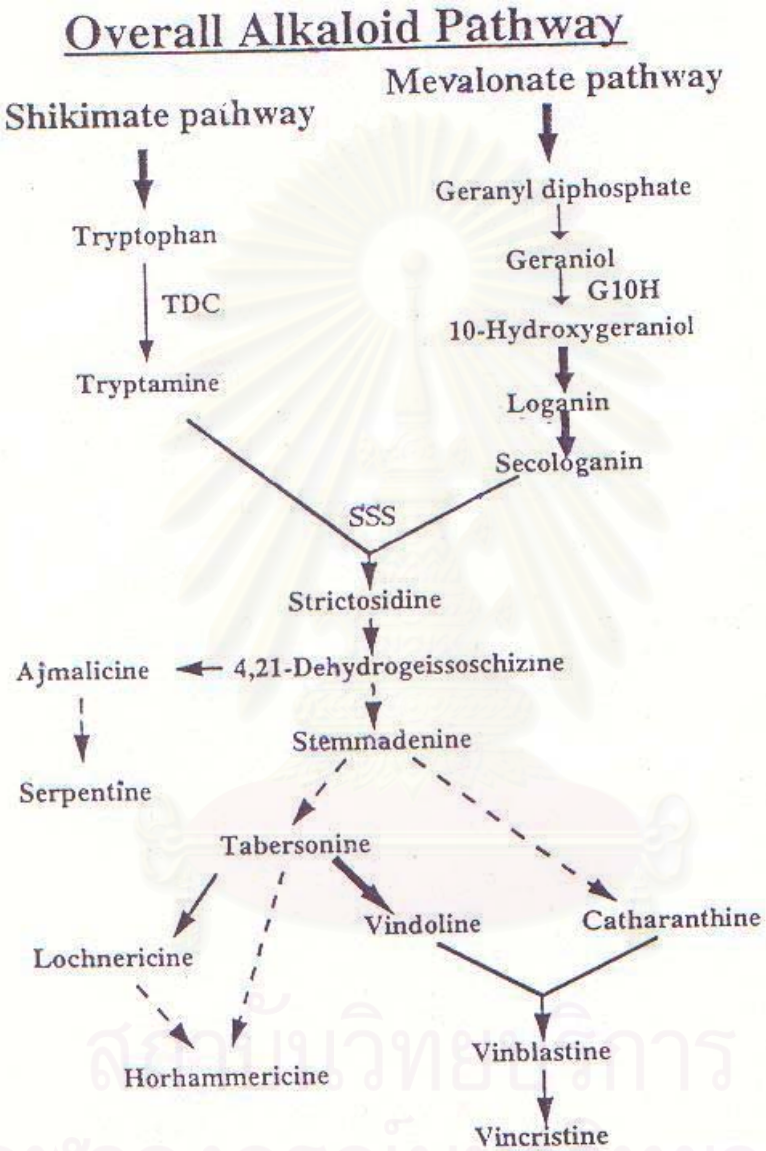

Figure 3.4 Schematic diagram of the biosynthesis of indole alkaloids in *Catharanthus roseus*. Thin arrows represent single enzymatic steps; thick arrows represent multiple steps: dashed arrows represent uncharacterized steps (From Shanks et al.,1998).

3.3 Direct model for elicitor binding

An elicitor might bind to a receptor, providing some function and then either diffusing away or being destroyed with other hormones.

The direct binding of elicitor (E) to the receptor (R) can be described by the Michaelis-Menten equation as follows.

$$E + R \underset{k_{-1}}{\longleftrightarrow} ER \xrightarrow{k_2} response \quad \text{..... ....(1)}$$

Rate of response

$$rate = \frac{d[response]}{dt} = k_2[ER] \text{..... .... (2)}$$

The rate of variation of the ER complex is

$$\frac{d[ER]}{dt} = k_1[E][R] - k_{-1}[ER] - k_2[ER] \text{..... ....(3)}$$

Since the receptor is not consumed, the conservation equation on the receptor yields

$$[R] = [R_0] - [ER] \text{..... ....(4)}$$

$$\frac{d[ER]}{dt} \approx 0$$

Assume the quasi-steady-state assumption to (3)

$$[ER] = \frac{k_1[E][R]}{k_{-1} + k_2} \text{..... ....(5)}$$

Substituting (4) to (5)

$$[ER] = \frac{k_1[E]([R_0] - [ER])}{k_{-1} + k_2} \text{..... ....(6)}$$

Solving equation(6) for [*ER*]

$$[ER] = \frac{[R_0][E]}{\frac{k_{-1} + k_2}{k_1} + [E]} \text{..... ....(7)}$$

Substituting (7) to (2) yields

$$rate = \frac{d[response]}{dt} = \frac{k_2[R_0][E]}{\frac{k_{-1} + k_2}{k_1} + [E]} \text{..... ....(8)}$$

$$rate = \frac{V_m[E]}{K_m + [E]} \text{..... ....(9)}$$

When $V_m = k_2[R_0]$

And $K_m = (k_{-1} + k_2)/k_1$ constant

Equation (9) can be linearized in double-reciprocal form:

$$\frac{1}{rate} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{[E]} \text{..... ....(10)}$$

A plot of 1/*rate* versus 1/[*E*] yields a linear line with a slope of K_m/V_m and y-axis intercept of $1/V_m$.

CHAPTER 4

MATERIAL AND METHODS

This chapter describes the apparatus and experimental methods used for alkaloid analysis from *C. roseus* leaves.

4.1 Apparatus

The apparatus used are listed as follows.

- Sonicate bath model 2210 of Branson, USA
- Centrifuge model J-25 of Beckman, USA
- Rotary evaporator model R-114 of Buchi, Switzerland
- High performance liquid chromatography (HPLC) model LC-3A from Shimadzu, Japan
  Detector model 4100 of LDC, USA

4.2 Chemicals

The chemicals used are listed as follows.

- Standard vindoline from Faith Eagle Ltd., Shenzhen, China.
- HPLC-grade Methanol from Labscan, Thailand
- Chitosan from Bioprocess Technology Programme, Asian Institute of Technology, Thailand
- Methyl jasmonate from Professor James C. Linden, Department of Chemical and Bioresource Engineering, Colorado State University, USA
- Analytical-grade $(NH_4)_2HPO_4$ from Univar, Australia
- Analytical-grade Glacial acetic acid from Labscan, Thailand
- 95.5% (V/V) ethanol commercial grade from Liquor organization, Thailand

4.3 Plant material

The plant material used is as follows.

- *C. roseus* seeds from Dr. Muenduen Phisalapong, Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Thailand

C. roseus plants were grown from seeds collected from the same maternal plant in pots every month for four months. The six-month old periwinkle plants were used in the experiments. Each plant was applied for only 1 treatment. The experiments were done in triplicate, using 3 plants for each treatment. The samples for analysis were from 3 leaves of each plant at the top, middle, and bottom position of the plant.

4.4 Experimental Methods

4.4.1 Elicitation experiments

Two elicitors were used for the study:

4.4.1.1 Chitosan 10, 20 , 30 ppm in 0.5%acetic acid solution

Chitosan was sprayed directly over the periwinkle plants approximately 5 ml/plant each time at 6 AM and 6 PM for 7 consecutive days.

4.4.1.2 MeJA 10, 50, 100, 1000 ppm in 95.5%(V/V) ethyl alcohol

5 ml of MeJA was poured into a plate and fumed under closed system for 2 hours at 6 PM for 7 or 14 consecutive days.

4.4.2 Alkaloid extraction

The leaf samples (0.15 gram) were ground in a mortar and rinsed with 45 ml methanol. The leaf suspension was transferred to the 100-ml test tubes. Then they were immediately sonicated for 5 hours and centrifuged at 12,000 rpm for 10 minutes to separate the cell debris and the supernatant. The supernatant was evaporated using a rotary evaporator at 70 – 90 ^{o}C to approximately 4 ml. The volume of the concentrated portion was recorded. The concentrated aliquot were then filtered through 0.45 micron

cellulose acetate filter paper to get rid of the minute particles in order to be ready for HPLC analysis.

