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BIOSYNTHETIC STUDIES OF NAPHTHOQUINONES IN *IMPATIENS BALSAMINA* ROOT CULTURES

ABSTRACT

Impatiens balsamina root cultures have been established in order to be used as a model for biosynthetic studies of lawsone-derived naphthoquinones. Chemical analysis of the ethyl acetate and methanolic extracts of the cultured roots by TLC revealed the presence of naphthoquinone and coumarin derivatives as major components. These compounds were isolated and their structures were elucidated by various spectroscopic data, as lawsone, 2-methoxy-1,4-naphthoquinone, methylene-3,3'-bilawsone (diphthicol), scopoletin, isofraxidin, 4,4'-biisofraxidin and a plant sterol, spinasterol (Panichayupakaranant et al., 1995). Among these, methylene-3,3'-bilawsone (bisnaphthoquinone) and 4,4'-biisofraxidin (biscoumarin) appeared to be new compounds (Panichayupakaranant et al., 1998). All these compounds could be effectively separated from one another by HPLC technique. Based on this technique, the chemical pattern and content of the secondary products produced by the cultured roots were investigated and compared with those of the intact plants (leaves and roots). The relationship between the root culture growth and the formation of each compound was also determined. Formation of the naphthoquinones was highly active in the late linear phase of the growth cycle. *In vivo* feeding experiments with [^{14}C]α-ketoglutarate showed that the radiolabelled precursor was incorporated directly into lawsone and 2-methoxy-1,4-naphthoquinone without detection of any radioactively labelled intermediates (e.g. *o*-succinylbenzoic acid). With cell-free extract prepared from the root cultures, [^{14}C]α-ketoglutarate was also converted directly to 2-methoxy-1,4-naphthoquinone, without detection of any labelled intermediates. These results suggest that various enzymes involved in the biosynthetic pathway of lawsone and 2-methoxy-1,4-naphthoquinone in *I. balsamina* are organized as either multifunctional enzyme or multienzyme complex. The enzyme complex was eluted with void volume of Superose 12 gel filtration column confirming its high molecular weight nature and complexity. The partially purified enzyme complex could use α-ketoglutarate and chorismic acid as substrates for the formation of 2-methoxy-1,4-naphthoquinone, with coenzyme A, ATP and Mg^{2+} as essentials for the enzyme activity. Lawsone-O-methyltransferase seemed not to be part of the enzyme complex. Methionine appeared to be the methyl source for 2-methoxy-1,4-naphthoquinone, but not for the methylene bridge of methylene-3,3'-bilawsone.

INTRODUCTION

There is a continued commercial demand for a wide range of secondary compounds, particularly in the food and pharmaceutical industries. The fact that plant cell cultures are not utilized to any great extent industrially for the direct production of such compounds is due to economic reasons. One of the problems associated with plant cell cultures is the usually low obtained yields in the production of some particular secondary products generally, the production of secondary metabolites in plants is a complex process highly coordinated in space and time. Its main components are biosynthesis and accumulation which are usually modified by tissue- and cell-specific compartmentation. Depending on the developmental stage, transport and degradation can be additional factor. In view of the complexity of secondary metabolism, it is not surprising that most cell culture systems have failed to produce a given compound in large quantities. Thus, it is exciting that several cell cultures have been established which produce a higher amount of a secondary metabolite than the respective intact plants (Ellis, 1988). Most successful compounds are synthesized by root tissue and the site of accumulation is either within the producing cell or the neighboring cells. On the other hand, compounds synthesized in roots, which are transported *via* the xylem to other plant parts, usually fail to accumulate in plant cell suspension cultures. This means that secondary metabolites whose biology shows a higher degree of complexity are usually not yet

successfully produced by plant cell cultures (Wink, 1987). It is interesting that when cultured plant cells differentiate into tissues and organs, their capacity to produce the secondary metabolites set in immediately. Thus, a possible approach to increase yields of secondary compounds is to allow differentiation of tissue by production of shoot or root cultures.

A detailed understanding of the enzymatic formation of secondary products, however, is a prerequisite for rational strategies to improve product formation. It is essential that their control and regulation mechanisms can be understood if they are to be manipulated. To manipulate plant secondary metabolism according to our needs, we have to understand the basic biochemical principles of product formation. Plant cell cultures have proved to be useful sources of enzyme which catalyze specific biosynthetic steps. Thus, this could be accomplished by using enzymology techniques which involve purification and characterization of the biosynthetic enzymes.

Among various secondary metabolic pathways in plants, the biosynthesis of naphthoquinone is of particular interest. There are at least four different routes of naphthoquinone formation established in the higher plants, including *p*-hydroxybenzoate, homogentisate, *o*-succinylbenzoate and polyketide pathways. In this study, emphasis was put on the naphthoquinones that accumulated in *Impatiens balsamina* L. Lawsone (2-hydroxy-1,4-naphthoquinone) and its methyl ether, 2-methoxy-1,4-naphthoquinone are the two main naphthoquinones found naturally in *I. balsamina* (Little *et al.*, 1948; Bohm and Towers, 1962). Both compounds have been reported to exhibit strong antifungal activity (Tripathi, Srivastava and Dixit, 1978; Farnsworth and Cordell, 1976; Thatree Phadungcharoen *et al.*, 1988). Biosynthetically, it has been proposed based on feeding experiments that lawsone is formed in plant *via* *o*-succinylbenzoic acid (Dansette and Azerad, 1970; Grotzinger and Campbell, 1974), a key intermediate arising from chorismic acid and α-ketoglutarate (Chen and Bohm, 1966; Grotzinger and Campbell, 1972). However, none of the enzyme involved in the formation of the naphthoquinones has been found in plants. This prompted us to investigate the biosynthetic pathway of lawsone and its methyl ether. We first examined for a suitable enzyme source by establishing various type of *in vitro* cultures of *I. balsamina* (Panichayupakaranant and De-Eknamkul, 1992). It was subsequently found that the root cultures of *I. balsamina* could produce a number of natural products, mostly naphthoquinone and coumarin derivatives (Figure 1) (Panichayupakaranant et al., 1995; 1998). In addition, the content of lawsone in the root cuiture was found to be higher than in the intact plants. This type of culture is therefore a suitable source of enzyme for the study on the biosynthesis of lawsone and its methyl ether.

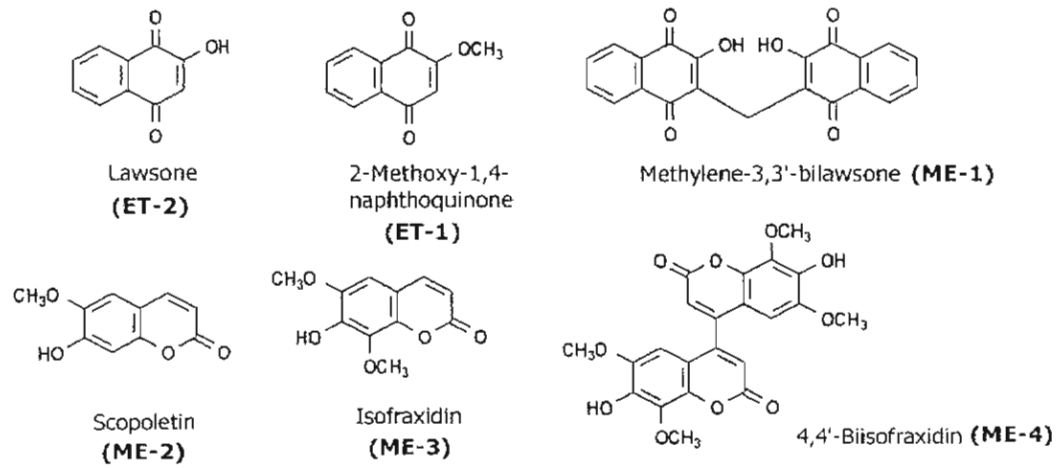

Figure 1

As part of our interest in investigation of the biosynthetic pathway of lawsone and its methyl ether, we first aimed to know the type and content of secondary metabolites produced in the root cultures. Thus, the isolation and structural elucidation of the secondary compounds produced by the root cultures were firstly performed. Consequently, the potential of the root cultures as a suitable enzyme source was investigated by the study on the chemical patterns and contents of the isolated compounds compared with those of the intact plant. For this aspect, the kinetic of growth and the formations of naphthoquinone and coumarin derivatives were also examined. Finally, the potential enzyme system involved in the biosynthesis of lawsone and its methyl ether was examined by both *in vivo* feeding and *in vitro* cell-free system with isotropic precursors, followed by partial purification and characterization of the enzyme.

MATERIALS AND METHODS

1. General

Standard B5 media and various plant hormones were purchased from Gibco Laboratories. All other chemicals were reagent or analytical grade, as available. TLC plates: silica gel precoated Al sheets (Merck); Column chromatography: silica gel (40-63 μm, Merck) and Sephadex LH-20 (Pharmacia); MPLC: prepacked Lobar column (LiChroprep, Diol, 40-63 μm, Merck); TLC densitometer: Shimadzu Dual Wavelength Model CS-930; HPLC: constaMetric 4100 (Thermo Separation Products); UV: Shimadzu UV-160A; IR: Perkin Elmer 16 PC FT-IR or Jasco; MS: Finnigan MAT TSQ 70 triple stage quadrupole instrument and JEOL HX-110; NMR: 500 MHz (^{1}H) and 125 MHz (^{13}C); TLC-radioscanner: Automatic TLC-Liner Analyzer (Tracemaster 20); Liquid scintillation counter: Wallac 1409; FPLC: FPLC^{R} system (Pharmacia); Chromatographic material for enzyme purification: Superose 12 HR.

2. Root culture of *Impatiens Balsamina*

Root cultures of *I. balsamina* L. were initiated from young leaf explants on B5 solid medium containing 0.1 mgl^{-1} NAA, 0.1 mgl^{-1} kinetin, 1.0 mgl^{-1} BA, and 20 gl^{-1} sucrose (Panichayupakaranant and De-Eknamkul, 1992). The roots were formed after 2 weeks of incubation (25°C, continuous light 2,000 lux). After 3 weeks, the root culture was transferred into B5 liquid medium containing the same supplements as the solid medium (100 ml medium in 500 ml Erlenmeyer flask). The culture was maintained by transfer of 2.0 g fresh weight tissue into 100 ml fresh medium every 3 weeks and maintained at 25°C, 80 rpm, in continuous light.

3. Chemical Pattern and Content of Secondary Products Produced by the cultured roots and the Intact Plants

3.1 Extraction and sample preparations

Each dried sample (0.2 g) including the cultured roots, the leaves and the roots of the intact plants was extracted as described below. The sample was ground and then sonicated with 10 ml petroleum ether for 1 hours (x2). The petroleum ether extract was discarded and the remaining residues were then refluxed with 20 ml methanol for 1 hour. The methanolic extract was dried *in vacuo* and then re-dissolved in methanol and the volume was adjusted to 5 ml. The obtained sample was then filtered through millipore filter (0.45 μm) before use in qualitative and quantitative analysis by HPLC.

3.2 Qualitative and quantitative analysis of secondary products by HPLC

Each filtered sample preparation was subjected to HPLC analysis. The conditions of HPLC system were described below. For the qualitative analysis, the sample peaks were identified by comparison of their R_f values and UV absorption spectra with authentic compounds. The areas under peaks of each secondary product were used for the quantitative analysis by converting to concentrations using their calibration curves. The calibration curves were established from the

authentic lawsone, 2-methoxy-1,4-naphtho-quinone and scopoletin at the concentration in the range of 0.003-0.050 mg/ml. In this way, the semi-quantitative determination of diphthicol was based on the calibration curve of lawsone, while, those of isofraxidin and diisofraxidin were based on the calibration curve of scopoletin.

HPLC conditions		
Column	:	Octyl-80Ts (4.6 x 150 mm)
Mobile phase	:	Solvent A : 0.15 M H_3PO_4 in methanol-water (1:9) Solvent B : 0.15 M H_3PO_4 in methanol-water (6:4)
Elution	:	0.15 M H_3PO_4 in methanol-water 1:9 to 6:4, in 50 min
Flow rate	:	0.7 ml/min
Detector	:	UV 275 nm for naphthoquinones; UV 365 nm for coumarin derivatives
Injection volume	:	20 μl

4. Kinetic Studies of Growth and the Formation of naphthoquinone and Coumarin Derivatives

Fresh roots (*ca* 2.0 g), subcultured on B5 medium, were transferred to fresh B5 medium and cultured for 30 days. The roots were harvested every 2 or 3 days. The harvested roots were, then, dried at 60°C for 12 hours and the dry weights were recorded. The dried roots were extracted by refluxing with methanol and the amounts of naphthoquinones and coumarin derivatives were determined by HPLC analysis as described in section 5.2. These data were then plotted to obtain growth and production curves.

5. Biosynthetic Studies of Lawsone and 2-Methoxy-1,4-Naphthoquinone

5.1 Synthesis of OSB and [2,3-^{14}C]-OSB

OSB was synthesized according to the method of Roser (Roser, 1984). Succinic acid and phthalic acid (both 3.0 g) and sodium acetate (1.05 g) were mixed and heated at 240°C for 2 minutes. The mixture was then extracted with 800 ml of water (x4) on the steam bath and filtered. The dilactone formed was hydrolyzed with a small amount of sodium carbonate on the water bath for 45 minutes. In order to purify the hydrolyzed product, it was partitioned with ethyl acetate. The aqueous part was then acidified and OSB was extracted by ether. The ether part was evaporated *in vacuo* and applied on to a Sephadex LH-20 column, eluted with ethanol. Fraction 3 was evaporated and recrystallized from a mixture of ether and petroleum ether to yield 184 mg of OSB. In the synthesis of [2,3-^{14}C] OSB, [2,3-^{14}C]-succinic acid was used instead of succinic acid.

5.2 Identification of OSB

OSB was converted into the form of dimethyl ester by refluxed with methanol in the presence of sulfuric acid. The obtained OSB dimethyl ester was then identified by ^{1}H NMR and ^{13}C NMR data as follows: ^{1}H NMR (500 MHz): δ 3.71 (3H, s), 3.88 (3H, s), 2.80 (2H, t, J = 6.8 Hz), 3.14 (2H, t, J = 6.8 Hz), 7.43 (1H, d, J = 7.5 Hz), 7.49 (1H, td, J = 7.51, 1.0 Hz), 7.58 (1H, td, J = 7.51, 1.0 Hz), 7.90 (1H, d, J = 8.0 Hz); ^{13}C NMR (125 MHz): δ 28.2, 37.5, 51.8, 52.6, 126.3, 128.2, 129.8, 129.9, 132.3, 143.0, 167.0, 173.3, 203.8

5.3 Preparation of cell-free extract for OSB-CoA ligase assay

The root culture of one-week old (25 g) were ground in liquid nitrogen. The root powder was then added into 20 ml of 0.1 M potassium phosphate buffer (pH 7.5), containing 0.2 mM DTT, 10 % PVP and 0.1 % protamine sulphate. After homogenized for 30 seconds (2 min interval, 5 times) and passed through 4-layer cheese-cloth, the obtained enzyme extract was centrifuged at 10,000g, for 20 min at 4°C. Thereafter, the supernatant was passed through a PD-10 column, eluted with the same buffer containing 0.2 mM DTT. The desalted enzyme solution was used for the assay of OSB-CoA ligase activity.

5.4 Assay for OSB-CoA ligase activity

The routine assay mixture contained, in a total volume of 140 μl: 0.7 mM OSB or 23,000 cpm [^{14}C]-OSB, 7.1 mM ATP, 1.8 mM CoASH, 7.1 mM $MgCl_2$ and 100 μl cell-free extract. The incubation mixture was incubated at 30°C for 30 minutes. The reaction was stopped by adding 20 μl formic acid and then analyzed by HPLC and TLC radioscanner.

HPLC conditions

Column	:	ODS-120T (4.6 x 150 mm)
Mobile phase	:	Solvent A : 0.15 M H_3PO_4 in methanol-water (1:9)
		Solvent B : 0.15 M H_3PO_4 in methanol-water (3:7)
Elution	:	0.15 M H_3PO_4 in methanol-water 1:9 to 3:7, in 35 min
Flow rate	:	1.0 ml/min
Detector	:	UV 257 nm
Temperature	:	40°C
Injection volume	:	50 µl

TLC conditions

Technique	:	one way, ascending, double development
Absorbent	:	aluminium sheet silica gel 60 F254
solvent system	:	Chloroform-methanol-water (65:35:10-lower phase)
Sample size	:	5 µl
Distance	:	10 cm
Detection	:	Automatic TLC-Liner Analyzer

5.5 [^{14}C-U]α-Ketoglutarate *in vivo* feeding experiment

Sodium salt of [^{14}C-U]α-ketoglutaric acid (3.5 mCi) was fed into one-week and four-week-old *I. balsamina* root cultures. After further incubated for 3 days, the root culture was harvested and refluxed with 50 ml methanol. The methanol extract was then concentrated *in vacuo* before partitioned between ethyl acetate and water. The ethyl acetate part was adjusted to 1 ml, and 100 ml aliquot was subjected to TLC using double development solvent system of petroleum ether-chloroform (20:80) and benzene-acetic acid (98:2). The TLC plates were then scanned to produce radiochromatograms by TLC-radioscanner.

5.6 [^{13}C]-Methionine *in vivo* feeding experiment

Sterile [^{13}C]-methionine (50 mg/ml) was administered to the three-week old root culture by aseptic technique. After 3-day incubation, the root culture was harvested and extracted by refluxing with 30 ml methanol for 1 hour. The crude extract, after concentrated in vacuo, was partitioned between ethyl acetate and water. The ethyl acetate fraction was then concentrated and subjected to preparative TLC (silica gel 60 F254). Double development using the solvent systems of (I) chloroform and (II) chloroform-methanol-water (65:35:10-lower phase) was performed for the separation of 2-methoxy-1,4-naphthoquinone, scopoletin, isofraxidin and diphthicol. These compounds were eluted with chloroform and subjected to ^{13}C NMR measurement.

5.7 Preparation of cell-free extract for the enzyme complex assay

I. balsamina root culture (4-week-old) was ground in liquid nitrogen. Ten ml of 0.1 M potassium phosphate buffer pH 7.0 containing 0.2 mM DTT was added to extract proteins. The crude protein extract was filtered through 4-layer chese-cloth. The filtrate was centrifuged at 100,000g for 30 min. The supernatant was separated and used for the enzyme assay.

5.8 Superose 12 column chromatography

A superose 12 HR 16/50 column (1.6 x 50 cm) was equilibrated with elution buffer, 0.1M potassium phosphate buffer (pH 7.0), containing 0.2 mM DTT. The supernatant fraction, after concentrated with Centricon-30 to 1 ml was applied to a Superose 12 column in the FPLC^R system. The enzyme was eluted with elution buffer at the flow rate of 0.5 ml/min. The eluates were collected into 1 ml fractions. After the determination of enzyme activity, the active fractions were pooled.

5.9 Assay for enzyme complex activity

The routine assay mixture contained, in a total volume of 250 µl: 8.8 mM ATP, 2.0 mM CoASH, 8.8 mM $MgCl_2$, 5.8 mM [^{14}C-U]α-ketoglutarate and 200 µl cell-free extract. Boiled enzyme was used for the control experiment. The mixture was incubated for 1 hour at 30 °C. The reaction was terminated by the addition of 700 µl ethyl acetate, and then vortex for 1 min. A 500 µl aliquot was concentrated to 20 µl and subjected to TLC using the TLC system as described in section 7.5. The TLC plates were then scanned to produce radiochromatograms by TLC-radioscanner. The radioactivity of a 100 µl aliquot was also measured by scintillation counter.

5.10 Determination of protein concentration

Protein concentration was estimated by the dye-binding method (Bradford, 1976), with bovine serum albumin as standard. Five dilutions of the standard BSA in the concentration range of 100-500 μg/ml were used for the calibration curve establishment. A 160 μl portion of each fraction was mixed with 40 μl of dye reagent. After incubation for at least 5 min at room temperature, the absorbance at 595 nm was measured.