4.4.3 HPLC analysis

The HPLC analysis was performed by using Hypersil C18 250 mm X 4.6 mm column. The alkaloids were separated by using isocratic condition of methanol 40 % and mobile phase 60 % with the overall flow rate 1 ml/min. The mobile phase was 41:59 by volume of Methanol: 5 mM diammonium hydrogen phosphate. The UV detection wavelength was 254 nm. Peak was qualified and quantified based on the comparison of retention times and under-peak area with authentic standards of vindoline.

CHAPTER 5

RESULTS AND DISCUSSIONS

This chapter presents the experimental results and the discussion dealing with elicitation of *C. roseus* leaves. After applying elicitors for a period of time, vindoline was extracted from the leaf samples and the concentration was measured by HPLC. The vindoline concentration was qualified and quantified against the standard curve of vindoline as in Appendix B. The experiments were done in triplicate and the samples for analysis were obtained from 3 leaves at different specified position of each plant. For each treatment, three plants without any treatment were kept separately as the control plants.

5.1 Effects of chitosan on the elicitation of *C. roseus* leaves.

The average microgram of vindoline per gram leaf responses to the concentration of chitosan was shown in Table 5.1 and 5.2. The comparison of vindoline levels of acid-treated plants and control plants was presented in Figure 5.2 (a). The vindoline levels of 0.5%-acetic-acid treated plants were not significantly different from those of the control plants. The relationship between the time of elicitation and the vindoline concentration of the control plants and the plants treated with 20-ppm chitosan is presented in Figure 5.1. As can be seen in Figure 5.1, the vindoline concentrations of the control plants and the treated plants behaved in the same ways. Therefore, the experiment was further carried out to confirm the result by applying 10, 20, and 30 ppm of chitosan in 0.5% acetic acid solution to plants, comparing with control plants. The time course of vindoline concentrations of the control plants and the plants treated with 10, 20 ,and 30 ppm chitosan solution is shown in Figure 5.2(b). As can be seen in Figure 5.2(b), the vindoline concentrations of all the treated plants and the control plants were not distinctively different with insignificant *t* test. This can be stated that chitosan at the applied range had no significant effect on the vindoline production of *Catharanthus roseus* leaves.

The degree of acetylation and molecular weight of chitosan are important to elicitation ability. For this research study, the chitosan of DP 7190, MW 1200 kDa, and %DA 14 was used, which had higher MW than the ones used by Ben-Shalom et al (2000) (MW 500kDa) in elicitation of tomato leaves and was nearly the same DP and MW as the one used by Kauss et al (1989) (DP 7567, MW 1666 kDa) in elicitation of *C.roseus* cells to produce callose. However, the %DA of the chitosan used in this experiment (%DA 14) was lower than the one used by Kauss (%DA 25). From the observation by Kauss et al., the chitosan of %DA 0 gave better result than the chitosan of %DA 23. Although, this study did not involve in callose formation, the secondary metabolite production was found by other groups to be anticipated in several plants after the callose induction according to Kauss et al.(1989). The insignificant affecting of chitosan elicitation on vindoline biosynthesis might be due to other factors, apart from the DP, the MW, and the %DA factors. However, the cells or the species of this study were different from those of Ben-Shalom et al. (2000) which used tomato leaves and those of Kauss et al. (1989) which used *C.roseus* suspension culture.

There are several reasons why chitosan cannot elicit the vindoline biosynthesis. First, chitosan might not be able to elicit the enzymes in the vindoline-biosynthesis pathway. In the case of chitin as an elicitor in suspension-cultured rice cells, chitinase is produced by host plants during the early steps of the invasion of the pathogenic fungi to derive N-acetylchitooligosaccharides from chitin. This oligosaccharide can induce phytoalexin formation in rice cells. Presence of chitinase relates to the induction of the enzyme activity by chitin (Yamada et al.,1993). In the same way, if chitosan cannot induce any enzyme in the plants that can digest chitosan to produce a substance that can stimulate vindoline biosynthesis in *C.roseus* leaves, the plants may not be able to synthesize more vindoline. In other words, *C.roseus* leaves might not be responsive to the external signals, of which chitosan induces a capable enzyme, to synthesize more vindoline. Second, chitosan might not be able to penetrate from the outest part of the leaves eg., wax, the long chain hydrocarbons crystallized on the surface of cuticle, and cutin, the long chain fatty acids covering the cellulose fibrils of the walls, to cell wall and from cell wall to plasma membrane. Comparing with tomato leaves studied by Kauss et al. (1989), *C.roseus* leaves are thicker and have more wax covering the leaves. In

addition, since the plant cell wall partially protects the plant cells against chitosan, presumably, ionic binding of chitosan to the plant cell wall polymers might be necessary (Young and Kauss, 1983). So, if there is no ionic binding of chitosan to the plant cell wall, chitosan then might not be able to penetrate through the plant cell wall to the plant cell. The elicitation of vindoline biosynthesis; therefore, can not take place. In the case of polymers such as chitin, they are supposed to be broken down into fragments that can penetrate the cell wall before the activation of plant cells (Yamada et al., 1993). Due to the fact that the chitosan used in this experiment is polymer with the high DP of 7190, it probably needs to be broken down to be shorter chains in order to be able to penetrate through barriers to the plasma membrane. Third, as external Ca^{2+} plays a role as a pool for that small fraction of Ca^{2+} required to enter cells as a second messenger of secondary metabolite productions in soybean and *C.roseus* (Kohle et al.,1985; Kauss et al., 1989), it might be necessary to have Ca^{2+} in the activation process of callose formation and DNA transcription to synthesize further proteins and enzymes. Consequently, if there is not enough Ca^{2+} amount in the cytoplasm, the Ca^{2+}-dependent callose synthesis together with other protein synthesis might not occur. Then the following up phenomenon; secondary metabolite synthesis, vindoline synthesis in this case, cannot take place at the elevated level.

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Table 5.1 Average vindoline concentrations and standard deviations of the control plants and the plants treated with 20-ppm chitosan solution and the time of elicitation.

Day	Vindoline concentration ($\mu g_{vindoline}/g_{leaf}$ DW)	
	0ppm	20ppm
0	899±190	797±211
3	742±223	1389±162
5	822±285	1691±279
7	2397±405	2842±955

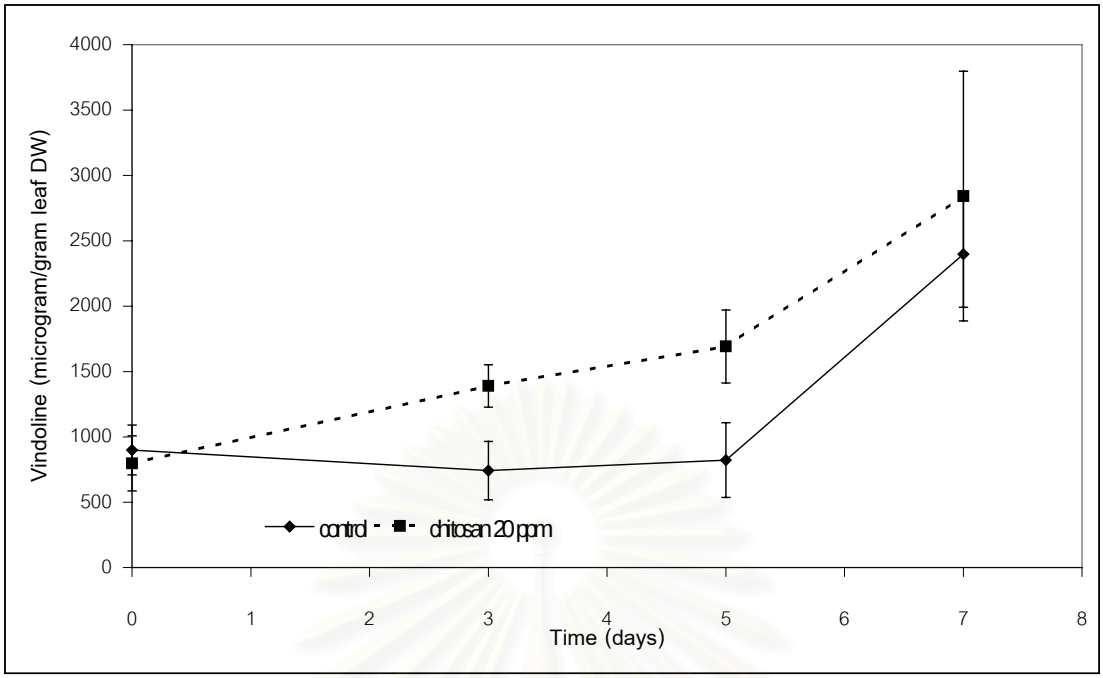

Figure 5.1 Effect of 20-ppm chitosan on vindoline concentration of *C. roseus* leaves.

Table 5.2 Average vindoline concentrations and standard deviations of the control plants and the plants treated with 10, 20, and 30 ppm chitosan solution and the time of elicitation.

Day	Vindoline concentration ($\mu g_{vindoline}/g_{leaf}$ DW)				
	control	acid control	10 ppm	20 ppm	30 ppm
0	1696±161	1472±141	1773±190	1649±154	1282±263
3	1429±296	963±261	1017±214	1540±239	1465±271
5	1173±381	1826±478	1789±336	2086±376	1094±384
7	1914±690	2175±351	2216±588	1412±395	2086±323

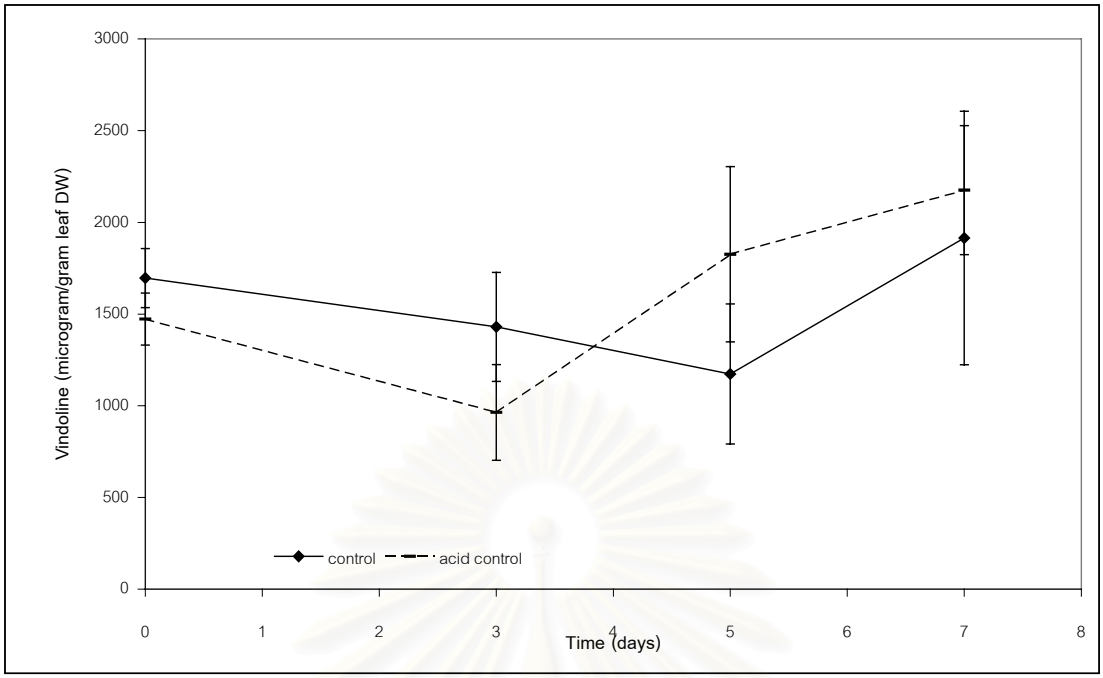

Figure 5.2 (a) Vindoline concentration from the control plants and the acid-treated control plants.

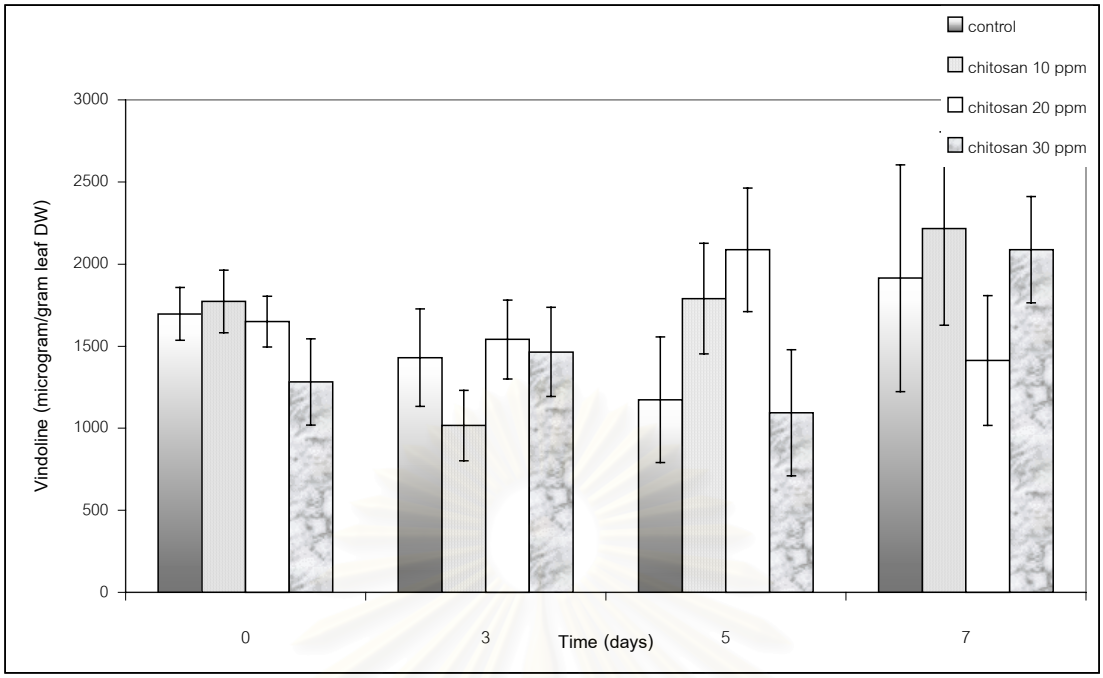

Figure 5.2(b) Effect of 10, 20, 30-ppm chitosan on vindoline concentrations of *C. roseus* leaves.

5.2 Effects of MeJA on the elicitation of *C. roseus* leaves.

The comparison of vindoline levels between control plants and ethanol-control plants was shown in Figure 5.4(a). As can be seen from Figure 5.4(a), vindoline concentrations of the control plants were similar to those of the ethanol-treated control plants, which confirmed that the amount of ethanol applying for this study did not have significant influence on vindoline level of the *C. roseus* leaves. The accumulations of vindoline from the plant leaves treated with 10, 100, and 1000-ppm MeJA were shown in Table 5.3. The time courses of the MeJA-induced vindoline formation were shown in Figure 5.3(a-c). As can be seen from Figure 5.3 (d), vindoline biosynthesis of the *C. roseus* leaves was dependent on the relative concentration of MeJA. The maximum vindoline concentration was obtained when the plant was exposed to 100-ppm MeJA. In this case, after 7 days of elicitation, the vindoline concentration was 2.3 mg/g DW or more than 4 fold over concentration for unelicited conditions. With statistical analysis, the mean vindoline levels of the 100-ppm MeJA treated group were significantly more than those of the control group at a 99% confidence interval by one-tailed *t*-test distribution.