5.11 Identification of enzymatic product

For the identification of the enzymatic product, TLC technique was used with two different solvent systems: (I) petroleum ether-chloroform (2:8), then benzene-acetic acid (98:2), (II) chloroform. The Rf value and UV absorption spectrum of the product were compared with those of the authentic 2-methoxy-1,4-naphthoquinone. The product was further identified by recrystallization method as described below. Spots of the product on TLC were cut off and eluted with chloroform. The chloroform fraction was, then, diluted with unlabelled 2-methoxy-1,4-naphthoquinone, and recrystallized from a mixture of chloroform and methanol to a constant specific activity.

RESULTS AND DISCUSSION

1. Secondary Product Composition of The Cultured Roots and Intact Plants

1.1 Chemical patterns of secondary products

Based on the results of the isolation and structure elucidation of the secondary products produced by the cultured roots of *I. balsamina*, it appeared that the root cultures contained essentially two main groups, namely naphthoquinones and coumarins (Fig. 1). The naphthoquinones include lawsone, 2-methoxy-1,4naphthoquinone and methylene-3,3'-bilawsone, and the coumarins include scopoletin, isofraxidin and 4,4'-biisofraxidin (Panichayupakaranant et al., 1995). Among these, methylene-3,3'-bilawsone (**ME-1**) was found to be the novel natural products found in this plant whereas (Panichayupakaranant et al., 1998) 4,4'-biisofraxidin (**ME-4**) was found to be the new compound that has not been found before. To study in more detail about the nature of secondary products produced by the root culture, their chemical pattern were compared with those in the intact plants. In doing this, the methanolic extracts of the cultured roots, leaves and the whole roots of *I. balsamina* were prepared and analyzed by high performance liquid chromatography (HPLC) equipped with a photodiode array detector.

It appeared that all the secondary compounds, including lawsone (**ET-2**), 2-methoxy-1,4-naphthoquinone (**ET-1**), methylene-3,3'-bilawsone (**ME-1**), scopoletin (**ME-2**), isofraxidin (**ME-3**) and biisofraxidin (**ME-4**) could be effectively separated from one another (Fig. 2) by the solvent system of methanol/water, containing 0.15 M H_3PO_4 (gradient system) using RP-18 column. The acidity of the solvent system could overcome the problem of broad tailing peaks by suppressing the ionization effect of these compounds. According to their UV absorption maxima, both the napthoquinones and coumarins were detected at UV 275 nm, whereas, only the coumarin derivatives were detected at 365 nm. The identification of each peak was performed by comparison with the retention times (R_t) and UV absorption spectra of authentic compounds. The results showed that the naphthoquinones lawsone, 2-methoxy-1,4-naphthoquinone and methylene-3,3'-bilawsone exhibited the R_t values of 38.17, 40.98 and 59.58 min while the coumarin derivatives, scopoletin, isofraxidin and biisofraxidin showed the R_t values of 31.00, 31.90 and 48.58 min, respectively.

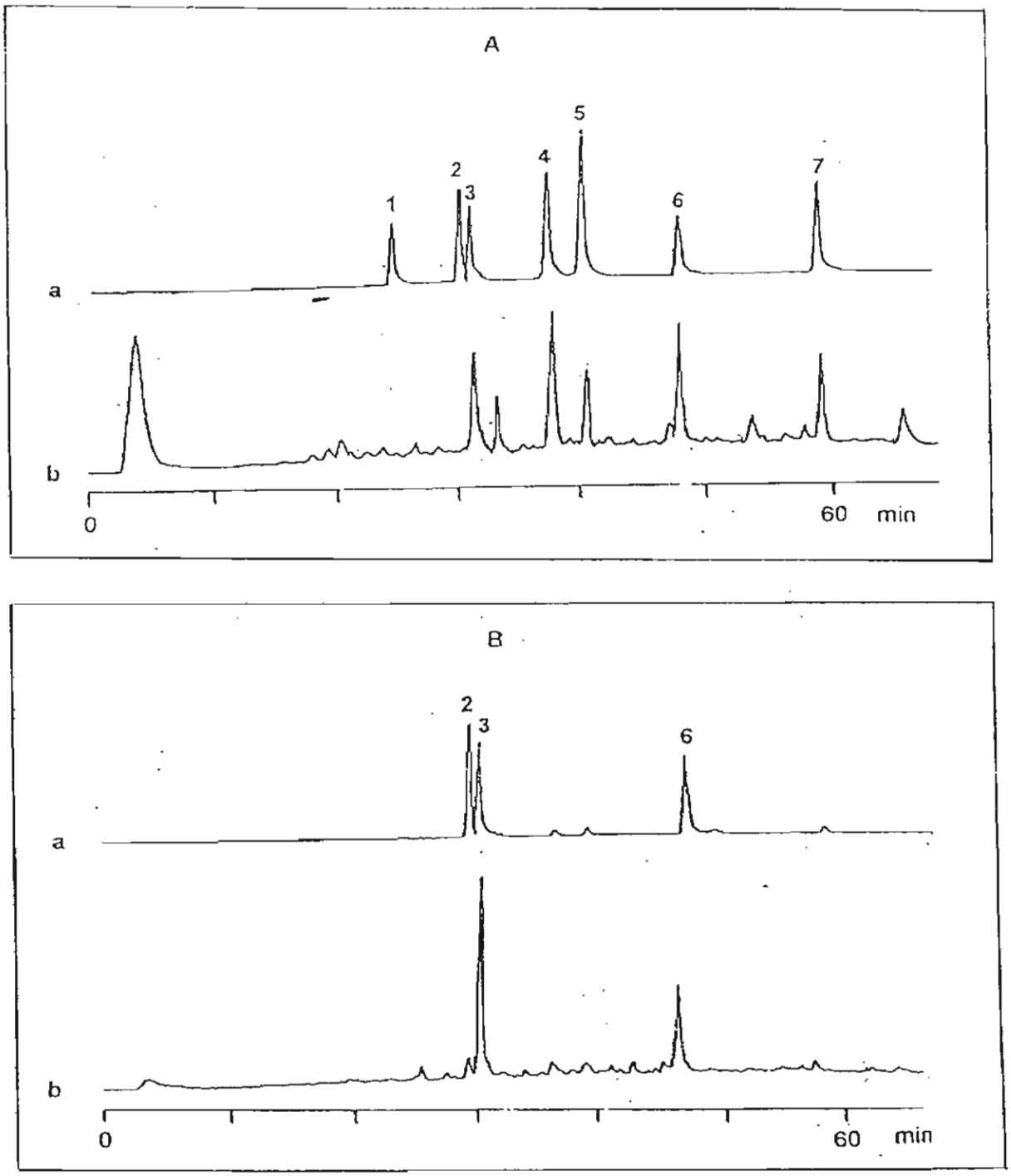

Fig. 2 HPLC chromatograms detected with UV 275 nm (A) and 350 nm (B) of the authentic compounds (a) and the extract of cultured roots (b). 1 : O-succinylbenzoic acid; 2 : scopoletin; 3 : isofraxidin; 4: lawsone; 5 : 2-methoxy-1,4-naphthoquinone; 6 : 4,4'-biisofraxidin; 7 : methyl-3,3'-bilawsone

HPLC chromatograms of the methanolic extracts confirmed that the naphthoquinone and coumarin derivatives are the two major groups of secondary products of the root culture. However, its chemical composition was found to be considerably different from those of the leaves and the whole roots of the plant. (Fig. 3, 4). With respect to the naphthoquinones (Fig. 3), the root cultures appeared to contain lawsone as the major constituent, while 2-methoxy-1,4-naphthoquinone seemed to be the major naphthoquinone in the leaves and the roots. Furthermore, the root cultures showed higher content of lawsone but lesser content of 2-methoxy-1,4-naphthoquinone than the leaves and the roots of the intact plant. Interestingly, it was found that the root cultures could produce a certain amount of methylene-3,3'-bilawsone which could not be detected in the leaves and the roots.

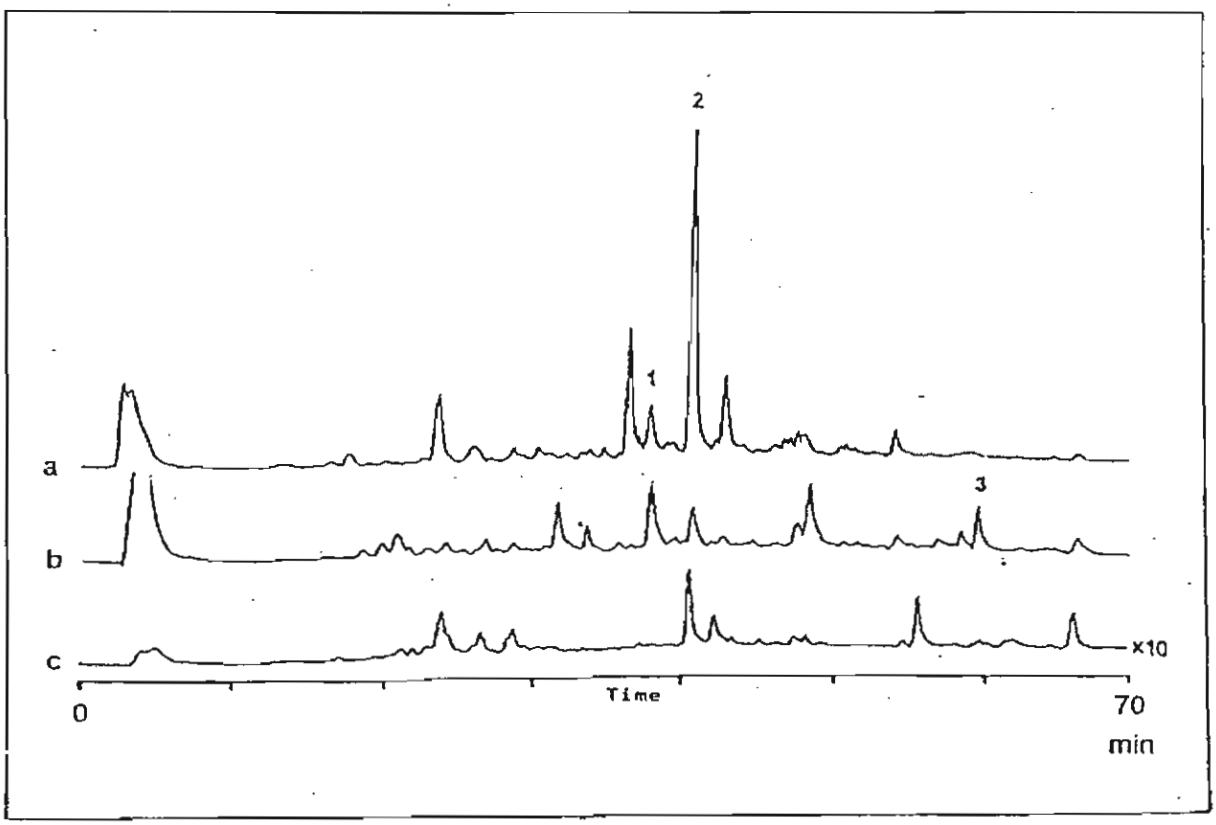

Fig. 3 HPLC chromatograms, detected with UV 275 nm, of the methanolic extracts of leaves (a), the cultured roots (b) and the whole roots (c) of *I. balsamina* ; 1 : lawsone, 2 : 2-methoxy-1,4-naphthoquinone, 3 : methylene-3,3'-bilawsone

For the coumarin derivatives, the HPLC chromatograms (Fig. 4) showed that the root cultures could accumulate much higher amount of the coumarin derivatives than the leaves and the roots. Isofraxidin appeared to be the major coumarin derivative accumulated in the root cultures whereas it was detected only in trace amount in the leaves and the roots. Although scopoletin was detected in both root cultures and the intact plants (leaves and roots), it was found also in small amount. As was found for the bisnapthoquinone, 4,4'-biisofraxidin could be detected only in the root cultures not in the intact plant. These results indicate that the secondary metabolism in the root cultures is different from that in the intact plants.

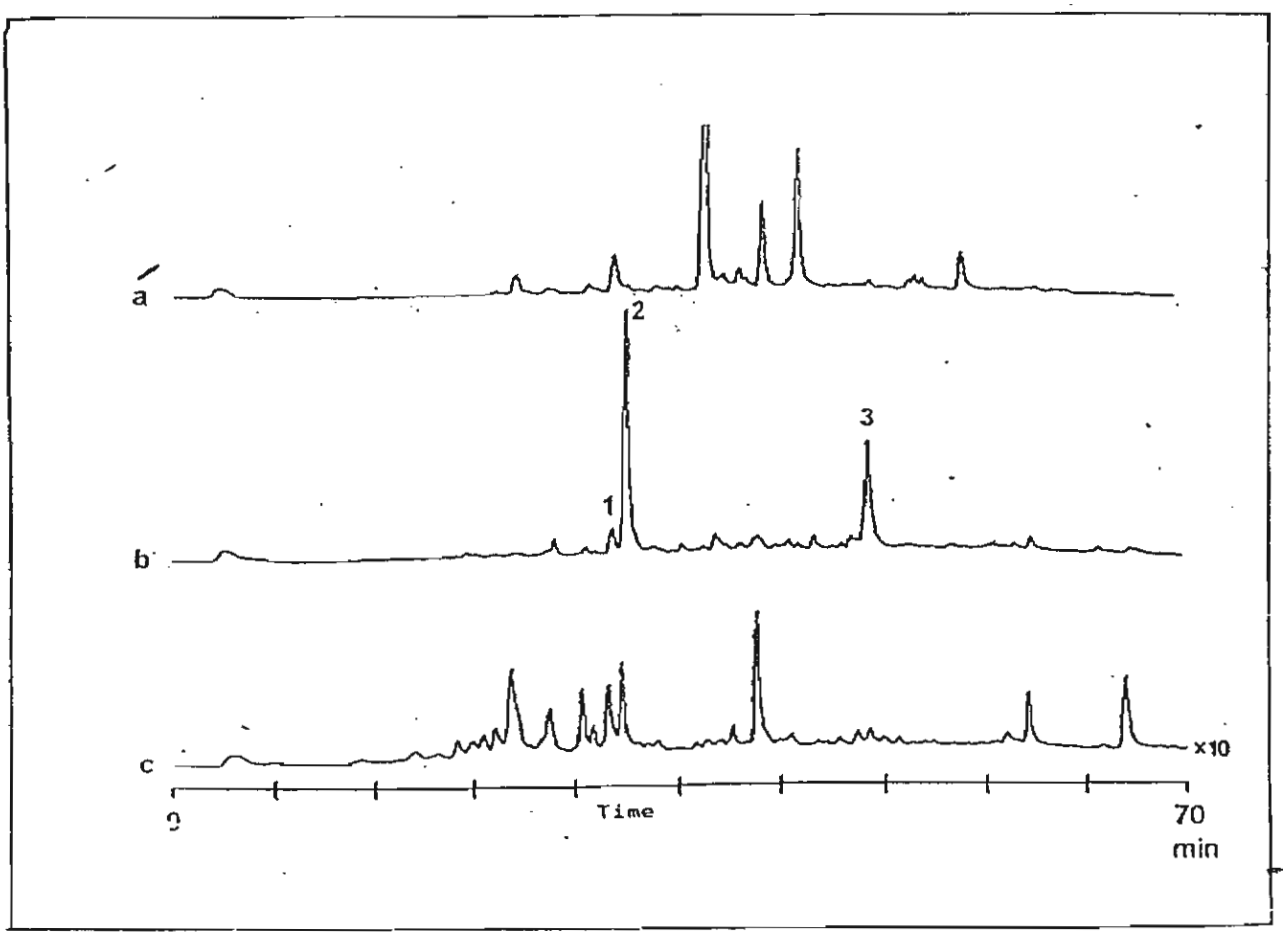

Fig. 4 HPLC chromatograms, detected with UV 350 nm, of the methanolic extracts of the leaves (a), the cultured roots (b) and the whole roots (c) of *I. balsamina* ; 1 : scopoletin, 2 : isofraxidin, 3 : 4,4'-biisofraxidin

Therefore, the root cultures of *I. balsamina* have a potential to produce the secondary metabolites with structures similar to or even more complex than those original found in the intact plants. In this case, the step of dimerization to form methylene-3,3'-bilawsone and 4,4'-biisofraxidin hardly found in the intact plants, appears to take place in the root cultures of *I. balsamina*. The reason for this difference in secondary metabolism is still unknown.

This HPLC conditions are also effective for the analysis of *o*-succinylbenzoic acid (OSB), a key intermediate in lawsone biosynthesis. This study, therefore, also put an attempt for the detection of OSB in the extracts of cultured root and the intact plants. Although, it has been reported that OSB is a key intermediate in lawsone biosynthesis (Dansette and Azerad, 1970; Grotzinger and Campbell, 1974), it could not be detected in all these extracts. These results suggested that the cellular pool of OSB is extremely small, thus it could not be detected in all these extracts.

1.2 Quantitative analysis of naphthoquinone and coumarin derivatives

The complete separation of each naphthoquinone and coumarin derivative by the HPLC method, allowed the compounds be quantitated by using the calibration curves of the authentic compounds. Although the amount authentic methylene-3,3'-bilawsone, isofraxidin and 4,4'-biisofraxidin were not sufficient to make their own calibration curves, their quantitative determinations were performed by using calibration curves of lawsone for methylene-3,3'-bilawsone, and of scopoletin for isofraxidin and biisofraxidin because of their similar chromophores. A linear relationship between the amount of lawsone (Fig. 5), 2-methoxy-1,4-naphthoquinone (Fig. 6) and scopoletin (Fig. 7) and their corresponding peak integrated areas were obtained in the range between 0.03 and 0.50 μg in each case. The correlation coefficients were found to be 0.9987, 0.9990 and 0.9995 for lawsone, 2-methoxy-1,4-naphthoquinone and scopoletin, respectively.