In order to confirm the result, the experiment for MeJA elicitation was further carried on. The result was shown in Table 5.4 and the responses to the concentrations of MeJA were shown in Figure 5.4(b-f). After 14 days of elicitation with MeJA concentrations 0-100 ppm, vindoline accumulation was roughly linear with MeJA concentration. Elicitation with MeJA concentration 1000 ppm did not increase more vindoline formation; but resulted in lower vindoline concentration comparing to those with 100-ppm MeJA. The effect of MeJA on vindoline formation in the second experiment was similar to that in the first one. The optimal MeJA concentration for elicitation of vindoline production in both experiments was the same (at 100-ppm MeJA). The maximum concentration of vindoline after the elicitation with 100 ppm of MeJA for 14 days was 4 mg/g DW (1.5 fold over concentration for unelicited condition). When tested with one-tailed *t*-test distribution, it was found that 1000-ppm MeJA significantly gave the vindoline concentrations more than ethanol did at a 90% confidence

interval.Therefore, it can be concluded that MeJA was an effective elicitor to stimulate vindoline production in *C.roseus* leaves.

Table 5.3 Average vindoline concentrations and standard deviations of the control plants and the plants treated with 10, 100, and 1000-ppm methyl jasmonate and the time of elicitation.

Day	Vindoline concentrations ($\mu g_{vindoline}/g_{leaf}$ DW)			
	Control	MeJA 10 ppm	MeJA 100 ppm	MeJA 1000 ppm
0	868±142	788±240	1159±110	675±82
3	1708±146	1633±480	701±212	958±48
5	762±96	1003±63	1139±118	1078±40
7	459±86	742±367	2278±394	1123±497

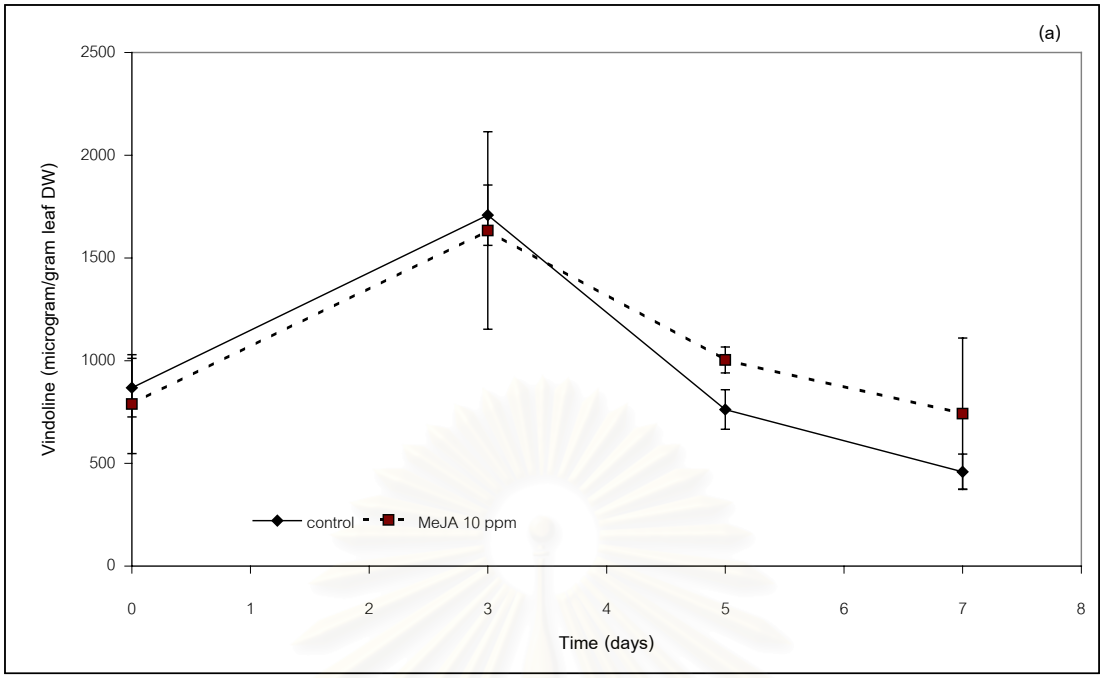

Figure 5.3 (a) Effects of 10-ppm MeJA on vindoline concentrations of *C. roseus* leaves.

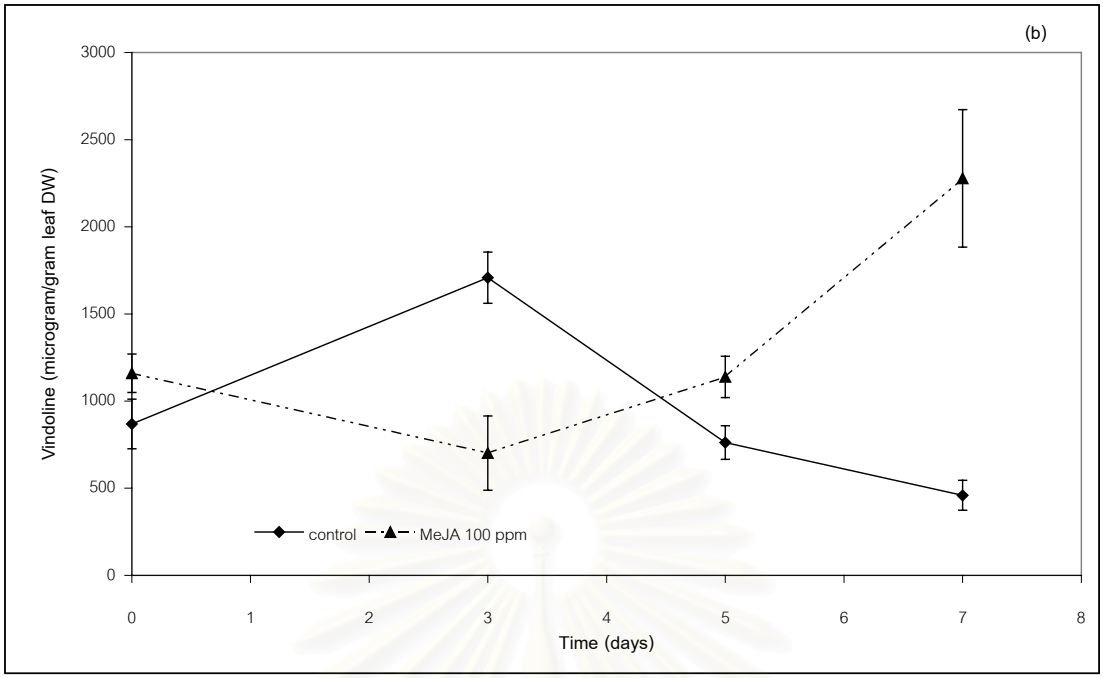

Figure 5.3 (b) Effects of 100-ppm MeJA on vindoline concentrations of *C. roseus* leaves. The 100-ppm MeJA gave the significantly higher vindoline content than control at a 99% confidence interval of *t*-test distribution.

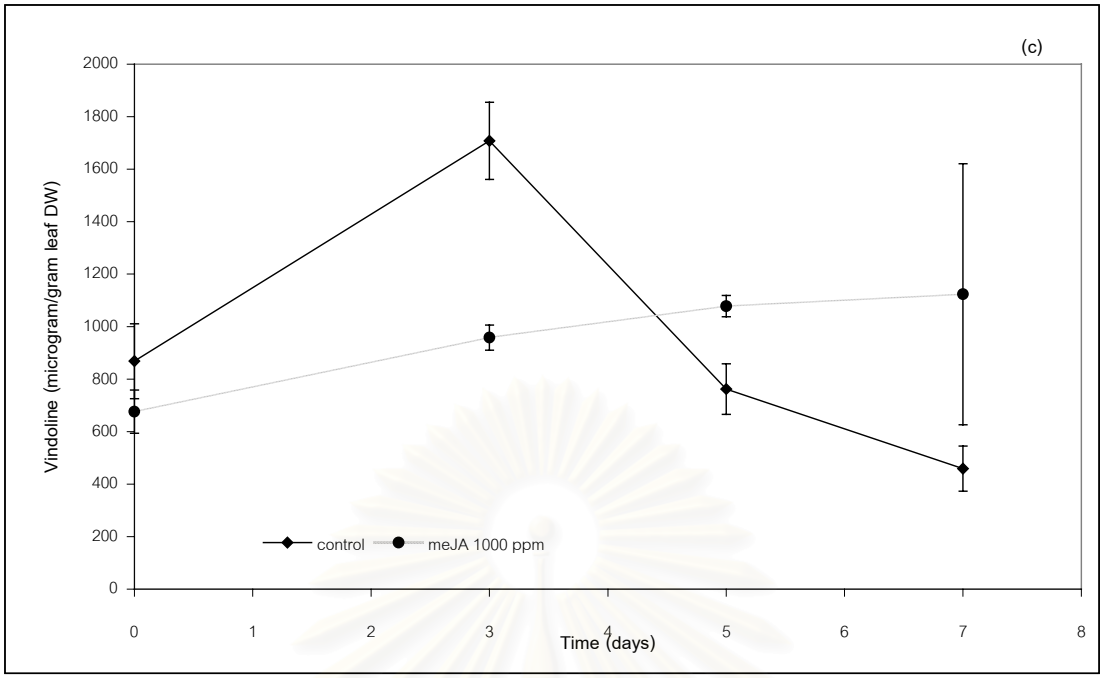

Figure 5.3 (c) Effects of 1000-ppm MeJA on vindoline concentrations of *C. roseus* leaves.

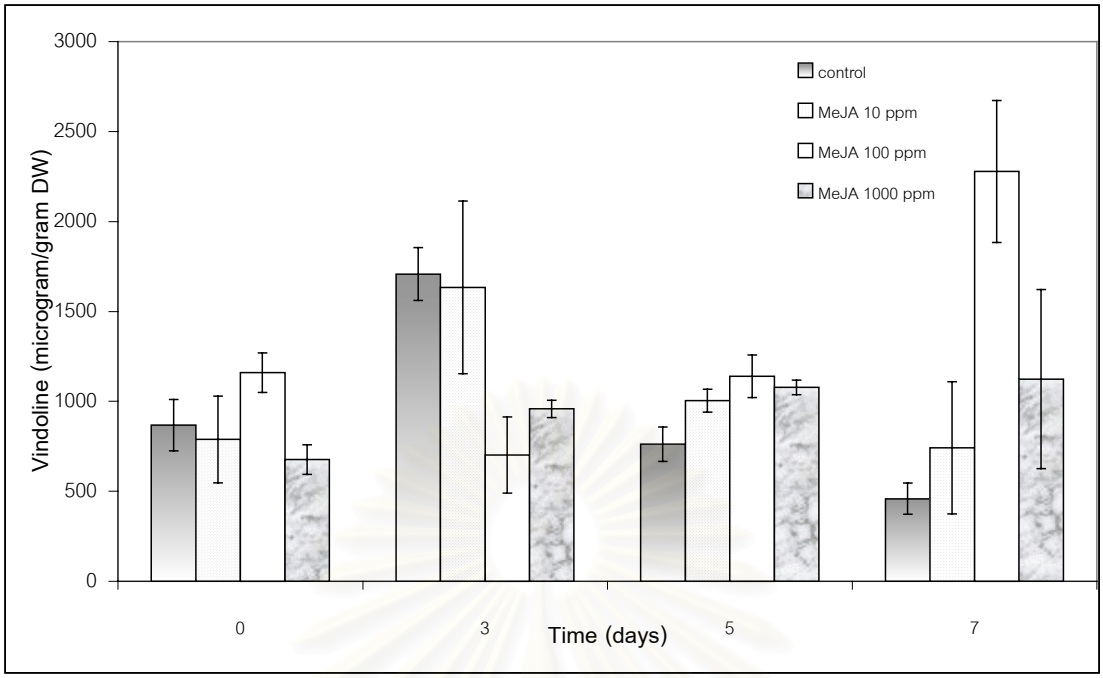

Figure 5.3 (d) Comparison between vindoline concentrations of plants treated with 10, 100, 1000-ppm MeJA.

Table 5.4 Average vindoline concentrations and standard deviations of the control plants and the plants treated with 10, 50, 100, and 1000-ppm MeJA and the time of elicitation.

Day	Vindoline concentrations ($\mu g_{vindoline}/g_{leaf}$ DW)					
	control	ethanol control	MeJA 10 ppm	MeJA 50 ppm	MeJA 100 ppm	MeJA 1000 ppm
0	1176±34	921±250	890±52	830±151	806±201	678±202
3	1835±51	1821±56	2728±714	1161±156	2596±434	3198±694
5	1100±266	1113±215	1603±381	637±69	1566±261	1360±492
7	987±308	1601±459	2125±341	1593±140	2044±114	2130±85
9	1786±184	1915±171	2028±498	1321±55	1641±110	2873±984
11	2425±339	2182±458	2294±368	2035±127	3143±339	2639±437
14	2711±1022	2625±101	2852±789	3367±1077	4159±1350	4136±827

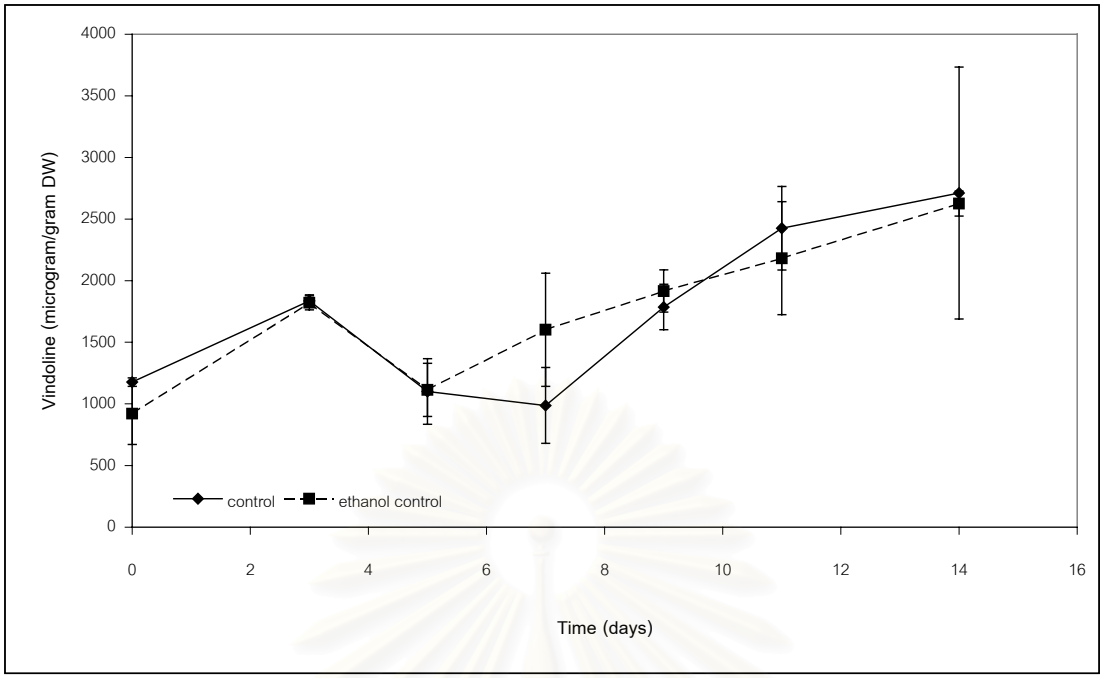

Figure 5.4 (a) Comparison of vindoline concentrations between the control plants and the ethanol-treated control plants.