Amount (μg)	Peak area ($x10^4$)
0.031	0.26
0.062	0.42
0.125	0.87
0.25	1.75
0.5	3.27

Regression Output:	
Constant	0.058946904
Std Err of Y Est	0.050140494
R Squared	0.998768281
No. of Observations	5
Degrees of Freedom	3
X Coefficient(s)	6.482712273
Std Err of Coef.	0.131437566

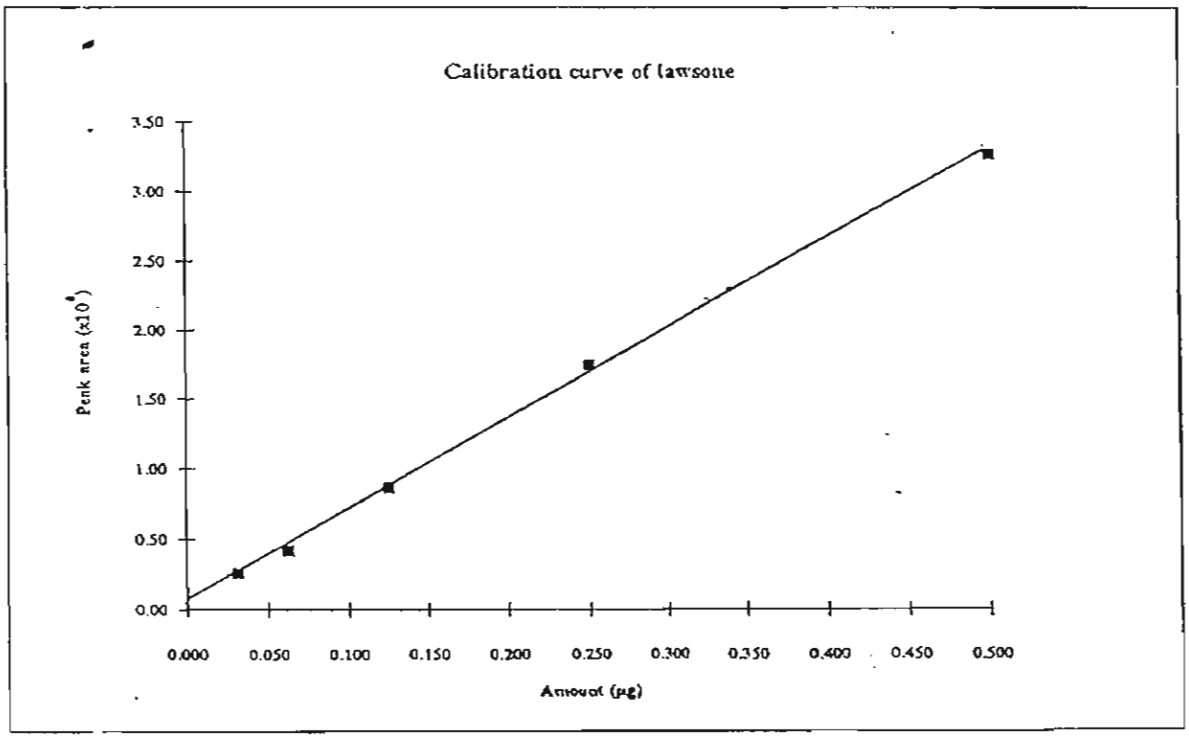

Fig. 5 Calibration curve of lawsone

Amount (μg)	Peak area ($x10^4$)
0.032	0.32
0.064	0.51
0.128	1.05
0.255	2.03
0.51	3.82

Regression Output:	
Constant	0.086602015
Std Err of Y Est	0.049859643
R Squared	0.999093558
No. of Observations	5
Degrees of Freedom	3
X Coefficient(s)	7.378149569
Std Err of Coef.	0.128308016

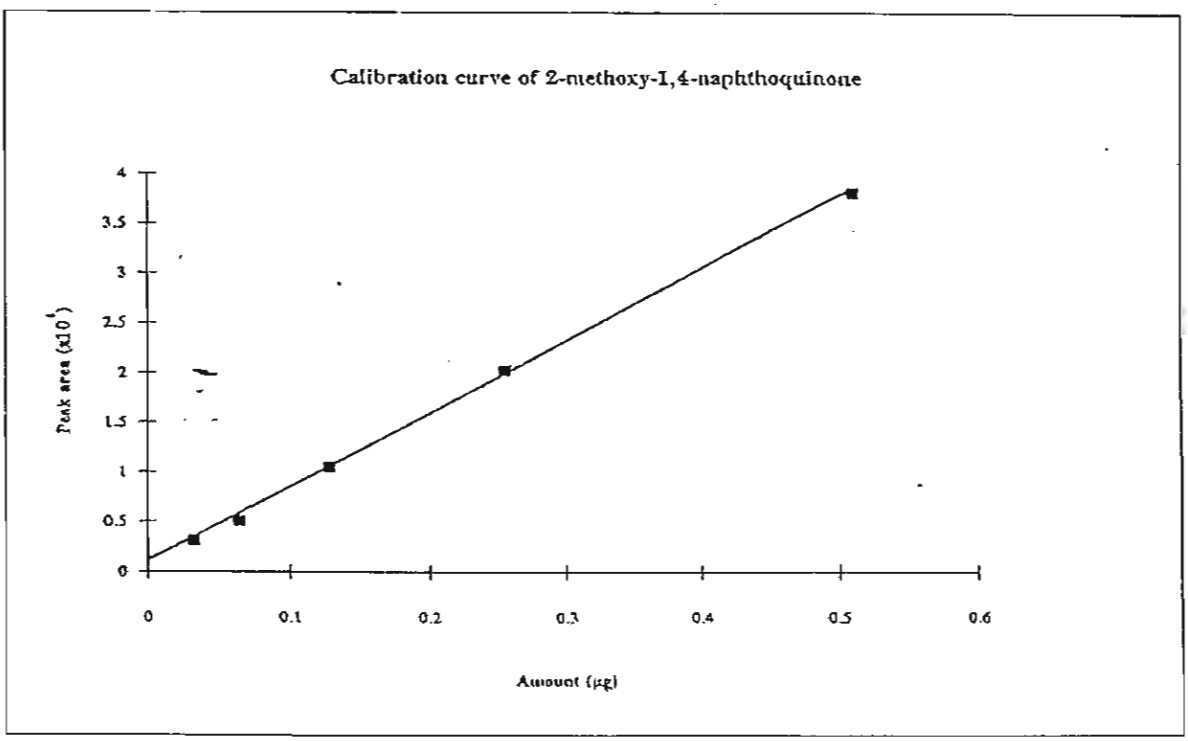

Fig. 6 Calibration curve of 2-methoxy-1,4-naphthoquinone

Amount (μg)	Peak area ($x10^4$)
0.033	0.22
0.066	0.35
0.132	0.72
0.265	1.36
0.53	2.61

Regression Output:	
Constant	0.060580207
Std Err of Y Est	0.024684415
R Squared	0.99952116
No. of Observations	5
Degrees of Freedom	3
X Coefficient(s)	4.8314S0471
Std Err of Coef.	0.061054642

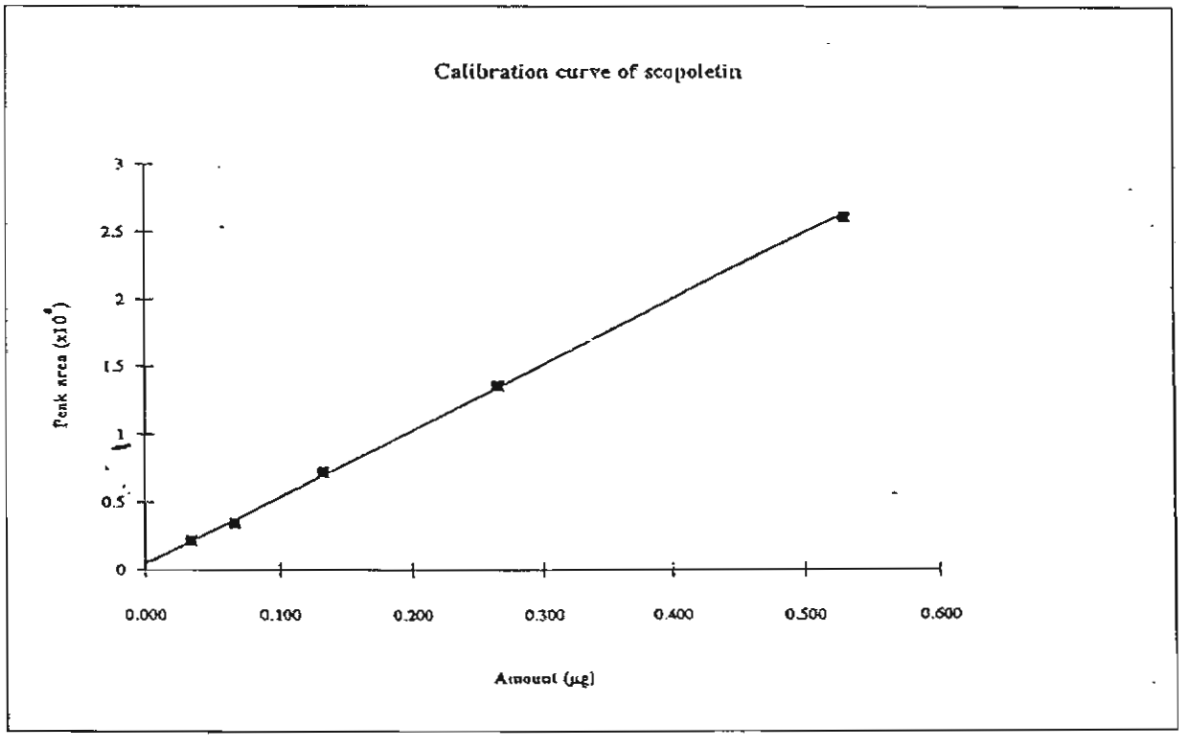

Fig. 7 Calibration curve of scopoletin

Based on this HPLC quantitative analysis, the content of lawsone, 2-methoxy-1,4-naphthoquinone, methylene-3,3'-bilawsone, scopoletin, isofraxidin and 4,4'-biisofraxidin, accumulated in the root cultures, the leaves and the roots of intact plants, were determined (Table 1). With respect to naphthoquinones, it was found that the root cultures accumulated higher content of lawsone (0.032 %) than the leaves (0.025 %) and the roots (less than 0.001%). The content of 2-methoxy-1,4-naphthoquinone accumulated in the root cultures (0.019 %), however, was less than that in the leaves (0.107 %) and the roots (0.028 %). These results suggested that the enzyme lawsone-O-methyltransferase better operate in the intact plants than in the root cultures. In contrast, methylene-3,3'-bilawsone was found to be accumulated in the root cultures (0.020 % base on lawsone) nearly the same amount as 2-methoxy-1,4-naphthoquinone, but not in the leaves and the whole roots. These results suggested that in the root cultures the enzyme system functioning on the formation of methylene 3,3'-bilawsone is as active as lawsone-O-methyltransferase while this enzyme system is not operated in the intact plants.

Table 1 The content of naphthoquinones and coumarin derivatives in *I. balsamina* root cultures (4-week old), the leaves and whole roots of the intact plants

Compound	Root culture (% dry weight)	Leaves (% dry weight)	Roots (% dry weight)
Lawsone	0.032	0.025	< 0.001
2-methoxy-1,4-naphthoquinone	0.019	0.107	0.028
Methylene-3,3'-bilawsone	0.020[a] 0.005	-	-
Scopoletin	0.032[b]	0.07	0.01
Isofraxidin	0.010[b]	< 0.001[b]	0.02[b]
Biisofraxidin		-	-

[a] Calculated from the calibration curve of lawsone, [b] calculated from the calibration curve of scopoletin.

For coumarin derivatives, the root cultures were found to accumulate scopoletin (0.005 %) in a rather smaller amount than the leaves (0.007%), though higher than the roots (0.001 %). On the other hand, the root cultures were found to contain in the highest amount of isofraxidin (0.035 % base on scopoletin) compared with the leaves (less than 0.001% base on scopoletin) and the roots (0.002 % base on scopoletin). These results suggested that the enzyme systems (oxidase and O-methyltransferase) functioning on the formation of isofraxidin are better operated in the root cultures than in the intact plants. As methylene-3,3' bilawsone, 4,4'-biisofraxidin was also accumulated only in the root cultures (0.010 % base on scopoletin) though in the higher amount than scopoletin. These results also suggested that the enzyme systems after scopoletin formation are well operated.

The ability of *I. balsamina* root cultures to produce the naphthoquinones and coumarin derivatives indicate that they can be a material of choice for biosynthetic studies of these naphthoquinones and coumarin derivatives. Such ability implies that various enzymes involved in these biosynthetic pathways are operating under these control conditions.

2. Kinetic Studies of Growth and The Formation of Naphthoquinone and Coumarin Derivatives

In a batch culture system, the biosynthetic activity of cultured cells usually varies with the stage of cell growth. Clarification of the relationship between cell growth and product formation is, therefore, an essential step in developing a better understanding of the controls operating in secondary metabolite production. Thus, in this study, the relationship between growth and the formation of naphthoquinones and coumarins of *I. balsamina* root cultures was examined. The dry weight , in gram per flask, of the harvested root was used as a parameter

for expressing the culture growth. Fig. 8A shows the growth cycle of the root cultures during a period of 30 days. It can be seen that there was a short period of the lag phase followed by a rapid growth of the exponential and linear phases. This resulted in a continuous increase in the biomass throughout the period of 19 days. The most active period of the increase in dry weight was observed from days 5 to 15. The growth rate was then slowing down to a zero-increase rate, which made the root culture reach a stationary growth phase at day 22. Thereafter, the dry weight of the biomass began to be constant and then declined, presumably due to the nutrient depletion of the medium. The root cultures attained their highest dry biomass weight of 1.02 g at day 22, equivalent to approximately 6 times of the inoculated root culture mass.

During this 30-day period of the culture growth, the formation of naphthoquinone and coumarin derivatives was also monitored and the results were expressed in the unit of milligram per gram dry weight. For the naphthoquinones (Fig. 8B), it was found that lawsone was initially accumulated in the early linear phase and actively biosynthesized throughout the linear phase, whereas methylene-3,3'-bilawsone was initially formed in the middle of the linear phase and actively biosynthesized until reaching the stationary phase. In contrast, 2-methoxy-1,4-naphthoquinone began to form in the late linear phase and was actively biosynthesized throughout the stationary phase. The highest level of lawsone was observed at day 15 and then the content slowly declined until the end of the growth cycle, while the formation of methylene-3,3'-bilawsone and 2-methoxy-1,4-naphthoquinone reached the maximum at days 22 and 28, respectively. These results suggested that the biosynthesis of lawsone is well operated in the early stage of growth, resulting in its accumulation in the middle growth stage. Subsequently, when the enzymes utilizing lawsone (e.g. methyl-transferase or condensing enzyme) were induced to be active, the cellular lawsone was used for the formation of 2-methoxy-1,4-naphthoquinone and methylene-3,3'-bilawsone in the middle or late stage growth phase.

As for the kinetic formation of coumarin derivatives (Fig. 8C), it was found that scopoletin began to be accumulated in the lag phase and still actively biosynthesized until the early linear phase, whereas isofraxidin and biisofraxidin were initially biosynthesized in the early linear phase and actively biosynthesized through the late linear phase until the stationary phase. The formation of scopoletin reached the maximum at day 7, while those of isofraxidin and biisofraxidin were at days 26 and 22, respectively. These production patterns also suggest that the biosynthesis of scopoletin is very active in the very early stage of growth, whereas the formations of isofraxidin and its dimer are actively operated in the late stage of growth. These are, again, probably due to the expression of the enzymes involved in the utilization of scopoletin at the middle growth phase.

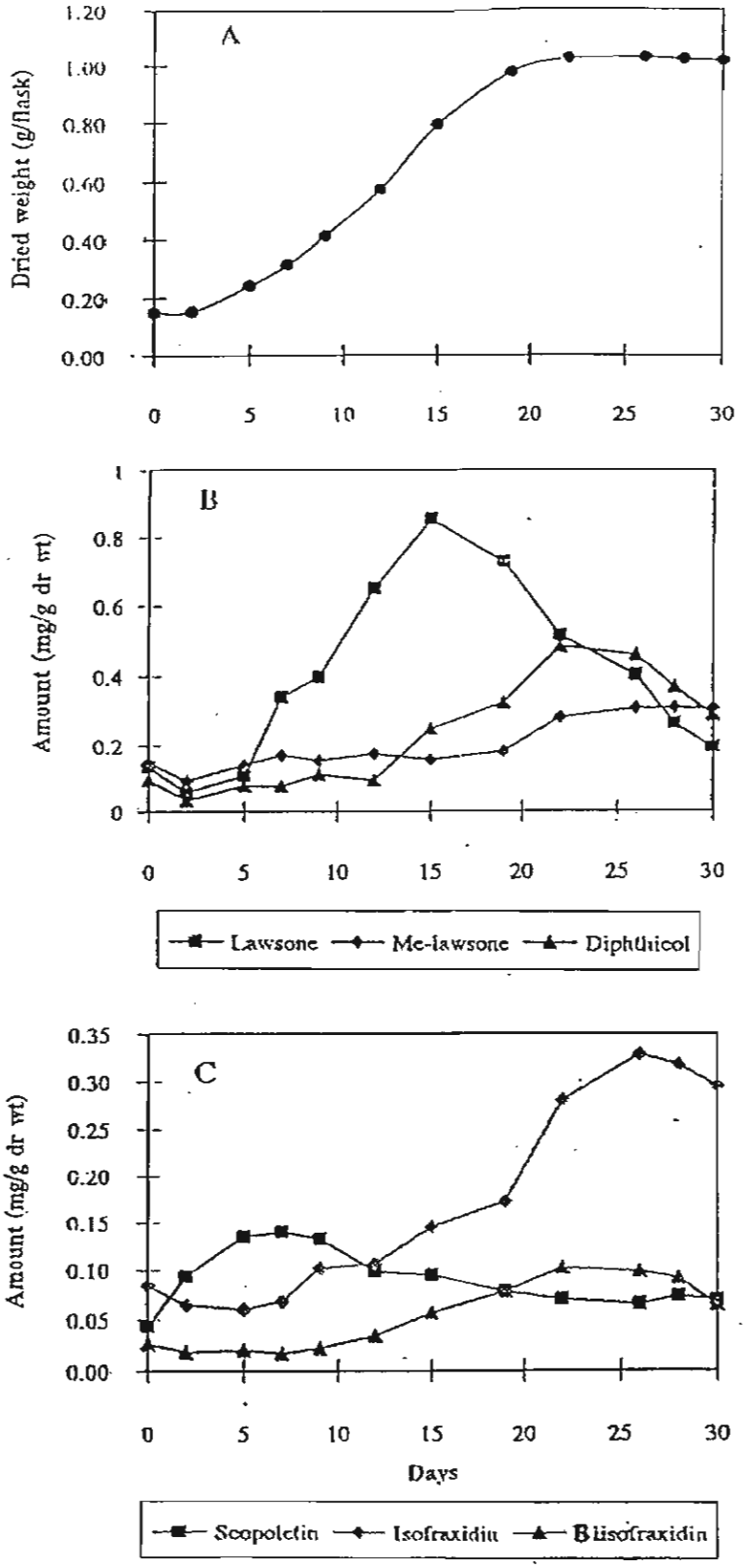

Fig. 8 Time-course of growth (A) and the formation of naphthoquinone (B) and coumarin (C) derivatives in *I. balsamina* root cultures

3. Studies on The Biosynthesis of Lawsone and 2-Methoxy-1,4-Naphthoquinone

3.1 Detection of OSB-CoA ligase activity

A study on the biosynthetic enzyme of lawsone and 2-methoxy-1,4-naphthoquinone in *I. balsamina* was first emphasized on the detection of the enzyme activity of *o*-succinylbenzoyl-CoA ligase activity which converts *o*-succinylbenzoic acid (OSB) to be its activated form, OSB-CoA ester (Fig. 9). This enzyme has been found previously in microorganisms and anthraquinone producing plants (Sieweke and Leistner, 1991; Sieweke and Leistner, 1992).

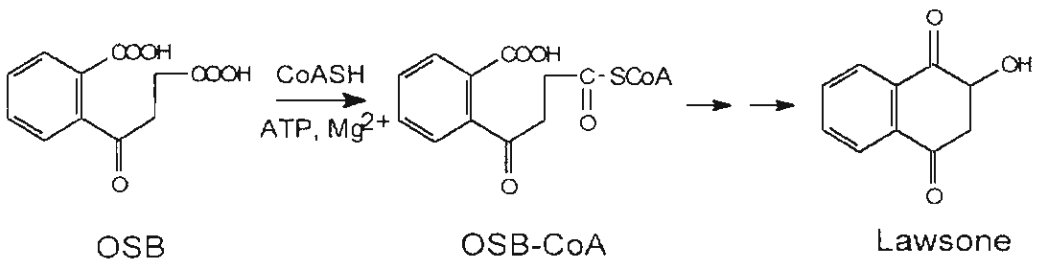

Fig. 9 Biogenesis of lawsone from OSB *via* its activated form, OSB-CoA ester.