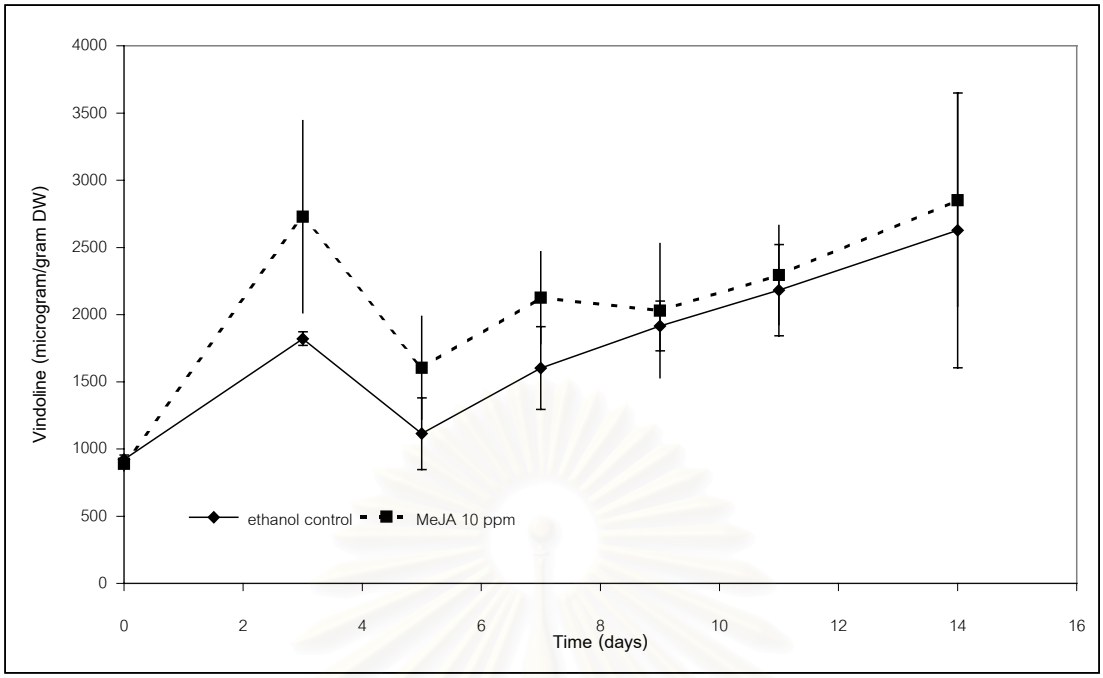

Figure 5.4 (b) Effects of 10-ppm MeJA on vindoline concentrations *of C. roseus* leaves.

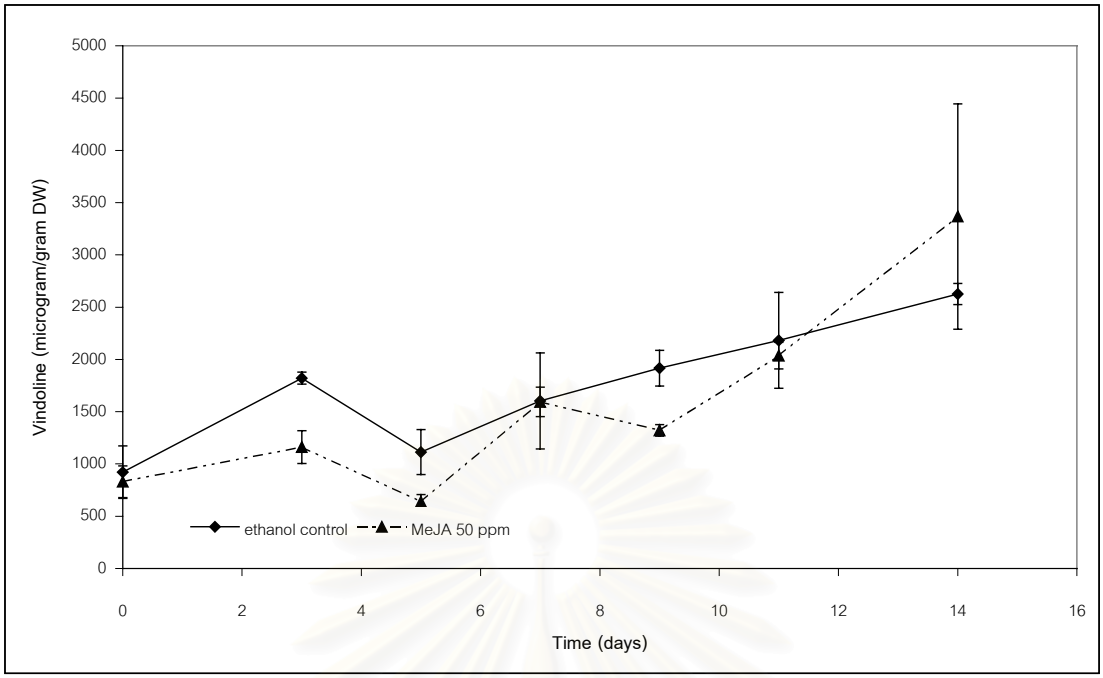

Figure 5.4 (c) Effects of 50-ppm MeJA on vindoline concentrations *of C. roseus* leaves.

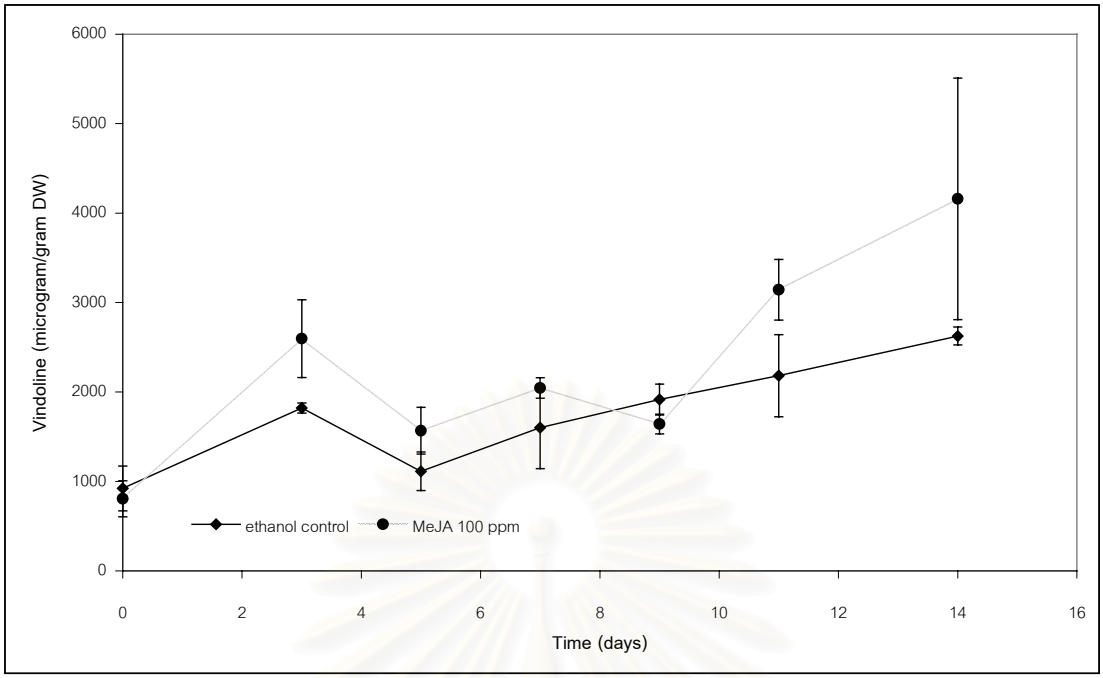

Figure 5.4 (d) Effects of 100-ppm MeJA on vindoline concentrations *of C. roseus* leaves.