In this study both [2,3-^{14}C]-OSB and nonlabelled OSB were used for the detection of OSB-CoA ligase activity in cell-free extract of *I. balsamina* root cultures. The routine assay mixture contained 0.7 mM OSB or 23,000 cpm [2,3-^{14}C]-OSB, 7.1 mM ATP, 1.8 mM CoASH, 7.1 mM $MgCl_2$ and 100 µl cell-free extract in a total volume of 140 µl. The incubation mixture was incubated at 30°C, for 30 minutes. Thereafter, the reaction was stopped by adding 20 µl formic acid and then analyzed for OSB-CoA ester. The techniques of both HPLC and TLC-radioscanning were used for the detection of this enzymatic product. No activity of the OSB-CoA ligase, however, was detected in the cell-free extract of *I. balsamina* root cultures with both techniques (Fig. 10). Furthermore, detection of the endogeneous OSB by HPLC revealed that no cellular OSB was presented in the root cultures (Fig. 2). These results suggested two possibilities. First, the biosynthetic pathway of lawsone in *I. balsamina* might not involve OSB as the intermediate. Second, the enzymes involved in the biosynthetic pathway of lawsone might be organized in such a way that they were closely associated to each other as a tight multienzyme complex and, thus, the added OSB could not reach the enzyme OSB-CoA ligase in the pathway.

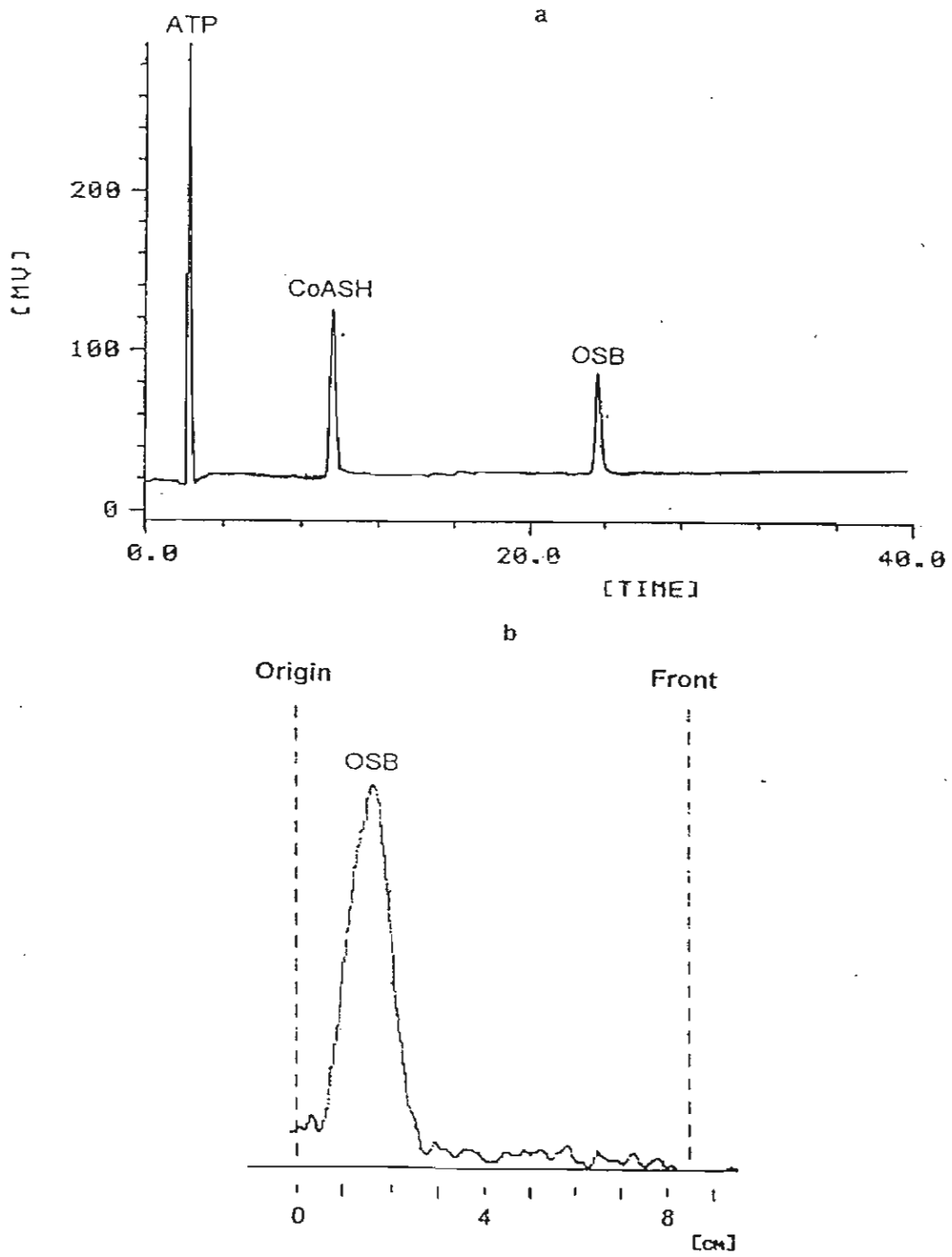

Fig. 10 HPLC chromatogram (a) and TLC-radiochromatogram showing no OSB-CoA ester was detected in the incubation mixtures of the OSB-CoA ligase assay.

3.2 *In vivo* feeding experiments with [^{14}C-U]α-ketoglutarate

In order to examine the possibilities mentioned above, *in vivo* feeding experiments were carried out using radiolabelled α-ketoglutarate as a precursor. In doing this, [^{14}C-U]α-ketoglutarate was feeded to the one-week and four-week-old root cultures and they were incubated for 3 days. After the incubation, an aliquot of the medium was taken and counted for the remaining radioactivity. It was found that 40 % and 76% of the radiolabelled α-ketoglutarate disappeared in the media of the one-week and four-week-old root cultures, respectively. This suggested that α-ketoglutarate was taken up by the old cultured roots (four weeks old) more effectively than the young roots (one week old). Both cultured roots were then harvested, extracted and the crude extracts were analyzed for their radioactive patterns. The results with the young root cultures showed that the radiolabelled substrate, [^{14}C-U]α-ketoglutarate, taken up by the root culture was incorporated directly into lawsone (Fig. 11). In contrast, feeding of [^{14}C-U]α-ketoglutarate to the old root cultures revealed that the labelled precursor was incorporated into 2-methoxy-1,4-naphthoquinone, interestingly, without significant detection of radiolabelled lawsone (Fig. 12). These results suggested that the enzymes involved in the biosynthesis of lawsone might be active in both the young and old root cultures, but the enzyme O-methyltransferase which methylates lawsone to form 2-methoxy-1,4-naphthoquinone might be active only in the old root cultures. This assumption was confirmed by the kinetic studies of lawsone and 2-methoxy-1,4-naphthoquinone formation in *I. balsamina* root cultures (Fig. 69) which showed that lawsone was initially formed in the early linear phase and biosynthesized throughout the linear phase, whereas 2-methoxy-1,4-naphthoquinone began to form in the late linear phase and actively biosynthesized throughout the stationary phase. Furthermore, it was also found that the incorporation of radioactive α-ketoglutarate into lawsone was smaller than in 2-methoxy-1,4-naphthoquinone. This might be explained by the uptake radiolabelled precursor was diluted by the primary metabolism which more active in young roots.

It should also be noted that the incorporation of [^{14}C-U]α-ketoglutarated into both naphthoquinones were taking place without any significant appearance of radiolabelled intermediates of the proposed biosynthetic pathway. These results suggested that the cellular pools of various intermediates in the biosynthetic pathway of lawsone and 2-methoxy-1,4-naphthoquinone are extremely small and those enzymes involved in the pathway should be organized in a complex form.

3.3 *In vivo* feeding experiments with [^{13}C]-methionine

To search for the chemical source of the methyl group of 2-methoxy-1,4-naphthoquinone and the methylene bridge of methylene-3,3'-bilawsone, another feeding experiment using [^{13}C]-methionine was carried out. Methionine is an amino acid in which its activated form, S-adenosylmethionine (SAM) acts as a general methyl group donor to methylate specific acceptors to form corresponding methylated products. The feeding experiment was performed as described in section 7.6, Chapter 3. After the feeding, 2-methoxy-1,4-naphthoquinone and methylene-3,3'-bilawsone were isolated. The identity of 2-methoxy-1,4-naphthoquinone and methylene-3,3'-bilawsone were confirmed with their Rf values (0.72 and 0.55, respectively) and UV absorption spectra compared with those of the authentic compounds. The results showed that the ^{13}C NMR spectrum of 2-methoxy-1,4-naphthoquinone exhibited a ^{13}C-enriched signal at δ 56.4 (Fig 13), indicating that [^{13}C]-methionine was incorporated into the methoxy group of 2-methoxy-1,4-naphthoquinone. In contrast, ^{13}C NMR spectrum of methylene-3,3'-bilawsone gave no ^{13}C-enriched signal of the methylene carbon at δ 18.4 (Fig. 14), indicating that [^{13}C]-methionine was not incorporated into the methylene bridge of methylene-3,3'-bilawsone. These results suggest that S-adenosylmethionine is served as the methyl source in the O-methylation of 2-methoxy-1,4-naphthoquinone biosynthesis, but not in C-methylation of methylene-3,3'-bilawsone biosynthesis.

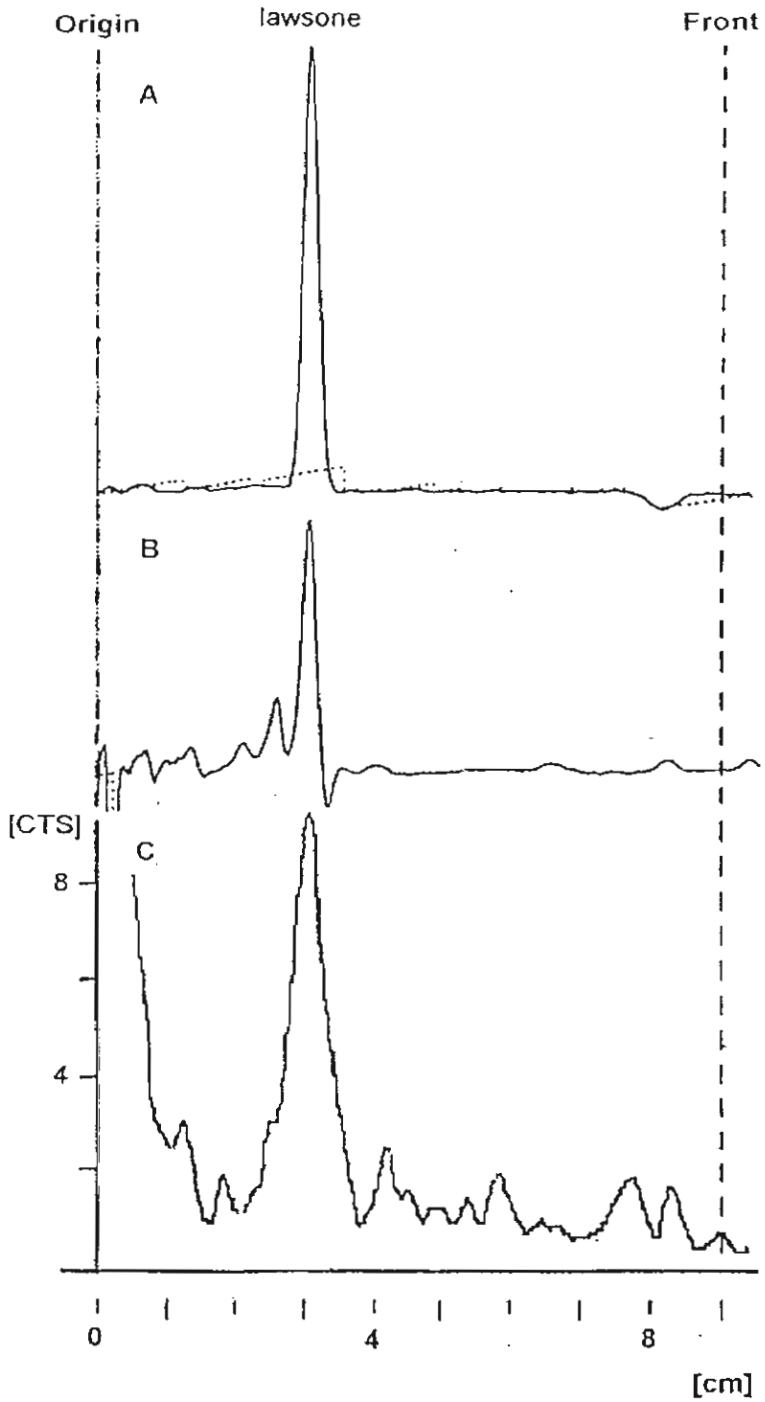

Fig. 11 TLC chromatograms of the authentic lawsone (A) and the cultured root extract (B) and the radiochromatogram of the cultured root extract (C)

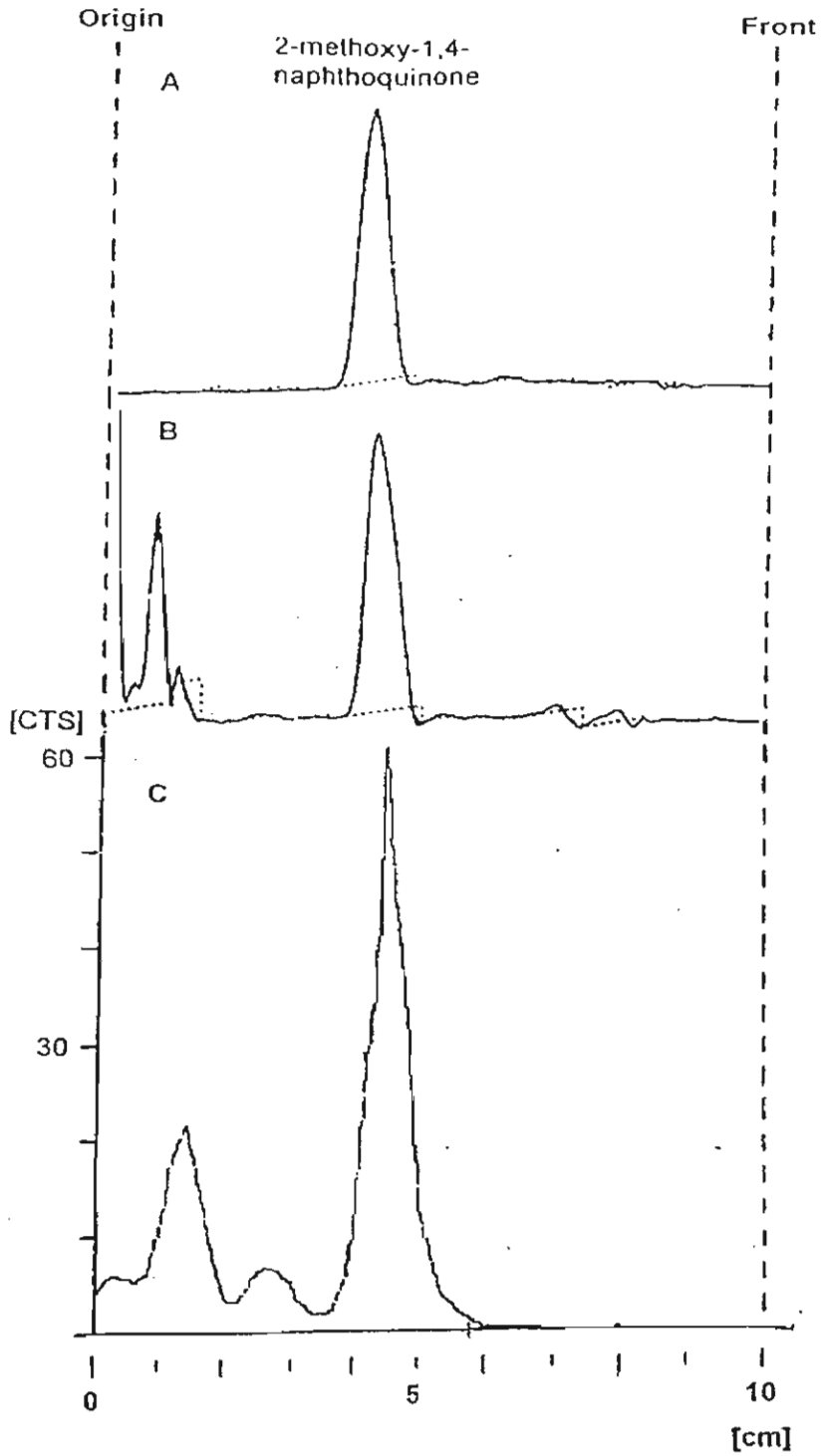

Fig. 12 TLC chromatograms of the authentic 2-methoxy-1,4-naphthoquinone (A) and the cultured root extract (B) and the radiochromatogram of the cultured root extract (C)

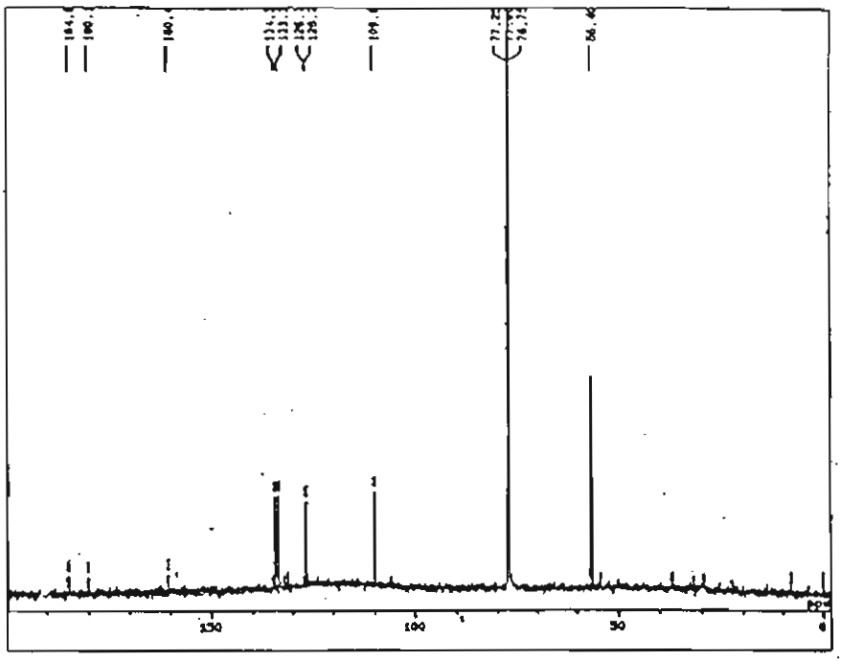

Fig. 13 ^{13}C NMR (125 MHz) spectrum of 2-methoxy-1,4-naphthoquinone showing the ^{13}C-enriched signal at δ 56.4

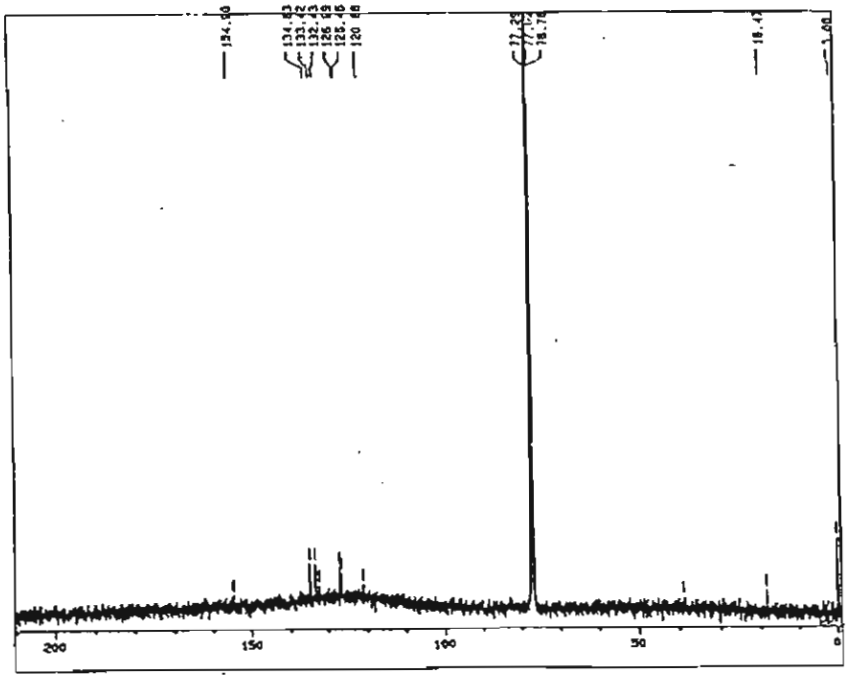

Fig. 14 ^{13}C NMR (125 MHz) spectrum of methylene-3,3'-bilawsone showing no ^{13}C-enriched signal at δ 17.9

3.4 Enzymatic formation of 2-methoxy-1,4-naphthoquinone by cell-free extracts of root cultures

To search for the enzyme activity involved in the biosynthetic pathway of lawsone and 2-methoxy-1,4-naphthoquinone, the cell-free extraction of four-week-old *I. balsamina* root cultures was prepared and examined for the enzyme activity using a radioactive substrate. The results showed that incubation of a crude cell-free extract of *I. balsamina* root cultures with [^{14}C-U]α-ketoglutarate in the presence of coenzyme A, ATP and Mg^{2+} led to rapid formation of the radioactive labelled 2-methoxy-1,4-naphthoquinone. This radioactive product was detected by TLC-radiochromatography (Fig! 15).