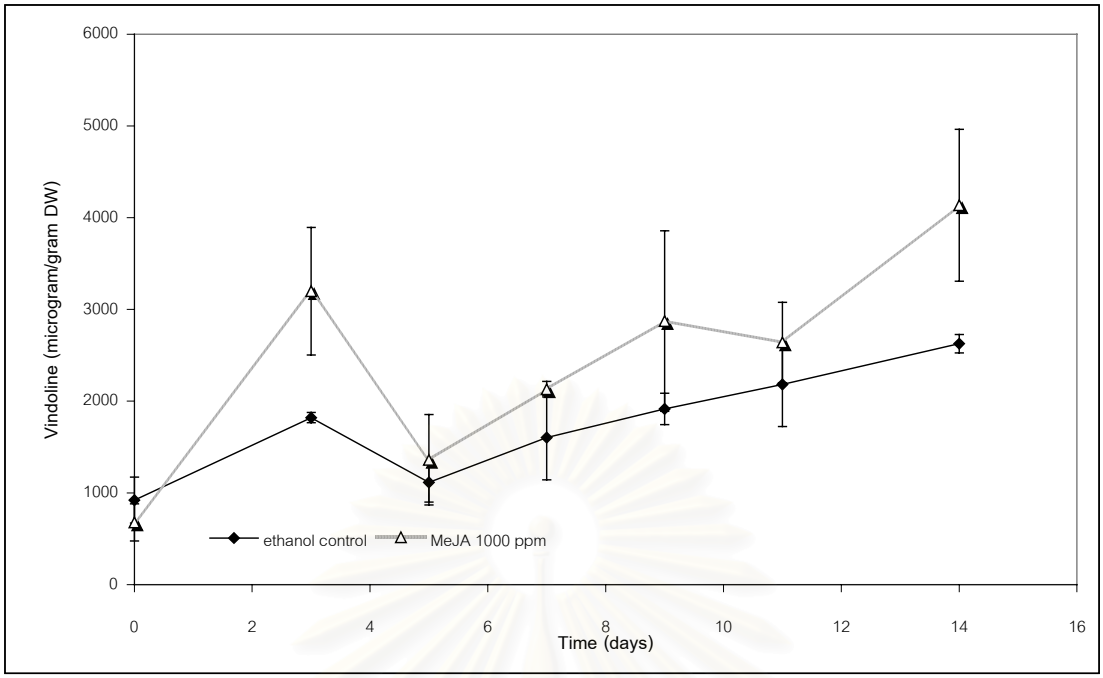

Figure 5.4 (e) Effects of 1000-ppm MeJA on vindoline concentrations *of C. roseus* leaves. The 1000-ppm MeJA gave the higher vindoline content than the ethanol-treated at the significance level 0.1 of t-test ditribution.

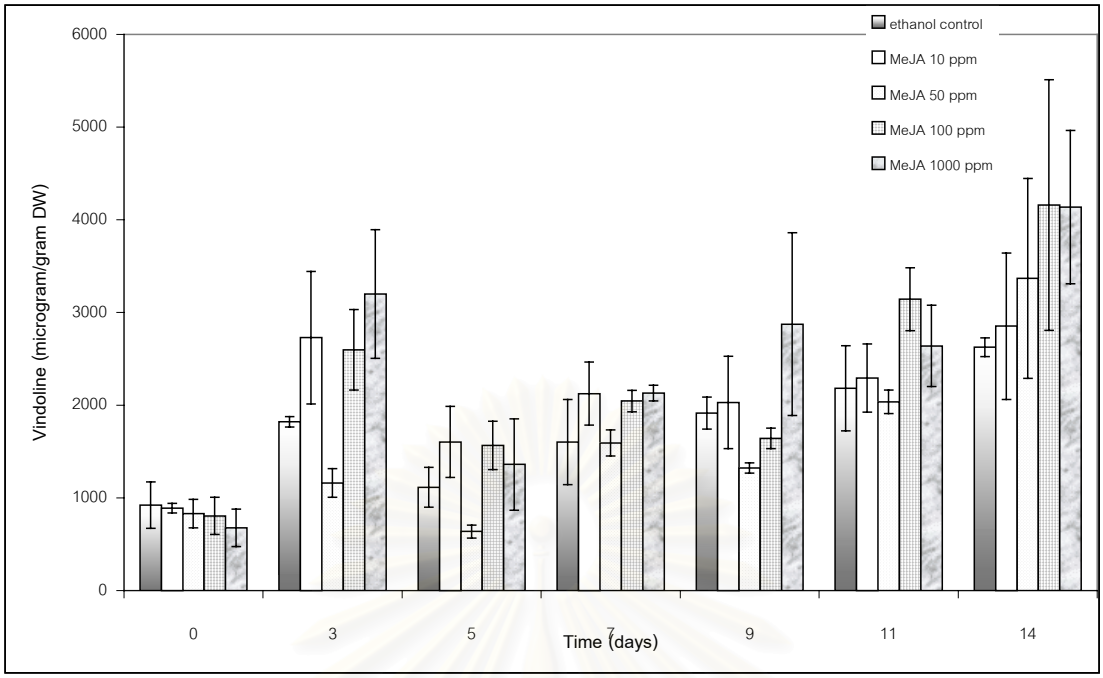

Figure 5.4 (f) Effects of 10, 50, 100, and 1000-ppm MeJA on vindoline concentrations of *C. roseus* leaves.

Considering the results in the experimental study, fluctuation of the data was observed. This may be due to the variations of vindoline concentrations in *C. roseus* leaves picked in the experimental study. According to Westkemper et al.(1980), the youngest leaves in the upmost apical region showed about a 10-fold lower concentration occurred in older leaves. The highest concentration of vindoline was found in just fully matured, non growing-old leaves. In this study, despite the 6-month old plants were used, the growth among the plants is not the same. Some plants have more leaves than others. Although the three positions of the leaves for vindoline analysis were picked at the top, the middle, and the bottom of the plants, assumably, to represent the whole plant, the collection of the leaf samples at the exactly same position sometimes was not possible. Furthermore, the leaf samples might not be in the same stage of development.

Jasmonic acid (JA) and its ester, MeJA, have been reported as effective universal elicitors for enhancement of defense-related secondary metabolite levels in a plant cell or tissue culture process. Exogenously applied JA may be recognized by a plasma membrane receptor (Ishihara et al., 2002). A hypothetical mechanism of action of MeJA was proposed by Gundlach et al. (1992): An elicitor-receptor complex activates a lipase releasing α-linolenic acid. Constitutive enzymes then transform the α-linolenic acid to jasmonic acid and methyl jasmonate. Then jasmonate-induced proteins are synthesized. A multitude of species-specific genes involved in several compound formation are expressed in response to these signal transducer molecules. MeJA was shown to increase vindoline level in *C. roseus* seedlings (Aerts et al.,1996). In this study, MeJA was able to increase the vindoline level in *C.roseus* leaves. It could be the same metabolism occurred as Gundlach et al.,(1992) proposed.

There are several possible reasons that why MeJA can induce the vindoline biosynthesis in *C.roseus* leaves. First, MeJA may enlarge the vindoline precursor pool in the alkaloid pathway. So, there are more substances to transform to vindoline. Second, MeJA may enhance several enzyme activities in alkaloid biosynthesis. Then the higher-acitivity enzyme can induce more vindoline. For example, strictosidine synthase (SSS) and tryptophan decarboxylase (TDC) were up-regulated in response to low levels of exogenous MeJA in *C.roseus* cell suspension culture (Menke et al.,1999). They

demonstrated that MeJA-induced activation of SSS gene expression occurred at the transcriptional level. It might be possible that MeJA induced the activation of SSS gene expression at the transcriptional level of *C.roseus* leaves as well. Third, MeJA might be recognized by a plasma membrane receptor or integrated directly in the octadecanoid pathway and then stimulate terpenoid indole alkaloid (TIA) metabolism via induction of the biosynthetic genes in the *C.roseus* leaves. Since *C.roseus* seedlings and suspension-cultured cells could accumulate terpenoid indole alkaloids in response to MeJA, this implied that the octadecanoids stimulated TIA metabolism via induction of the biosynthetic genes (Menke et al.,1999). Therefore, the consequence of the octadecanoid pathway in the alkaloid biosynthesis, including vindoline, could increase.

From the data of elicitation day 3, the rising of the vindoline level were noticed from the control plants and all the MeJA treated plants. This might be due to the stress effect of experimental treatment to induce the plant defense system.

Moreover, there is some evidence for protein kinases of the protein phosphorylation steps acting upstream of the octadecanoid pathway in elicitor-induced responses. In addition, one or more protein kinases are involved in transducing the JA signal downstream of the octadecanoid pathway (Menke et al.,1999). In the same way, in *C.roseus* leaves, the protein kinases may play important role acting on protein phosphorylation pathway.