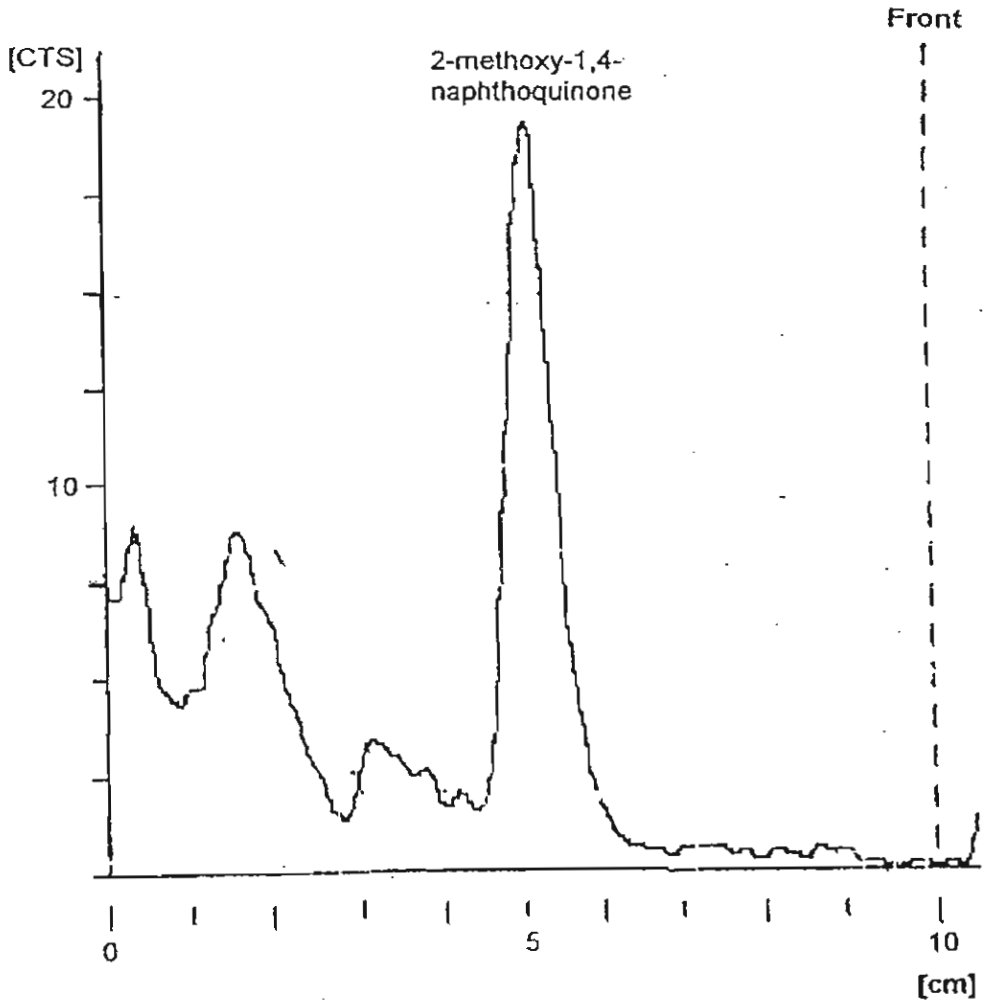

Fig. 15 TLC-Radiochromatogram showing the conversion of [^{14}C-U]α-ketoglutarate into 2-methoxy-1,4-naphthoquinone by cell-free extract of *I. balsamina* root cultures

In contrast, the boiled control reaction mixture did not show any reaction product of the labelled α-ketoglutarate. These results indicate that there is an enzyme activity involved in the biosynthesis of 2-methoxy-1,4-naphthoquinone in the cell-free extract of root cultures. It was also observed that no significant intermediate was detected in TLC-radiochromatogram. This confirms our assumption that the enzymes of lawsone and 2-methoxy-1,4-naphthoquinone are organized as an enzyme complex.

3.5 Identification of the enzymatic product

Identification of the enzymatic product was, first, carried out by TLC. The Rf values of the reaction product, 0.52 and 0.38, are the same as those of the authentic 2-methoxy-1,4-naphthoquinone in two solvent systems, (I) petroleum ether/chloroform (2:8) and then benzene/acetic acid (98:2); and (II) chloroform, respectively. Its UV absorption spectrum, measured by TLC densitometric method, was also similar to that of the authentic 2-methoxy-1,4-naphthoquinone (Fig. 16).

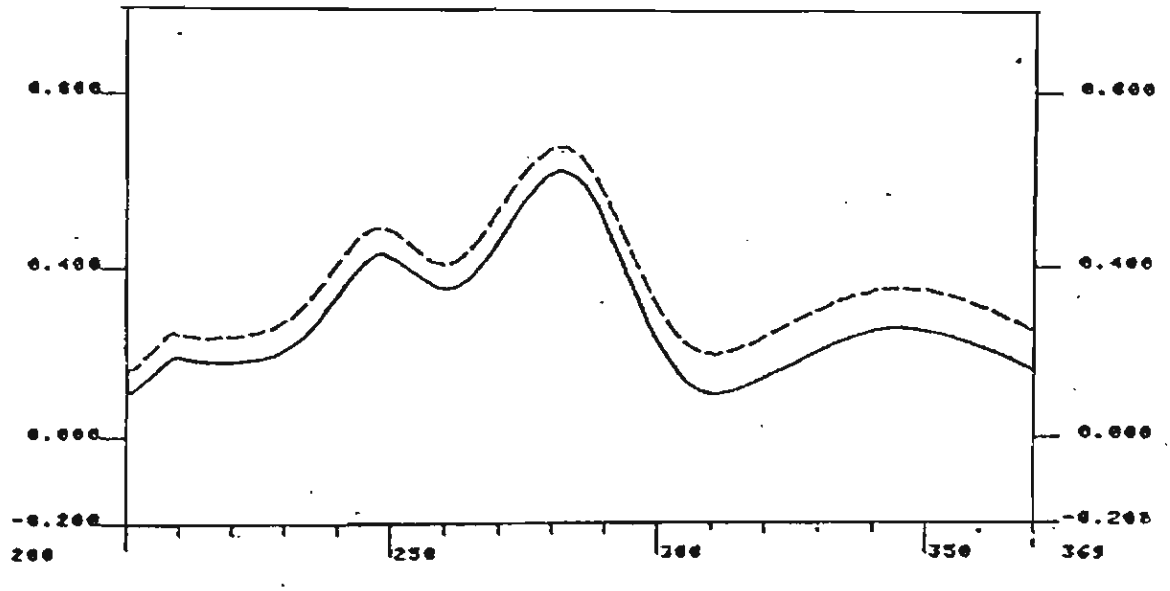

Fig. 16 UV spectra of the enzymatic product (———) and the authentic 2-methoxy-1,4-naphthoquinone (- - - -) measured by TLC-densitometric method

Moreover, the enzymatic product was further identified by recrystallization method. The spots of the enzymatic product on TLC were cut off and eluted with chloroform. This enzymatic product was then diluted with unlabelled 2-methoxy-1,4-naphthoquinone (10 mg) and recrystallized (62.50 cpm x μmol^{-1}). Additional recrystallization from chloroform-methanol (x3) showed a constant specific activity of 62.23 cpm x μmol^{-1}. These results confirm that the enzymatic product is 2-methoxy-1,4-naphthoquinone.

3.6 Partial purification of the enzyme system producing 2-methoxy-1,4-naphthoquinone

In order to confirm that the enzymes responsible for lawsone biosynthesis are organized as an enzyme complex system, the technique of gel filtration was introduced. This technique allows different protein molecules to be separated from one another based on their molecular sizes. The very big protein sizes would be eluted with void volume whereas the ones with smaller sizes would be eluted later. The purpose of this experiment was to observe whether the activity of lawsone biosynthesis disappeared or still remained after the crude extract was subjected to the step of gel filtration. The disappearance of the enzyme activity would oppose our suggestion on the multienzyme complex while the retaining of enzyme activity would support this proposal. In this experiment, the crude cell-free preparation was first subjected to the ultracentrifugation (100,000g, for 30 min). Both the supernatant and pellet fractions obtained by 100,000g ultracentrifugation were assayed for the enzyme activity. It was found that when the labelled α-ketoglutarate was incubated with 100,000g supernatant

fraction, it was converted rapidly into 2-methoxy-1,4-naphthoquinone (Fig. 17). No enzyme activity was observed with the pellet fraction. Therefore, the 100,000g supernatant part was used for further experimentation. By desalting the supernatant fraction using a Sephadex PD-10 column, however, the enzyme activity could not be detected anymore. The possible reason might be due to the deficiency of some cofactors needed in the biosynthetic pathway. Various cofactors which potentially required in the biosynthetic pathway, including NADPH, NADP and thiamine were therefore added into the incubation mixture. The enzyme activity, however, still could not be detected in the desalted enzyme solution. This suggested that there are other undefined cofactors in the crude enzyme extracts that are essential for the biosynthesis of 2-methoxy-1,4-naphthoquinone. The crude salt fraction eluted lately from PD-10 column was therefore saved for checking whether this small molecule fraction could restore the enzyme activity of the desalted extract. This was done by adding the small molecule fraction into the incubation mixture during the enzyme assay. Indeed, the desalted crude extract appeared to gain the enzyme activity back after the reconstitution. This suggested that the small molecular fraction from PD-10 contains some unknown cofactors which are required for the biosynthesis of 2-methoxy-1,4-naphthoquinone. These findings were useful for the further purification of the enzyme complex.

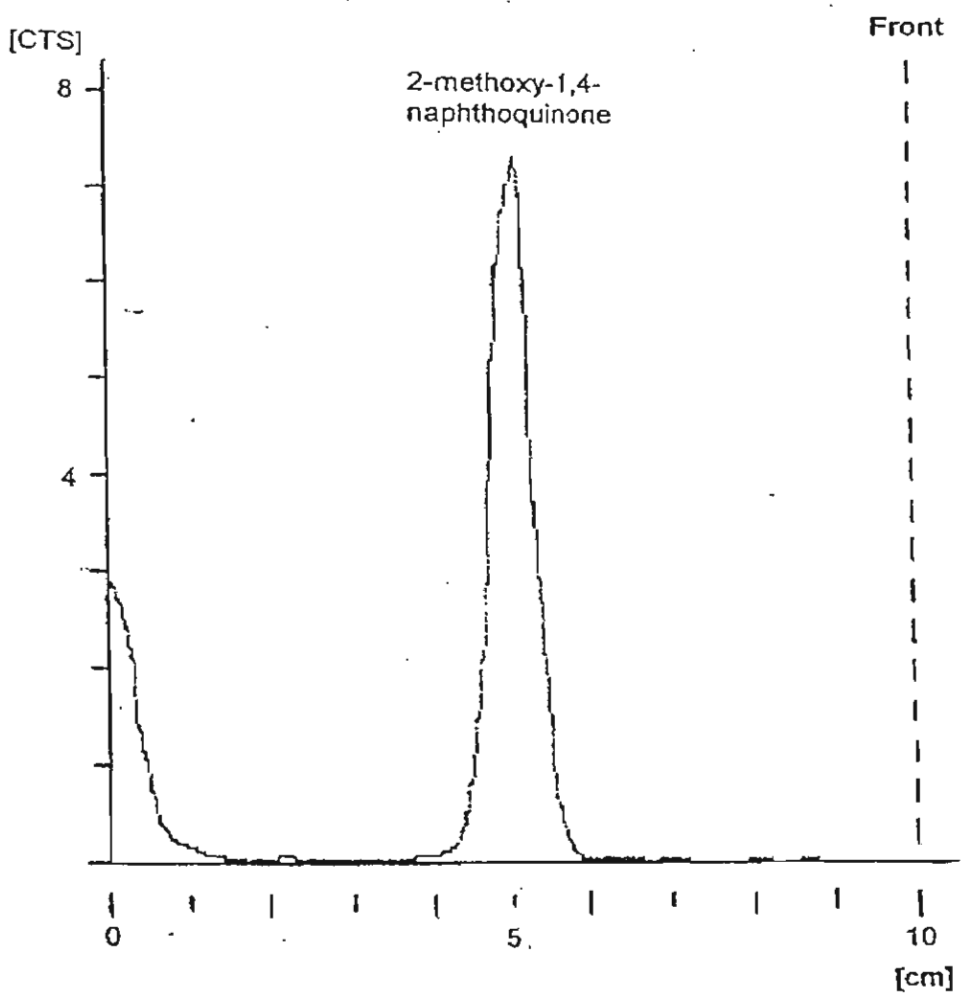

Fig. 17 TLC-radiochromatogram showing the conversion of [^{14}C-U]α-ketoglutarate into 2-methoxy-1,4-naphthoquinone by 100,000g supernatant part of the cell-free extract

In the step of gel filtration, the desalted 100,000g supernatant fraction was first concentrated by Centricon-30, and then fractionated on a Superose 12 HR column. The column was eluted with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.2 mM DTT. The amount of protein in each collected fraction was determined by Bradford's method (Bradford, 1976). The eluted profile (Fig. 18) of protein showed a major single peak eluted with the void volume which covered the fractions 30-32. Enzyme assay of all the fractions also showed that the enzyme activity was present only in the fractions of the same major protein peak (fractions 31 and 32). The void volume of Superose 12 HR 16/50 column was determined as 30 ml, suggesting that the enzyme fractions 31 and 32 contained high molecular weight proteins These results confirm our proposal that the enzymes involved in lawsone biosynthesis are organized as a high-molecular-weight enzyme complex.

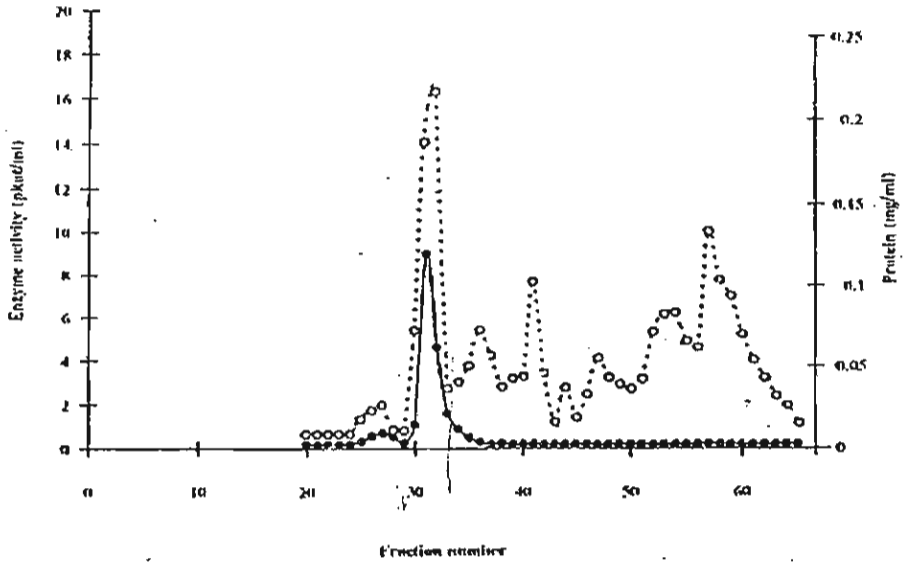

Fig. 18 The elution profile of enzyme activity (—○—) and protein (- - ○ - -) on Superose 12 gel filtration column

3.7 Some characteristics of the enzyme complex

With the partially purified enzyme preparation, the involvement of some cosubstrates or cofactors in the functioning of enzyme complex was examined. In this experiment, the relative enzyme activity was calculated based on the incorporation of the labelled α-ketoglutarate into 2-methoxy-1,4-naphthoquinone in the complete incubation (100 %) As shown in Table 2 it was found that the conversion of the labelled α-ketoglutarate to 2-methoxy-1,4-naphthoquinone was accelerated by the addition of chorismic acid, but not affected by the addition of OSB or benzoic acid into the incubation mixture. The relative enzyme activity could be increased to 122.6 % by chorismic acid, suggesting that chorismic acid, but not OSB or benzoic acid, could be channeled by the enzyme complex, as a cosubstrate, into 2-methoxy-1,4-naphthoquinone In addition, the decrease in the radioactivity of 2-methoxy-1,4-naphthoquinone was observed by the addition of unlabelled lawsone into the incubation mixture. This suggested that the labelled lawsone could be diluted by the added unlabelled lawsone prior to the step of methylation by the enzyme O-methyltransferase. Therefore, the enzyme O-methyltransferases seem to be loosely bound to the enzyme complex due to the ability of the unlabelled lawsone in interfering the formation of 2-methoxy-1,4-naphthoquinone in the methylation step.

Table 2 Relative incorporation of [^{14}C]α-ketoglutarate into 2-methoxy-1,4-naphthoquinone by cell-free preparation of *I. balsamina* root cultures

Condition	Relative enzyme activity (%)
Complete incubation	100.0
boiled enzyme	0.2
+ OSB	105.2
+ chorismic acid	122.6
+ benzoic acid	100.1
+ lawsone	53.4
Incomplete incubation	
- CoASH	60.6
- ATP	36.0
- $MgCl_2$	43.7

In the absence of CoASH or ATP, or Mg^{2+}, the radioactivity of 2-methoxy-1,4-naphthoquinone, compared with the complete incubation , was also decreased. The relative enzyme activity decreased to 60.6 %, 36.0 % and 43.7 %, when coenzyme A or ATP or Mg^{2+} were omitted, respectively. This suggested that CoASH, ATP and Mg^{2+} were also required as cosubstrate or cofactor in the biosynthesis of lawsone and 2-methoxy-1,4-naphthoquinone.

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APPENDIX

"Publications"

Dedicated to Nikolaus Amrhein, Zurich, on the occasion of his 60th birthday.

Molecular characterization of root-specific chalcone synthases from *Cassia alata*

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The nucleotide sequences reported in this paper have been submitted to the GenBank™/EBI Data Bank with accession numbers AF358430 (*CalCHS1*). AF358431 (*CalCHS2*), AF358432 (*CalCHS3*).
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Abstract Three cDNAs encoding very similar but unique isoforms of chalcone synthase [EC 2.3.1.74] were isolated from a cDNA library prepared from RNA from root tissue of the Thai medicinal plant *Cassia alata* L. (ringworm bush, Leguminosae). Gene transcript for these three type III polyketide synthases was found to accumulate predominantly in root. The heterologously expressed enzymes accepted acetyl-, *n*-butyryl-, isovaleryl-, *n*-hexanoyl-, benzoyl-, cinnamoyl-, and *p*-coumaroyl-CoA as starter molecules and together with the co-substrate malonyl-CoA, formed multiple products. With the exception of the assay in which acetyl-CoA was used as the starter molecule, all substrates yielded a phloroglucinol derivative resulting from three sequential condensations of acetate units derived from three malonyl-CoA decarboxylations. Every substrate tested also produced two pyrone derivatives, one resulting from two acetate unit condensations (a bis-noryangonin-type pyrone derailment product) and one resulting from three acetate unit condensations (a 4-coumaroyltriacetic acid lactone-type pyrone derailment). *C. alata* accumulates the flavonoids quercetin, naringenin and kaempferol in roots, suggesting that the *in planta* function of these enzymes is the biosynthesis of root flavonoids.

Keywords *Cassia*, Leguminosae, Ringworm Bush, Polyketide Synthase, Chalcone Synthase, Flavonoids

Abbreviations CE: collision energy, CHS: chalcone synthase, CID-MS: collision-induced dissociation mass spectrometry, CoA: coenzyme A, DTT: dithiothreitol, HEPES: *N*-2-hydroxypiperazine-*N*-2-ethane sulfonic acid, LC/ESI-MS: liquid chromatography/electrospray ionization-mass spectrometry, PCR: polymerase chain reaction, 2-PS: 2-pyrone synthase, RT: retention time, RT-PCR: reverse transcription-polymerase chain reaction, PKS: polyketide synthase, SRM: selected reaction monitoring, TLC: thin layer chromatography, Tris: tri(hydroxymethyl)methylamine

Introduction

Plant polyketide synthases are encoded by a multigene family that has chalcone synthase as a prototype. Gene family evolution in plants appears to occur by gene duplication followed by nucleotide substitution that can lead to biochemical diversity. Plant polyketide synthases are presumably derived from a common ancestor that diverged to perform different reactions. This is clearly demonstrated by the highly similar genes that encode chalcone synthase and resveratrol synthase in a single species, such as found in *Vitis vinifera* (Melchior and Kindl 1990; Sparvoli et al. 1994), or by the occurrence of the closely related, but enzymatically unique chalcone and acridone synthases in *Ruta graveolens* (Springob et al. 2000).

Detailed crystal structure and site-directed mutagenesis studies have provided insight into the structural control of polyketide formation in plant homodimeric iterative (type III) polyketide synthases (Jez et al. 2000a; 2000b; Suh et al. 2000). The active site cavity can serve as a size-based filter to sterically exclude bulkier starter molecules (Jez et al. 2000a). This explains why pyrone synthase cannot accept *p*-coumaroyl CoA as a starter molecule, whereas chalcone synthase can use acetyl CoA as a starter molecule, albeit inefficiently, and forms a small amount of methylpyrone (Schüz et al. 1983). Modification of as few as three amino acids can change chalcone synthase into a pyrone synthase (Jez et al. 2000a). This demonstrates how readily plant type III polyketide synthases can be modified by nucleotide substitution to produce new natural products. Plants produce a variety of polyketide-derived secondary metabolites such as naphtha- and anthraquinones that could potentially be biosynthesized from type III polyketide synthases, such as has been demonstrated for the formation of tetrahydroxynaphthalene in the microorganism *Streptomyces griseus* (Funa et al. 1999).

Cassia alata L. (ringworm bush, Leguminosae), distributed mainly in the tropics and subtropics, is used to treat superficial mycosis of the skin (Crockett et al. 1992). Anthraquinones accumulate in aerial plant parts as well as in roots (Kelly et al. 1994; Yadav and Kalidhar 1994) and are the principal laxative constituents in *Cassia* species used as purgatives (Elujoba et al. 1989). *C. alata* also finds use in preventative veterinary medicine and is used to deworm dogs (Lans et al. 2000). In an effort to characterize polyketide synthase genes from medicinal plants native to Thailand and to search for polyketide synthases catalyzing anthraquinone formation, we have isolated and functionally expressed three cDNAs encoding isoforms of a root-specific polyketide synthase from *C. alata*. We report herein on the molecular characterization of these three polyketide synthase isoforms.

Materials and methods

Plant material

Seeds of *Cassia alata* L. were obtained from plants growing in an open field of The Walai Rukhavej Botanical Research Institute, Mahasarakham University, Mahasarakham, Thailand. They were sown in the greenhouse at the Institute of Plant Biochemistry, Halle, Germany, at 24 °C with 18 h of light and 50% humidity. Young plant material was used for the following experiments.

cDNA isolation

Partial cDNA clones encoding putative polyketide synthases were generated by PCR using cDNA produced by reverse transcription of total RNA. Total

RNA (10 μg) was reverse transcribed using a cDNA synthesis kit (Life Technologies) as described by the manufacturer. DNA amplification using degenerate primers, (5'-AA(AG)GC(CT)AT(AC)GAIGA(AG)TGGGG-3') as the forward primer and (5'-CCACCIGG(AG)TGI(AG)CAATCC-3') as the reverse primer, was performed under the following conditions: 3 min at 94 °C, 3 min for 1 cycle, then 30 cycles of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 1 min. The last step was followed by an additional elongation step at 72 °C for 7 min. The amplified DNA was resolved by agarose gel electrophoresis. The band of approximately the desired size (584 bp) was eluted from the gel using a gel extraction kit (Qiagen). The DNA was subsequently ligated into pGEM-T Easy (Promega) and was transformed into *E. coli* DH5α. Sequencing of several clones confirmed that the PCR products showed similarity to polyketide synthases.

Poly(A)$^+$RNA was used as the template for first and second strand cDNA synthesis, using a ZAP-cDNA synthesis kit (Stratagene). The cDNA was extended with *Eco* RI and *Xho* I adaptors prior to ligation into the Uni-ZAP XR vector (Stratagene), which was then ligated to λ-ZAPII. The phagemids were packaged with a Gigapack III Gold Packaging Extract (Stratagene).

The partial clones encoding polyketide synthases that were generated by RT-PCR, as described above, were labeled with ^{32}P and used to screen a *C. alata* cDNA library of 100,000 phages for full-length clones. The library was plated and the plaque DNA was transferred to nitrocellulose membranes (Schleicher & Schuell). Filters were hybridized with the individual hybridization probes in a solution of 5x SSPE containing 50% formamide, 5% Denhardt's reagent, 0.5% SDS and 100 mg/ml salmon sperm DNA at 42 °C overnight. The hybridized filters were washed three times in 0.1x SSPE and 0.1% SDS for 20 min at 42 °C. Radioactivity was visualized on X-ray film using an intensifying screen. In this manner, three clones (*CalPKS1*, *CalPKS2* and *CalPKS3*) were identified and taken through several rounds of screening until the viruses were pure enough to convert to plasmids by excision. To ascertain the identity of the cDNAs as encoding polyketide synthases, the nucleotide sequences were determined on both strands and the translations were compared to those sequences available in the GenBank/EMBL databases.

Functional expression and enzyme assays

The CalPKS reading frames were amplified by PCR. CalPKS1 using 5'-TAGTCATATGGTGAAGGTGGAAGAG-3' as the forward primer (*Nde* I site is underlined and the translation start site is bold) and 5'-TTGGCTCGAGTTAAATAGCAATACTGT-3' as the reverse primer (*Xho* I site is underlined and stop codon is bold); CalPKS2 using 5'-ATTTTTCATATGGTGAGTGTTGAAGAG-3' as the forward primer (*Nde*

I site is underlined and the translation start site is bold) and 5'-TTTAAAGGATCCTTAGTTAACTGCCACACT-3' as the reverse primer (*Bam*H I site is underlined and stop codon is bold); CalPKS3 using 5'-TAGTCATATGGTGAAGGTGGAAGAG -3' as the forward primer (*Nde* I site is underlined and the translation start site is bold), and 5'-TTGGCTCGAGTTAGACAGCCACACTAT-3' as the reverse primer (*Xho* I site is underlined and stop codon is bold) with the cDNAs in pBluescript as template. The 1.2 kb PCR products were digested with *Nde* I and *Xho* I, gel purified and ligated into *Nde*I/*Xho*I-digested pET-14b (Novagen) to generate the expression plasmids pET*CalPKS1*, pET*CalPKS2* and pET*CalPKS3*.

pET*CalPKS1*, pET*CalPKS2* and pET*CalPKS3* were each transformed into *E. coli* BL21(DE3). Transformed *E. coli* were grown at 37 °C in Luria-Bertani medium containing 50 µg/ml ampicillin until $A_{600\ nm}$ = 0.6. After induction with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside, the cultures were grown at 28 °C for an additional 12 hrs. Cells were collected by centrifugation (10,000 x *g*, 10 min, 4 °C) and resuspended in a buffered solution containing 50 mM Tris-HCl (pH 7.0), 500 mM NaCl, 2.5 mM imidazole, 10 mM β-mercaptoethanol, 10% (v/v) glycerol, 1% (v/v) Tween-20 and 0.75 µg/ml lysozyme (47,000 units/mg solid). After sonication and centrifugation (10,000 x *g*, 10 min, 4 °C), the supernatant was passed over a Talon (Clontech) metal affinity resin column. The resin was washed with 10 bed volumes of a buffered solution containing 50 mM Tris-HCl (pH 7.0), 500 mM NaCl, 2.5 mM imidazole, 10 mM β-mercaptoethanol, 10% (v/v) glycerol, and the His-tagged protein was eluted with a buffered solution containing 50 mM Tris-HCl (pH 7.0), 500 mM NaCl, 10 mM β-mercaptoethanol, 10% (v/v) glycerol and 250 mM imidazole. The eluted protein solution was desalted on a PD-10 column (Pharmacia) equilibrated with 50 mM HEPES (pH 7.0), 5 mM DTT, 10% glycerol and was stored at 4 °C.

Polyketide synthase activity was measured by the conversion of various "starter" CoA molecules and [2-^{14}C]malonyl CoA into reaction products. The standard enzyme assay contained 100 mM HEPES buffer (pH 7.0), 20 µM "starter" CoA and 12,000 dpm malonyl CoA (2 GBq/mmol) (Biotrend Chemikalien, Cologne) and 1.5 µg polyketide synthase in a 50 µl reaction volume. The assay mixture was typically incubated for 30 min at 30 °C. The reaction was stopped by addition of 5 µl 10% (v/v) HCl and was extracted twice with 100 µl ethylacetate. The combined organic phase was evaporated to dryness, the residue dissolved in 10 µl ethylacetate and this entire volume was resolved by RP-C18 thin layer chromatography (Merck). The TLC plates were developed in methanol:H_2O:acetic acid (75:25:1). The radioactive products were quantified with a Rita Star TLC scanner (Raytest) or by phosphorimagery.

Mass spectrometric analysis

The positive and negative electrospray ionization (ESI) mass spectra were obtained with a Finnigan MAT TSQ 7000 instrument (electrospray voltage 4.5 kV; heated capillary temperature 220 °C; sheath and auxillary gas: nitrogen) coupled to a Micro-Tech Ultra-Plus MicroLC system equipped with an RP18-column (5 µm, 1x100 mm, SepServ, Berlin). For all compounds (**1-7**), a gradient system was used that ranged from H_2O:CH_3CN 90:10 (each containing 0.2% HOAc) to 10:90 over 15 min, followed by isocratic elution with a 10:90 mixture of both solvents for 10 min; flow rate 70 µl min^{-1}. The collision-induced dissociation (CID) mass spectra during an HPLC run were recorded with a collision energy of –20 or -25 eV for positive ions as well as +20 or +25 eV for negative ions, respectively (see Table 2); collision gas: argon, collision pressure: 1.8 x 10^{-3} Torr.

Flavonoid analysis

Plant tissue (1 g fresh weight) was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. To the powder was added 10 ml 80% methanol, 3% concentrated HCl. The suspension was heated to 100 °C for 60 min in a sealed tube. After cooling, the cell debris was removed from the extract by centrifugation at 10,000 x g for 10 min at room temperature. The extract was taken to dryness *in vacuo*, dissolved in 5 ml water and extracted twice with 2 volumes of ethylacetate. The ethylacetate was removed *in vacuo* and the residue dissolved in 50 µl 50% methanol. Isolubles were separated by centrifugation (10,000 x g, 10 min, room temperature). For resolution of the natural products, 10 µl of the supernatant was injected onto a Hewlett Packard Series 1100 HPLC equipped with a Knauer Eurospher-100 C18 column (5 µm, 250 x 4 mm). The solvent system was 97.8% (v/v H_2O), 2% CH_3CN, 0.2% H_3PO_4 (A) and 97.8% (v/v CH_3CN), 2% H_2O, 0.2% H_3PO_4 (B) with a gradient of 0-30 min 20-45% B, 30-35 min 45-55% B at a flow rate of 0.6 ml min^{-1}. The flavonoid content was monitored at 370 nm.

General methods

Young leaves and roots were harvested, frozen immediately in liquid nitrogen and stored at -80 °C. The isolation of total RNA was carried out as described by Salzman, et al. (1999). Poly(A)$^+$ RNA was isolated with Oligotex beads (Qiagen) according to the manufacture's instructions.

For RNA gel blot analysis, total RNA (10 µg) was resolved by electrophoresis on 1% agarose gels containing formaldehyde. The RNA was transferred to a nylon membrane (Hybond N, Amersham) by capillary blotting. The three *Cassia* polyketide synthase cDNAs were labeled with

^{32}P using a random primer DNA labeling kit (Life Technologies) and were then used as hybridization probes. Hybridization was performed at 65 °C overnight and the membranes were then washed three times for 15 min with 2x SSC and 0.1% SDS at 65 °C. Radioactivity was visualized by phosphorimagery.

Genomic DNA was isolated from young leaves according to Dellaporta et al. (1983). The DNA (10 μg) was incubated with each of the following restriction endonucleases: *Apo*I, *Bcl* I and *Bst*X I (one restriction recognition site within the reading frame), and *Dra* III, *Nde* I and *Xba* I (no restriction recognition site within the reading frame) and was then resolved by electrophoresis on a 0.8% agarose gel, and capillary blotted onto a Hybond N nylon membrane. The three *Cassia* polyketide synthase cDNAs were labeled with ^{32}P using a random primer DNA labeling kit and were then used as hybridization probes. Hybridization was performed at 65 °C overnight and the membranes were then washed three times for 15 min with 2x SSC and 0.1% SDS at 65 °C. Radioactivity was visualized by phosphorimagery.

Results and discussion

Isolation of cDNAs encoding *C. alata* polyketide synthases

Using oligodeoxynucleotide primers that were based upon conserved regions in plant polyketide synthases, RT-PCR was carried out using *C. alata* root total RNA as template. The PCR products were resolved by agarose gel electrophoresis and the band of approximately the correct size (584 bp) was purified and ligated into pGEM-T Easy. Nucleotide sequence analysis revealed that the PCR products were highly similar to plant chalcone synthases. These partial clones were then used as a hybridization probe with which to screen a cDNA library in λ ZAP II prepared from *C. alata* root mRNA. In this manner, three clones that encoded complete reading frames were isolated. Translation of the nucleotide sequences of the three cDNAs indicated that they were highly similar, but non-identical (Fig. 1). A comparison of the translation of the nucleotide sequences of these cDNAs with those present in the GenBank/EMBL databases indicated that the *Cassia* genes are most similar to chalcone synthase from other members of the Leguminosae, specifically *Glycine max* (Akada et al. 1993) and *Phaseolus vulgaris* (Ryder et al. 1987) (Table 1).

The complete nucleotide sequences of the three cDNAs *CalPKS1*, *CalPKS2* and *CalPKS3* were determined and contained 1554, 1536 and 1590 bp encoding complete reading frames of 389, 390 and 389 amino acid residues, respectively. The predicted molecular masses for the encoded proteins are 42.6, 42.9 and 42.6 kDa, respectively.

Functional expression and characterization

The open reading frames of the putative polyketide synthases from *Cassia* were ligated into a T7 expression vector containing an amino terminal hexahistidine fusion tag. The proteins were then purified from *Escherichia coli* BL21(DE3) in two steps (sonication / centrifugation, cobalt affinity-chromatography) to yield electrophoretically homogeneous enzyme of approximately 42 kDa (Fig. 2). Per liter, the bacterial culture typically produced 1.1 mg of recombinant enzyme. Electrophoretically homogeneous enzyme was used in enzyme assays to determine the substrate / product specificity of the putative polyketide synthases.

A series of seven coenzyme A esters were tested together with [2-^{14}C]malonyl CoA for their capacity to serve as polyketide synthase "starter" molecules. The compounds used were acetyl-, *n*-butyryl-, isovaleryl-, *n*-hexanoyl-, benzoyl-, cinnamoyl-, and *p*-coumaroyl-CoA. In each case, multiple products were formed as determined by phosphorimagery following resolution of the reaction mixtures by TLC (Fig. 3a). This broad product spectrum had been reported previously for several of the CoA-esters with chalcone synthase, for example, from *Pinus sylvestris*, *P. strobus* and *Sinapis alba* (Zuurbier et al. 1998) *Scutellaria baicalensis* (Morita et al. 2000) and from raspberry *Rubus idaeus* (Zheng et al. 2001). In order to thoroughly characterize the heterologously expressed *Cassia* cDNAs, the reactions were repeated with unlabeled substrates on a larger scale so that the reaction products could be analyzed by mass spectrometry. The chemical structures of the compounds identified by liquid chromatography electrospray ionization mass spectrometry (LC/ESI-MS) are summarized in Fig. 4 and the corresponding mass spectral data are shown in Table 2. With the exception of the assay in which acetyl-CoA is used as the starter molecule, all substrates yielded a phloroglucinol derivative resulting from three sequential condensations of acetate units derived from three malonyl-CoA decarboxylations. The tetraketide intermediate thus formed cyclizes by an intramolecular Claisen condensation into the hydroxylated aromatic ring system. Every substrate tested in the assay also produced two pyrone derivatives, one resulting from two acetate unit condensations (a bis-noryangonin-type pyrone derailment product) and one resulting from three acetate unit condensations (a 4-coumaroyltriacetic acid lactone-type pyrone derailment). Unlike the complete reaction that leads to the formation of naringenin chalcone, these condensations do not result in the formation of an aromatic ring. All three *Cassia* polyketide synthases produced the same spectrum of enzymatic products. This capacity to build multiple polyketides from a variety of CoA-substrates raised the question as to the physiological reaction catalyzed by these polyketide synthases *in planta*.

Tissue-specific expression

The accumulation of the *Cassia* polyketide synthase transcript was investigated in developing seedlings and plants of *C. alata*. Since the nucleotide sequences of the three polyketide synthase clones were more than 90% identical, no attempt was made to differentially detect the transcripts. RNA was isolated from seedlings of 0.5-5cm in length (Fig. 5a). RNA gel blot analysis indicated that transcript was present already in 0.5 cm long seedlings (Fig. 5b, Lanes 1-6). As the seedlings grow, the level of polyketide synthase transcript increases. A 5 cm long seedling contains approximately the same level of transcript as mature root (Fig. 5b, Lanes 7-8).

In the mature *C. alata* plant, the highest level of polyketide synthase transcript was found in young root, whereas much less accumulated in leaf and stem (Fig. 6a-c). It was surprising to find a root-specific polyketide synthase, with a substrate and product spectrum similar to that of chalcone synthase, in a plant known to accumulate anthraquinones in both aerial and underground tissue. To investigate the phenylpropanoid composition of leaf and root tissue, an acidic methanol extract was prepared and was analyzed by reversed-phase HPLC. Quercetin was found to be a major component of root (Fig. 7c) and was also present as part of a more complex mixture in leaf (Fig. 7b). Given the distribution of gene transcript and of flavonoids, it is reasonable to assume that the *C. alata* root-specific polyketide synthases are chalcone synthases that demonstrate broad substrate specificity *in vitro*. *CalPKS1*, *CalPKS2* and *CalPKS3* were thus renamed *CalCHS1*, *CalCHS2* and *CalCHS3*, respectively.