In this study, vindoline was found accumulated in the *C.roseus* leaves. However, the other two determined alkaloids, ajmalicine and catharanthine, were not formulated. From the overall alkaloid pathway, there are five branches stemmed from strictosidine. These branches lead to ajmalicine catharanthine, lochnericine, horhammericine, and vindoline. Therefore, if inhibitors were applied to the plants to block the undesired branches, there could be possibility to increase the desired vindoline level. Then all the precursor substances will then go directly to be transformed to vindoline. The enzymology and molecular biology surrounding the tabersonine branchpoint is an area worthy for investigation.

5.3 Direct-binding model fitting

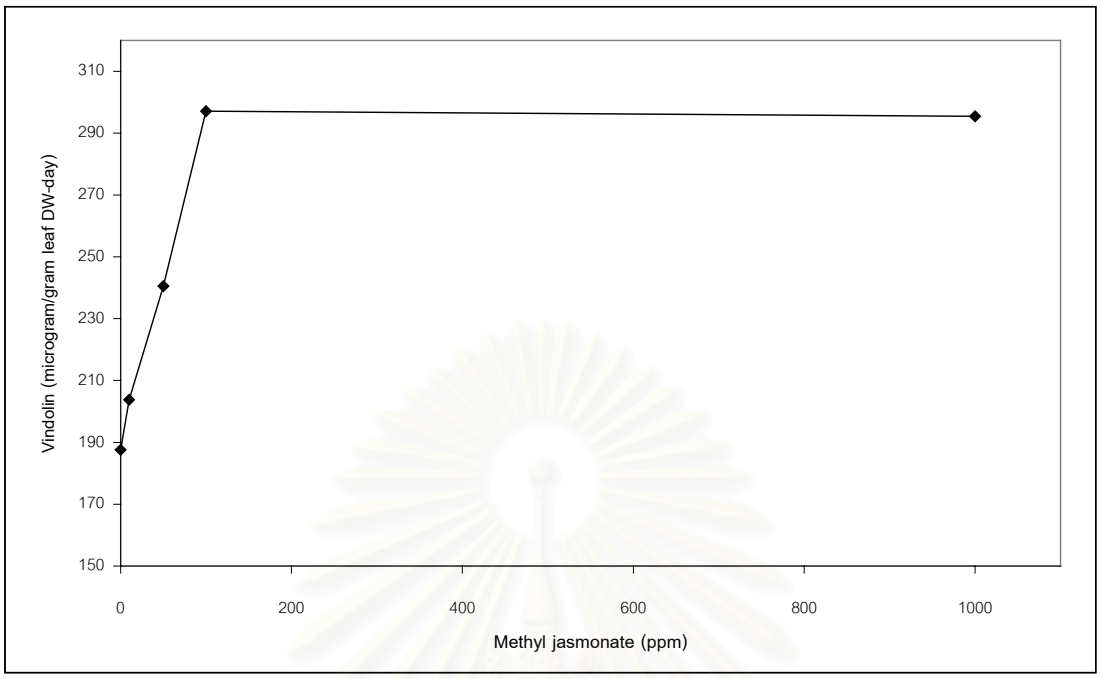

Figure 5.5 Vindoline production in *C.roseus* leaves elicited with MeJA at different concentrations after elicitation for 14 days.

The data obtained from the experimental study was plotted in dose-response curve as shown in Figure 5.5. The result of the elicitation day 14 was used because the treated plants began to be stable under MeJA effects. The formation of vindoline at the low MeJA level increased linearly with the MeJA concentrations and it leveled off at the high methyl jasmonate concentration (more than 100-ppm MeJA). Studies on the phenomenon in the binding of ligands to proteins led to observation that was analogous to cooperative rates with enzyme kinetics and the concept of allosteric regulation. This regulation included binding of ligands to some proteins by considering a ligand as a molecule binding to a receptor protein. The direct binding model was applied to suggest the binding of MeJA on protein receptor in this study.

$$E + R \xleftrightarrow{k_1} ER \xrightarrow{k_2} response$$

$$rate = \frac{V_m [E]}{K_m + [E]}$$

Where [*E*] is elicitor concentration, *R* is receptor, and *ER* is elicitor-receptor complex, which activates response. The unit of response is (μg/g DW) and the unit of rate is (μg/g DW)/(day).

Considering the direct binding model, at low elitcitor concentration, the denominator K_m + [*E*] becomes K_m , as the term [*E*] is negligible. Then the rate linearly relates to the elicitor concentration. When there is high elicitor concentration, the denominator term K_m + [*E*] becomes the term [*E*], as the constant term K_m is negligible. So, the rate is constant and equals to V_m. This agreed well with the observation for the response of vindoline accumulation to MeJA concentrations in this study.

To estimate the kinetic parameters, the data was plotted between 1/[r-r_0] and 1/[*E*] to yield the linear relationship. When *r* was the *rate* of vindoline biosynthesis, r_0 was the *rate* of vindoline biosynthesis without MeJA application, and [*E*] was the MeJA concentrations. Maximum velocity V_m was estimated from the intercept of the graph to be 334.6 microgram/gram DW-day. The K_m constant was estimated from the slope of the graph to be 80.764 ppm.

Mirjalili and Linden (1996) suggested the direct binding model for the response of the taxol productivity by ethylene induction with the kinetic constant $K_m = 4*10^{-8}$ M and V_{max} = 0.35 mg/(L day). The interaction and binding model of taxol formation in elicited suspension cell cultures of *T.canadensis* was also postulated for the explanation of allosteric relationships between MeJA concentration and taxol production (Phisalaphong and Linden, 1999).

From this study, MeJA, a signal transducer, can significantly induce the biosynthesis of vindoline in *C.roseus* leaves, while little, if any, induction of vindoline formation occurred when exposured to chitosan. Therefore chitosan may not integrate in the MeJA-induced vindoline synthesis pathway. However, it remains unclear on what regulatory level jasmonates exert their effect on secondary metabolism (Menke et al., 1999). Apart from the possibility that chitosan might not be able to elicit the enzymes in the vindoline biosynthesis pathway. This might either depend on whether it can penetrate to the site of the biosynthetic pathway or whether there is enough of Ca^{2+} in the activation process.

Study with the intact plant has the advantages in such a way that the subcellular compartmentation of enzymes is not the obstacles in the alkaloid production because the plant is in the complete and native condition. In this study, vindoline could be synthesized and stored in sufficiently developed leaves. On the other hand, plant cell culture or hairy root cultures do not contain complete parts of the plant and some enzymatic steps might be blocked. In cell suspension culture or hairy root culture, only very small amount of vindoline was found accumulated. As the regulatory mechanisms of the both systems might not be the same, the result of plant tissue culture may not be able to imply to the intact plant.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

This chapter presents the conclusions of this study and the recommendations

6.1 Conclusions

1. In this study, chitosan (DP 7190, MW 1200 kDa, 14% DA) did not show significant effect on vindoline accumulation in *C. roseus* leaves after spraying chitosan of 10, 20, and 30 ppm over the whole plant for 7 days.
2. MeJA showed significant increase of the vindoline accumulation in *C. roseus* leaves in the day 7 and 14 of elicitation with a 99% and a 90% confidence interval of *t*-test distribution, respectively.
3. A direct binding model of elicitor to receptor was well fitted with the data of vindoline accumulation in *C.roseus* leaves responded to MeJA concentration after 14 days of elicitation. The value of V_m was found to be 334.6 microgram/gram DW-day, and the K_m constant was 80.764 ppm.

6.2 Recommendations

1. Ca^{2+} could be applied to the whole plant together with chitosan solution in order to overcome the problem of lacking Ca^{2+} in the activation of DNA transcription to proteins after the callose formation.
2. The reason why *C.roseus* leaves were not responsive to the elicitation by chitosan may be due to its ability to penetrate from the outest part of the leaves e.g., wax, cutin, and cell wall to the plasma membrane. Increase of chitosan diffusivity by adjusting solution condition or using chitosan with lower DP should be applied for the further study of the effects of chitosan on the elicitation.

3. The use of inhibitors to block the undesired alkaloid synthetic branches in the pathway could be applied, together with the precursor feeding techniques, to enhance the secondary metabolism pathway.
4. The position and the age of leaves, and the age of plants should be highly considered to allow the least fluctuation to occur.

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