Conclusions

As part of our investigation of type III polyketide synthases that occur in Thai medicinal plants, we have isolated three cDNAs that encode chalcone synthase. The gene transcripts were found predominantly in roots, which also accumulate the flavonoids quercetin, naringenin and kaempferol. The heterologously expressed cDNAs show broad substrate specificity for non-physiological substrates and produce multiple aromatic and pyrone derailment products from each substrate. The enzymes can accept CoA-esters that are not derived from phenylpropanoid metabolism and convert them to compounds that are not known to occur in the *Cassia* plant. Jez et al. (2000a) compare the crystal structure of *Medicago sativa* chalcone synthase to that of *Gerbera hybrida* pyrone synthase. From that study, three catalytic residues are present in the active sites of both type III plant polyketide synthases (Cys 164, His 303, Asn 336 in chalcone synthase and Cys 169, His 308, Asn 341 in pyrone synthase). The catalytic mechanism for polyketide formation is expected to be equivalent in both enzymes.

Pyrone synthase, however, has a smaller initiation/elongation cavity that cannot accommodate the larger starter molecule *p*-coumaroyl CoA that is requisite to naringenin formation. Four amino acid residues have been identified as important to cavity size (Thr 197, Ile 254, Gly 256, Ser 338 in chalcone synthase and Leu 202, Met 259, Leu 261, Ile 343 in pyrone synthase). The *Cal*CHS cDNAs contain catalytic and initiation/elongation cavity residues identical to those of *M. sativa* chalcone synthase. Jez et al. (2000a) report methylpyrone formation by the *M. sativa* chalcone synthase, which is consistent with results reported herein. The initiation/elongation cavity of *Cal*CHS is, by sequence comparison, expected to be as large as that of the *M. sativa* chalcone synthase. It is therefore not surprising that starter molecules that are smaller than *p*-coumaroyl CoA can be accommodated by the enzyme with varying degrees of efficiency. The relative amount of products should depend on the assay conditions employed. Formation of multiple products from multiple starter molecules *in vitro* is likely a result of the large initiation/elongation cavity of *Cal*CHS and does not necessarily suggest *in vivo* activity. Additional three dimensional structure determinations for type III polyketide synthases with varying substrate and product specificities will certainly provide insight into the efficiency of various starter molecules and the formation of derailment products for this important class of enzymes.

Roots of *Cassia* were also found to accumulate the potentially polyketide-derived anthraquinones chrysophanol, emodin and rhein as well as the anthracenone germichrysone (data not shown). Since the only cDNAs that were isolated by the RT-PCR approach used herein clearly encoded chalcone synthase, the nature of the enzyme that catalyzes formation of the anthraquinone skeleton remains to be identified.

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Table 1 Amino acid similarity of *C. alata* root-specific type III polyketide synthases

	*Cal*PKS1[a]	*Cal*PKS2	*Cal*PKS3	*Psa*CHS	*Gma*CHS
*Cal*PKS1	100[b]	92	98	92	92
*Cal*PKS2		100	93	92	93
*Cal*PKS3			100	92	93
*Psa*CHS				100	92
*Gma*CHS					100

[a] *Cal*PKS1, *Cassia alata* type III polyketide synthase 1 (this work); *Psa*CHS, *Pisum sativum* chalcone synthase (Ichinose et al. 1992); *Gma*CHS, *Glycine max* chalcone synthase (Akada et al. 1991).

[b] % amino acid identity

Aromatic and pyrone polyketides synthesized by a stilbene synthase from *Rheum tataricum*

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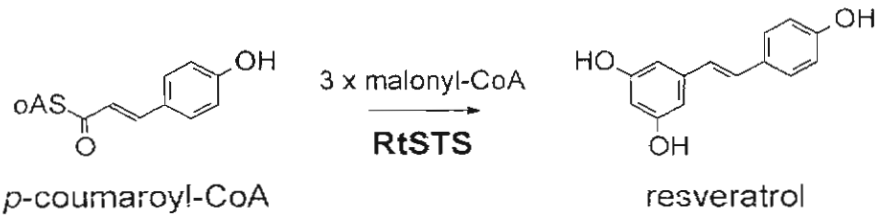
A plant polyketide synthase, RtSTS, was isolated as a cDNA clone from *Rheum tataricum* and functionally characterized as a resveratrol-forming stilbene synthase by *in vitro* assay. *RtSTS* transcript is expressed mainly in *R. tataricum* rhizomes, where resveratrol-type stilbenes occur in high concentration.



p-coumaroyl-CoA resveratrol

"In press" in ***Phytochemistry***

Aromatic and pyrone polyketides synthesized by a stilbene synthase from *Rheum tataricum*

Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday.

Supachai Samappito[a,b,c], Jonathan E. Page[a,*], Jürgen Schmidt[a], Wanchai De-Eknamkul[b], Toni M. Kutchan[a]

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The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL/DDBJ with the accession number AF508150.

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Abstract

A cDNA encoding a stilbene synthase, RtSTS, was isolated from the rhizomes of Tatar rhubarb, *Rheum tataricum* L. (Polygonaceae), a medicinal plant containing stilbenes and other polyketides. Recombinant RtSTS was expressed in *E. coli* and assayed with acetyl-coenzyme A (CoA), *n*-butyryl-CoA, isovaleryl-CoA, *n*-hexanoyl-CoA, cinnamoyl-CoA and *p*-coumaroyl-CoA as primers of polyketide synthesis. RtSTS synthesized resveratrol and a trace amount of naringenin chalcone from *p*-coumaroyl-CoA, supporting the enzyme's identification as a resveratrol-type stilbene synthase (EC 2.3.1.95). Bis-noryangonin and *p*-coumaroyl triacetic acid lactone (CTAL)-type pyrones were observed in minor amounts in the reaction with *p*-coumaroyl-CoA and as major products with cinnamoyl CoA. As well, such pyrones, and not aromatic polyketides, were identified as the only products in assays with aliphatic and benzoyl CoA esters. Acetonyl-4-hydroxy-2-pyrone, a pyrone synthesized from acetyl-CoA, was identified as a new product of a stilbene synthase. Using Northern blot analysis, *RtSTS* transcript was found to be highly expressed in *R. tataricum* rhizomes, with low transcript levels also present in young leaves. This expression pattern correlated with the occurrence of resveratrol, which was detected in higher amounts in *R. tataricum* rhizomes compared with leaves and petioles using

HPLC. Few stilbene synthases have been found in plants, and the identification of *RtSTS* provides additional sequence and catalytic information with which to study the evolution of plant polyketide synthases.

Keywords: *Rheum tataricum* L., Polygonaceae, Tatar rhubarb, polyketide synthase, stilbene synthase, chalcone synthase, resveratrol, pyrone.

Abbreviations:
ACS, acridone synthase.
BAS, benzalacetone synthase
BBS, bibenzyl synthase
BNY, bis-noryangonin
CoA, coenzyme A
CHS, chalcone synthase
CID, collision-induced decomposition
CTAL, *p*-coumaroyl triacetic acid lactone
CTAS, *p*-coumaroyl triacetic acid synthase
IPTG, isopropyl-β-D-thiogalactopyranoside
PKS, polyketide synthase
2-PS, 2-pyrone synthase
RACE, rapid amplification of cDNA ends
RtSTS, *Rheum tataricum* stilbene synthase
SDS, sodium dodecyl sulfate
SSC, saline sodium citrate
STS, stilbene synthase
UPM, universal primer mix
VPS, valerophenone synthase

Introduction

The chalcone synthase superfamily of polyketide synthases (PKSs) are key enzymes in the biosynthesis of many plant natural products including the ubiquitous flavonoids and related anthocyanins, and stilbenes, pyrones, phloroacylphenones and acridone alkaloids of more restricted distribution (Schröder, 2000). The enzymes responsible are homodimeric type III PKSs that, using aromatic or aliphatic CoA esters as reaction primers, carry out sequential decarboxylation and condensation reactions with one, two or three C_2 units derived from malonyl-CoA. The prototype enzyme of the superfamily is chalcone synthase (CHS) (EC 2.3.1.74), which has been extensively investigated at the biochemical (Jez and Noel, 2000) and structural level (Ferrer et al., 1999). CHS uses *p*-coumaroyl-CoA to form naringenin chalcone via a Claisen-type cyclization, with this compound further cyclizing to give naringenin. A closely related plant PKS, stilbene

synthase (STS) (EC 2.3.1.-), also uses phenylpropanoid CoA starter esters but cyclizes the tetraketide intermediate in an aldol-type reaction to yield resveratrol, from *p*-coumaroyl-CoA (EC 2.3.1.95), or pinosylvin, from cinnamoyl-CoA (EC 2.3.1.146).

Plant PKSs are catalytically flexible, usually forming several products *in vitro*, and accepting a variety of physiological and non-physiological substrates. Both chalcone and stilbene synthases convert *p*-coumaroyl-CoA to the major products naringenin chalcone or resveratrol, respectively, as well as to the byproduct pyrones bis-noryangonin (BNY) and *p*-coumaroyl triacetic acid lactone (CTAL) (Yamaguchi et al., 1999). The latter are formed by derailment after two or three condensation reactions with malonyl-CoA, respectively. Additionally, cross reactivity between CHS and STS has been demonstrated with CHS forming resveratrol and STS forming naringenin in assays with *p*-coumaroyl-CoA, albeit in small amounts (Yamaguchi et al., 1999). CHSs and STSs accept aliphatic CoA esters as reaction primers. For example, *Scutellaria baicalensis* CHS converts isovaleryl-CoA to phloroacylphenones (via a correct Claisen cyclization) (Morita et al., 2000), while *Arachis hypogaea* STS forms BNY-type pyrones rather than aromatic products with this substrate (Morita et al., 2001). CHS and STS also utilize thiophene, furan and halogenated analogues of *p*-coumaroyl-CoA to form unnatural polyketides (Abe et al., 2000; Morita et al., 2001). The ability of plant PKSs to accept different CoA primers and carry out various condensation and cyclization reactions (Jez et al., 2002) makes this enzyme class, along with terpene cyclases (Greenhagen and Chappell, 2001), one of the major generators of carbon skeleton diversity in natural products.

As part of a study of the biosynthesis of polyketides present in medicinal plants, we investigated the CHS superfamily members present in *Rheum tataricum* L. (Polygonaceae), or Tatar rhubarb. *Rheum* species are a rich source of polyketides including phenylbutanoids, anthraquinones, naphthalenes and stilbenes (Kashiwada et al., 1988). Members of this genus are also used as medicinal plants (Foust, 1992), particularly in Asian traditional medicine, and stilbenes have been reported to mediate the antioxidant activity of *Rheum* extracts (Matsuda et al., 2001). It is interesting to note that the first *in vitro* evidence for STS activity was shown with protein extracts of *Rheum rhaponticum* rhizomes (Rupprich and Kindl, 1978), although the enzyme responsible was never isolated using biochemical or molecular approaches. Abe et al. (2001) recently reported the isolation of a cDNA encoding benzalacetone synthase (BAS), a PKS involved in phenylbutanoid biosynthesis, from the leaves of *Rheum*

palmatum. In this paper, we describe the cloning of *RtSTS*, a resveratrol-type STS from *Rheum tataricum* rhizomes and the characterization of its *in vitro* reaction products.

Results and Discussion

A homology-based approach with degenerate oligonucleotide primers that correspond to conserved regions of plant PKSs was used to amplify gene fragments from *Rheum tataricum* rhizome RNA by RT-PCR. Rhizomes were targeted because many of the polyketides of interest, including stilbenes and anthraquinones, are synthesized in this tissue. In this manner, two 584 bp fragments, *RtPKS1* and *RtPKS2*, that showed similarity to CHS superfamily members were isolated. Since these fragments showed 98.5% similarity at the nucleotide level, only *RtPKS1* was selected for further analysis. The cDNA regions 5' and 3' from this fragment were obtained using RACE PCR. The full-length *RtSTS* cDNA was 1429 bp encoding a protein of 391 amino acids with a predicted molecular weight of 43.0 kDa. The 59 bp 5' untranslated region contained a stop codon in-frame with the presumed start codon indicating the cDNA was full length. The deduced amino acid sequence of *RtSTS* is shown in Fig. 1, as a multiple sequence alignment with other plant CHS superfamily members. Southern blot analysis (data not shown) found that three copies of *RtSTS* are present in the *R. tataricum* genome.

RtSTS was expressed in *E. coli* and purified by immobilized metal affinity chromatography on a cobalt resin to give homogeneous recombinant enzyme (Fig. 2). In order to determine the catalytic activity of RtSTS, recombinant enzyme was incubated with ^{14}C-malonyl-CoA and the potential starter CoA esters, acetyl-CoA, *n*-butyryl-CoA, isovaleryl-CoA, *n*-hexanoyl-CoA, cinnamoyl-CoA and *p*-coumaroyl-CoA. The resulting radioactive products were resolved by reversed-phase thin layer chromatography. We acidified enzyme assays before extraction to increase recovery of pyrones (Yamaguchi et al., 1999) and prevent cyclization of chalcones to their corresponding flavanones. As shown in Fig. 3, RtSTS accepted both aliphatic and aromatic CoA esters as primers for polyketide synthesis and produced multiple products from each. The major product formed in the *p*-coumaroyl-CoA assay co-chromatographed with resveratrol, suggesting that the *Rheum* PKS encoded an STS.

To conclusively identify the polyketide products, RtSTS was incubated with unlabelled malonyl-CoA and the starter CoA esters in scaled up *in vitro* reactions and the resulting product mixtures analyzed by LC-MS and LC-MS/MS. Products were identified by their parent ions and collision-induced

decomposition (CID) spectra. We have previously published the results from the LC-MS analysis of a series of polyketides formed enzymatically from CHS (Samappito et al. 2002); therefore, only a graphical summary of the enzymatic products of RtSTS is presented here (Table 1). RtSTS formed aromatic products only with *p*-coumaroyl-CoA and cinnamoyl-CoA. Incubation with *p*-coumaroyl-CoA gave resveratrol (**7e**) (*m/z* 229, $[M+H]^+$ and *m/z* 227, $[M-H]^-$) as the major product, demonstrating that RtSTS is an STS. Resveratrol was identified by its electrospray CID spectrum of the $[M-H]^-$ ion (*m/z* 227) displaying key ions at *m/z* 185 ($[M-H-CH_2CO]^-$) and *m/z* 143 ($[M-H-2CH_2CO]^-$) in comparison with an authentic sample. Smaller amounts of BNY-type (**7b**) and CTAL-type (**7c**) pyrones, and naringenin chalcone (**7d**), were also detected in the *p*-coumaroyl-CoA assay. This spectrum of four products, including the naringenin chalcone formed as a cross-reaction product, has been previously described for *Arachis hypogaea* STS (Yamaguchi et al., 1999). RtSTS synthesized BNY-type (**6b**) and CTAL-type (**6c**) pyrones as well as a small amount of pinocembrin chalcone (**6d**) and pinosylvin (**6e**) from cinnamoyl-CoA. Pinocembrin chalcone was clearly identified by its parent ion (*m/z* 257 $[M+H]^+$) and the typical collision-induced fragments at *m/z* 153 (trihydroxybenzoyl ion) and *m/z* 131 (cinnamoyl ion), while pinosylvin (**6e**) was detected by the LC-MS analysis showing a peak with masses of *m/z* 213 ($[M+H]^+$) and *m/z* 211 ($[M-H]^-$). Only three products were visible in the ^{14}C-labeled assay with cinnamoyl CoA (Fig. 3), the least polar (lower R_f value) of which likely corresponds to an unresolved mixture of pinocembrin chalcone and pinosylvin.

With benzoyl-CoA and the aliphatic substrates tested, RtSTS catalyzed only the formation of BNY-type (**1b**, **2b**, **3b**, **4b**, **5b**) and CTAL-type (**1c**, **2c**, **3c**, **4c**, **5c**) pyrones as a result of derailment after two and three condensation reactions. With some notable exceptions, the exclusive formation of pyrones by plant PKSs seems to be an indicator that the starter CoA ester in the reaction is not the physiologically relevant substrate for the enzyme under assay. This phenomena was observed in the assays of RtSTS with aliphatic and benzoyl CoA substrates. Pyrones also form as byproducts in reactions with correct substrates but the amounts are generally minor compared to the main aromatic product. In a limited number of cases, pyrone formation appears to be the true catalytic activity of plant PKS enzymes. 2-Pyrone synthase from *Gerbera hybrida* forms 6-methyl-4-hydroxy-2-pyrone as its major product *in vitro*, with the involvement of this enzyme in pyrone metabolism supported by antisense "knockout" of 2-pyrone synthase in *G. hybrida* (Eckermann et al., 1998). A *p*-coumaroyl triacetic acid synthase from *Hydrangea macrophylla* may also represent an example of a pyrone-

forming PKS, although this enzyme has been suggested to yield a linear tetraketide *in vitro* (Akiyama et al., 1999)

The formation of 6-acetonyl-4-hydroxy-2-pyrone (tetracetic acid lactone) (**1c**) (*m/z* 169 $[M+H]^+$), in addition to 6-methyl-4-hydroxy-2-pyrone (triacetic acid lactone) (**1b**) (*m/z* 127 $[M+H]^+$), when RtSTS was incubated with acetyl-CoA is notable. Compound **1b** has been previously described as the main product formed from the use of acetyl-CoA as a starter CoA ester by *Gerbera hybrida* 2-pyrone synthase (Eckermann et al., 1998) and CHS from *Petroselinum crispum* (Schuz et al., 1983). 6-Acetonyl-4-hydroxy-2-pyrone (**1c**) is a new enzymatic product from a plant PKS, although we have also observed it as an enzymatic product from *Senna alata* CHS (Samappito et al., 2002). Its synthesis is analogous to the formation of CTAL and likely involves the condensation of acetyl-CoA with three C_2 units derived from malonyl-CoA followed by a pyrone-type cyclization. This compound has not been reported previously from plants, although it has been isolated as a polyketide metabolite of *Penicillium stipatatum* (Bentley and Zwitkowits, 1967).

To confirm that stilbenes were present in the tissue from which the *RtSTS* cDNA was isolated, we analyzed leaf, petiole and rhizome extracts of *Rheum tataricum* by HPLC. Acid hydrolysis of extracts was used to cleave sugar residues and release stilbenes as their aglycones. Resveratrol was detected in all tissues, with higher amounts occurring in the rhizomes (Fig. 4). To the best of our knowledge, this is the first report of resveratrol from *R. tataricum*. The presence of this compound provides further evidence that the *in vivo* function of RtSTS is the biosynthesis of resveratrol. The distribution of resveratrol correlated with the expression pattern of the *RtSTS* transcript as determined by Northern blot analysis of total RNA isolated from young leaves, mature leaves, petioles and rhizomes (Fig. 5). Young leaves showed low expression levels, and the transcript was virtually absent from older leaves and petioles. A high expression level was detected in rhizomes where resveratrol levels were also highest.

A phylogenetic comparison of the relationships between *RtSTS* and other chalcone synthase superfamily members (Fig. 6) shows *RtSTS* to be closely related to *Rheum palmatum* BAS (82% amino acid identity) and forming a group with functionally diverse enzymes such as *Ruta graveolens* acridone synthase (ACS), *Gerbera hybrida* 2-pyrone synthase (2-PS) and *Vitis vinifera* STS. This group appears to be distinct from groups composed of angiosperm CHSs, and from PKSs from the Leguminosae (*Arachis* and *Medicago*), *Pinus* and *Psilotum*. Abe et al. (2001) analyzed the phylogenetic

relationships of *Rheum palmatum* BAS and characterized the group to which this enzyme belongs as more primitive than flavonoid forming CHSs. While the sequences and methods used for phylogenetic analysis may differ from those used in this study, the close relationship of *R. palmatum* BAS with *RtSTS* and *V. vinifera* STS and *Humulus lupulus* CHS suggests that no evolutionary conclusions as to the primitive or advanced nature of PKSs can be drawn on the basis of a simple comparison of sequence and enzyme function. The close relationship of *Rheum palmatum* BAS and *R. tataricum* STS supports a trend, first noted by Tropf et al. (1994), that functionally distinct enzymes that occur in the same plant taxa are more similar to each other than they are to functionally identical proteins that occur in more distantly related taxa.

STSs are not common in the plant kingdom. In comparison to the more than 100 genes encoding CHS that have been isolated from different species of higher plants (as reported in Swiss-Prot (2002)), STSs have been found in only four plant genera: *Arachis* (Schröder et al., 1988), *Vitis* (Melchior and Kindl, 1990), *Pinus* (Fliegmann et al., 1992) and *Psilotum* (Yamazaki et al., 2001). This is due bóth to the restricted distribution of stilbenes and to the inability to separate stilbene from CHSs based on sequence analysis; some sequences annotated as CHSs in sequence databases may in fact encode STSs. The cloning and characterization of a resveratrol-type STS from the medicinal plant *Rheum tataricum* provides a new example of a plant PKS participating in the biosynthesis of the pharmacologically and agriculturally important stilbene, resveratrol. It will be interesting to obtain the sequence of CHSs from *Rheum* and *Arachis*, so that a pairwise phylogenetic comparison of co-occurring CHS and STSs from *Vitis*, *Pinus*, *Arachis*, *Psilotum* and *Rheum* may be undertaken.

Experimental

Chemicals

Acetyl-CoA, malonyl-CoA, *n*-butyryl-CoA, isovaleryl-CoA, *n*-hexanoyl-CoA, benzoyl-CoA, naringenin and resveratrol were purchased from Sigma. [2-^{14}C]-malonyl-CoA was from Biotrend Chemikalien (Cologne, Germany). *p*-Coumaroyl-CoA and cinnamoyl-CoA were kindly provided by D. Knöfel of the Abteilung Sekundärstoffwechsel, Leibniz-Institut für Pflanzenbiochemie (IPB), Halle.

General methods

Rheum tataricum was grown from seed in the greenhouse at the IPB, Halle. Total RNA was isolated as described by Salzman et al. (1999). Poly(A)$^+$ RNA was isolated with Oligotex beads (Qiagen) according to the

manufacturer's instructions. For Northern blot analysis, 10 μg total RNA was resolved by electrophoresis and transferred to a nylon membrane (Hybond N, Amersham Pharmacia Biotech). The full-length cDNA was excised by restriction enzyme digest and labeled with ^{32}P using random primers. Hybridization was performed at 65° overnight and the membranes were then washed three times for 15 min with 2x SSC and 0.1% SDS at 65°. Radioactivity was visualized by phosphorimaging.

For Southern blot analysis, genomic DNA was isolated from young leaves according to Dellaporta et al. (1983). DNA (10 μg) was digested with *Apo*I, *Bcl*I and *Bst*XI (each cutting once within the reading frame), resolved by electrophoresis on a 0.8% agarose gel, and capillary blotted onto a Hybond N nylon membrane. The cDNA labeled as above was used as a hybridization probe as described for Northern blot analysis. Radioactivity was visualized by phosphorimaging.

Amplification of partial cDNAs from R. tataricum

First-strand cDNA was synthesized from 5 μg of total RNA isolated from *R. tataricum* rhizomes using Superscript II reverse transcriptase (Life Technologies). One μl of the first-strand cDNA was used as a PCR template with degenerate primers 5'-AA(A/G)GC(C/T)AT(A/C)GAIGA(A/G) TGGGG-3 and 5'-CCACCIGG(A/G)TGI(A/G)CAATCC-3' based on those described by Helariutta et al. (1995) and *Taq* polymerase (Promega). Cycling conditions consisted of an initial denaturation at 94°, 3 min; 30 cycles of 94°, 30 sec; 48°, 30 sec and 72°, 1 min, followed by 10 min at 72°. A PCR product of the expected length (575-600 bp) was purified by gel electrophoresis, ligated into pGEM-T Easy (Promega) and sequenced. Comparison of several fragments showed that two sequences of 98.5% similarity were amplified from *R. tataricum* rhizomes.

5' and 3' RACE PCR

A RACE PCR kit (SMART technology; Clontech) was used to synthesize cDNA from total RNA isolated from *R. tataricum* rhizomes. 3' RACE was performed using the gene-specific primer 5'-CATAGACTCCATGGTAGGGCAAGC-3' and universal primer A mix (UPM) supplied by the manufacturer to amplify a 755 bp DNA fragment. Cycling conditions consisted of an initial denaturation at 94°, 5 sec; 30 cycles of 94°, 5 sec; 68°, 10 sec and 72°, 3 min, followed by 7 min at 72° Advantage polymerase (Clontech). Similarly, 5' RACE used the gene-specific primer 5'-GCCGCACCGTCACCAAATATTGC-3' and UPM to amplify a 781 bp DNA fragment. Both 3' and 5' RACE PCR products were gel purified, ligated into pGEM T-Easy and sequenced.

Expression in E. coli

To express the cDNA in *E. coli*, the open reading frame was cloned into pET14b (Novagen), which contains a hexahistidine N-terminal fusion tag. Amplification was performed using primers 5'-AATAGTCATATGGCACCGGAGGAGTCG-3' (the NdeI site is underlined) and 5'-TTTAAAGGATCCTCAGGTAATTAGCGGCAC-3' (the *Bam*HI site is underlined) with the 5'-RACE cDNA as template and *Pfu* polymerase (Promega). The 1.2 kb PCR product was digested with *Nde*I and *Bam*HI, gel purified and ligated into *Nde*I/*Bam*HI-digested pET-14b. Cloning of the expression construct was confirmed by sequencing. The plasmid was transformed into *E. coli* BL21(DE3) cells, 1 l cultures in LB broth containing 50 µg/ml ampicillin were grown at 37° until OD_{600} ~0.6 and protein expression induced with 1mM IPTG. Induced cultures were grown at 28° for 12 hours, harvested by centrifugation and frozen at -80°.

Purification of recombinant RtSTS

Frozen *E. coli* cells were resuspended in buffer containing 50 mM Tris-HCl (pH 7), 500 mM NaCl, 2.5 mM imidazole, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 1% Tween-20 and 750 µg/ml lysozyme. Cells were incubated for 30 min on ice followed by a brief sonication. The lysate was clarified by centrifugation and the recombinant protein bound to Talon resin (Clontech). After washing with lysis buffer lacking detergent and lysozyme, recombinant protein was eluted with buffer containing 50 mM Tris-HCl (pH 7), 500 mM NaCl, 250 mM imidazole, 10% (v/v) glycerol, 10 mM β-mercaptoethanol. The protein-containing fractions were pooled and the buffer exchanged to 50 mM HEPES (pH 7), 10 mM β-mercaptoethanol and 10% (v/v) glycerol using a gel filtration column (PD10, Amersham Pharmacia Biotech). Recombinant RtSTS was determined to be pure by electrophoresis on a denaturing polyacrylamide gel (Laemmli, 1970). Purified enzyme was stored at -20° before use.

Enzyme assays

Polyketide synthase activity was measured by the conversion of starter CoA esters and [2-^{14}C]malonyl-CoA into reaction products. The standard enzyme assay contained 100 mM HEPES buffer (pH 7), 20 µM starter CoA and 12,000 dpm [2-^{14}C]-malonyl-CoA (55 mCi/mmol) and 1.5 µg RtSTS in a 50 µl reaction volume. The assay mixture was incubated for 30 min at 30°. The reaction was stopped by addition of 5 µl 10% (v/v) HCl and was extracted twice with 100 µl ethylacetate. The combined organic phase was evaporated to dryness and the products separated by thin layer chromatography (RP18, Merck) developed in MeOH-H_2O-acetic acid

(70:30:1). Selected reactions were co-chromatographed with a resveratrol standard. The ^{14}C-labelled products were visualized by phosphorimaging.

Mass spectrometric analysis of enzymatic products

To identify the enzymatic products, scaled up reactions containing 75 mM Tris-HCl (pH 7), 50 µM starter CoA, 100 µM malonyl-CoA and 5 µg purified enzyme in a 200 µl reaction volume were used. The reaction proceeded for 1 hour at 30° before acidification and extraction with ethyl acetate. After drying *in vacuo*, the residue was dissolved in methanol and analysed by LC-MS. Positive and negative electrospray ionization (ESI) mass spectra were obtained with a Finnigan MAT TSQ 7000 instrument (electrospray voltage 4.5 kV; heated capillary temperature 220 °C; sheath and auxillary gas nitrogen) coupled to a Micro-Tech Ultra-Plus MicroLC system equipped with an RP18-column (5 µm, 100x1 mm, SepServ, Berlin). For all compounds, a gradient system was used that ranged from H_2O:CH_3CN 90:10 (each containing 0.2% (v/v) acetic acid) to 10:90 over 15 min, followed by isocratic elution with a 10:90 mixture of both solvents for 10 min; flow rate 70 µl/min. The collision-induced dissociation mass spectra during an HPLC run were recorded with a collision energy of –20 or -25 eV for positive ions as well as +20 or +25 eV for negative ions, respectively; collision gas: argon, collision pressure: 1.8 x 10^{-3} Torr.

HPLC analysis of R. tataricum rhizomes

To measure resveratrol in the leaves, petioles and rhizomes of *R. tataricum*, 1 g fresh plant material was frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. Ten-ml of 80% (v/v) MeOH containing 3% (v/v) HCl was added and the extract was hydrolysed at 100° for 60 min in a sealed tube. After centrifugation (10,000 x g, 10 min) the clarified extract was evaporated, dissolved in water, and extracted with ethylacetate. The organic layer was dried *in vacuo*, the residue dissolved in 50 µl 50% (v/v) methanol and 10 µl were analysed by HPLC. The HPLC system consisted of a Hewlett Packard Series 1100 instrument with a Eurospher-100 RP18 column (5 µm, 250x4mm, Knauer). Chromatographic separation was performed using a solvent sysem of (A) H_2O containing 2% CH_3CN and 0.2% phosphoric acid and (B) CH_3CN containing 2%) H_2O and 0.2% phosphoric acid (B) with a linear gradient of 20-60% B over 30 min Flow rate was 0.6 ml/min with detection at 320 nm. All HPLC solvent percentages are expressed v/v.

Accession numbers

The GenBank accession numbers of the CHS superfamily members used to construct the phylogenetic tree are *Phalaenopsis* BBS (P53416), *Ruta*

graveolens ACS (S60241), *R. graveolens* CHS (CAC14059), *Gerbera hybrida* 2-PS (CAA86219), *G. hybrida* CHS (S56699), *Rheum palmatum* BAS (AAK82824), *Rheum tataricum* STS (AF508150), *Hydrangea macrophylla* CTAS (BAA32733), *H. macrophylla* CHS (BAA32732), *Humulus lupulus* VPS (BAA29039), *H. lupulus* CHS (BAB47196), *Vitis vinifera* STS (CAA54221), *V. vinifera* CHS (CAA53583), *Petroselinum crispum* CHS (CAA24779), *Oryza sativa* CHS (CAA61955), *Arabidopsis thaliana* CHS (BAB11121), *Arachis hypogaea* STS (P20178), *Medicago sativa* CHS (P30074), *Pinus densiflora* CHS (BAA94594), *P. densiflora* STS (BAA94593), *Pinus sylvestris* CHS (CAA43166), *P. sylvestris* STS (CAA43165), *Psilotum nudum* CHS (BAA87922), *P. nudum* VPS (BAA87923) and *P. nudum* STS (BAA87925).

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High Performance Liquid Chromatography in Phytochemical Analysis of *Impatiens balsamina* Root Cultures

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"Submitted" to ***Phytochemical Analysis***

Abstract : An analytical method using HPLC with UV detection was developed to investigate simultaneously the production of three naphthoquinones (lawsone, 2-methoxy-1,4-naphthoquinone and methylene-3,3'-bilawsone) and three coumarins (scopoletin, isofraxidin and 4,4'-biisofraxidin) was examined during the growth cycle of *Impatiens balsamina* root cultures. Among these compounds, scopoletin appeared to be produced in the lag and exponential phases, lawsone in the linear phase, and methylene-3,3'-bilawsone, 2-methoxy-1,4-naphthoquinone, isofraxidin and 4,4'-biisofraxidin were all actively produced in the stationary phase. Chemical contents of root cultures were significantly different from those found in leaves and roots of the intact plant. Bisnaphthoquinone, methylene-3,3'-lawsone, and the biscoumarin, 4,4'-biisofraxidin, were detected only in root culture extracts.

Key Words : *Impatiens balsamina*, Balsaminaceae, naphthoquinones, lawsone, 2-methoxy-1,4-naphthoquinone, coumarins, root culture, HPLC

Introduction

Impatiens balsmamina L. (Balsaminaceae), popularly known as Thian-Ban (Thai name), is an erect annual herb that grows widely in Thailand. The stems and leaves of the plant have been traditionally used for the treatment of abscesses, ingrown nails, glass-puncture wounds and chronic ulcers caused by allergic reaction to detergents (1). It has been reported that the leaves contain two antifungal naphthoquinones, lawsone and 2-methoxy-1,4-naphthoquinone (2) along with phenolic acids, flavonoids and a coumarin (3).

As part of our studies of biosynthesis of plant naphthoquinones, we established root cultures of *I. balsamina* and examined their ability to produce these compounds. It has been found that root cultures: produce 6 major secondary metabolites, including three naphthoquinones: lawsone, 2-methoxy-1,4-naphthoquinone, methylene-3,3'-bilawsone and three coumarins: scopoletin, isofraxidine and 4,4'-biisofraxidin (4,5) (Fig.1). Since clarification of the relationship between the culture growth and product formation is an essential step for biosynthetic study of these compounds, in this study, we examine changes in the product content during the culture cycle. Over the course of this investigation, we developed an HPLC system which is capable of simultaneous separation of all the six naphthoquinones and coumarins. This complete separation allowed the crude root culture extracts to be analyzed directly for their chemical composition, and thus allowed examination of the relationship between growth and product formation. Use of this technique also allowed direct comparison of secondary root culture metabolites to products of the whole plant.

Materials and Methods

Root cultures

Root cultures of *I. balsamina* L were initiated from young leaf explants on B5 solid medium containing 0.1 mg/l NAA, 0.1 mg/l kinetin, 1.0 mg/l BA and 20 g/l sucrose (4). The roots were formed after 2 weeks of incubation (25°C, continuous light 2000 lux). After 3 weeks, the root culture was transferred into B5 liquid medium containing the same supplements as the solid medium (55 ml medium in 250 ml Erlenmeyer flask). The culture was maintained by transfer of 2 g fresh weight tissue into 55 ml of fresh medium every 3 weeks, and maintained at 25°C. 80 rpm, in continuous light. For time-course studies of product formation, the cultured roots were harvested every 2 or 3 days in duplicate flasks. The harvested roots were dried at 60°C for 12 h and dry weights were recorded.

Chemicals

Lawsone, 2-methoxy-1,4-naphthoquinone, methylene-3,3'-bilowsone, scopoletin, isofraxidin and 4,4'-biisofraxidin used as authentic compounds were isolated from *I. balsamina* root cultures as described previously (4,5).

Sample preparation for HPLC analysis
Each dried sample (0.2 g) of either the root culture or the leaves or roots of the intact plant was ground into powder and sonicated twice with 10 ml petroleum ether for 1 h. Petroleum ether extract was discarded and the residues were refluxed with 20 ml methanol for 1 h. Each methanolic extract was dried in *vacuo* and re-dissolved in 5 ml methanol. The solution was then filtered through a 0.45 mm Millipore filter before it was subjected to HPLC analysis.

HPLC analysis
The naphthoquinones and coumarins in the crude methonolic extracts were separated and quantified by reverse-phase HPLC on an octyl-80TS (C_8) column (0.46 x 15 cm) eluting with a linear gradient from 0.15 M H_3PO_3 in methanol-water 1:9 to 0.15 M H_3PO_3 in methanol-water 6:4 in 50 min, at 0.7 ml/min flow rate and monitoring at 275 nm. Products were identified by co-elution and by the comparison of retention times. Quantitation of each compound was performed using peak area integration.

Results and Discussion

We have recently reported isolation and structure elucidation of 3 naphthoquinones and 3 coumarins which are produced as major secondary metabolites by *Impatiens balsamina* root cultures (4,5). Among these compounds, methylene-3,3' -bilawsone and 4,4'-biisofraxidin appear to be new compounds which have not been found in the whole plant of *I. balsamina*. The ability of the root culture to produce the naphthoquinones, coumarins and the unusual dimers of both groups make the culture a good model for biosynthetic studies of these compounds.

In order to characterize the formation of the naphthoquinones and coumarins during the culture cycle, an HPLC system was developed to simultaneously separate all six compounds in a single run. It was found that this could be accomplished by using a C-8 reverse-phase column and a gradient elution of 0.15 M H_3PO_4 in MeOH/H_2O from 1:9 to 6:4 in 50 min (Fig. 2). Under these conditions, the order of product elution from the column appeared to be scopoletin (Rt = 31.0 min), isofraxidin (31.9 min) lawsone (38.2 min), 2-methoxy-1,4-naphthoquinone (41.0 min) 4,4'-biisofraxidine (46.8 min) and methylene-3,3'-bilawsone. This allowed the methanolic extracts prepared from both the root culture and the whole plant of *I. balsamina* to be analyzed directly for these compounds.

Figure 3A shows the growth cycle of the root cultures during a period of 30 days. It can be seen that there was a short lag phase (1 day) followed by rapid exponential and linear growth phases. This resulted in a continuous increase in biomass throughout the 19 day period. The growth rate then slowed down and the root culture reached the stationary growth phase at day 22. Thereafter, the dry weight of the biomass began to be constant and then declined. The root cultures attained their highest dry biomass weight of 1.02 g at day 22, equivalent to about six times the inoculated root culture mass.

During the 30-day period of culture growth, it was found that lawsone initially accumulated in the early linear phase and was actively biosynthesized throughout the linear phase, whereas methylene-3,3'-bilawsone initially formed in the middle of the linear phase and was biosynthesized until the stationary phase (Fig. 3B). In contrast, 2-methoxy-1,4-naphthoquinone production began in the late linear phase and was actively biosythesized throughout the stationary phase. The highest content of lawsone was observed at day 15 while the formation of methylene-3,3'-bilawsone and 2-methoxy-1,4-naphthoquinone reached their maximum at days 22 and 28, respectively (Fig. 3B). From these results, it was possible that the biosynthesis of lawsone was quite acitve in the early stages of the growth cycle, leading to accumulation of the compound in the middle growth stage. Subsequently, when the enzymes utilizing lawsone became active, cellular lawsone was then utilized in the formation of 2-methoxy-1,4-naphthoquinone and methylene-3,3'-bilawsone in the later stages of the growth cycle.

For the formation of coumarin derivatives (Fig. 3C), it was found that scopoletin began to accumulate in the lag phase and was actively biosynthesized until the early linear phase. Isofraxidin and its dimer were, on the other hand, initially biosynthesized in the early linear phase and throughout the late linear phase until the stationary phase. The formation of scopoletin reached its maximum at day seven while that of isofraxidin and 4,4'-biisofraxidin

both reached their maximum around day 25. These production patterns, again, indicated that the enzymes involved in the utilization of scopoletin started to be expressed at the middle growth phase, and caused a continuous decrease of the pool size of scopoletin in the subsequent growth phases.

These results suggest that the coumarin pathway is first expressed in *I. balsamina* root culture right after subculturing followed by a rapid expression of the lawsone pathway. This results in the accumulation of scopoletin and lawsone in the early growth phase. The basic chemical skeletons of coumarin and naphthoquinone are then modified nearly simutaneously by their specific condensing enzymes, to form 4,4'-biisofraxidin and methylene-3,3'-bilawsone, or by their specific methyltranferases, to form isofraxidin and 2-methoxy-1,4-naphthoquinone. The coumarin and naphthoquinone pathways are known to be related to each other at the stage of the shikimic acid pathway (6). Therefore, we propose that in *I. balsamina* root cultures, the scopeletin formation involves the shikimate and the general phenylpropanoid pathways, whereas the lawsone formation branchs from the shikimate pathway at the intermediate of isochorismic acid followed by the O-succinylbenzoate pathway (Fig. 4). These pathways are likely operate in the whole plant as well, since both scopoletin and lawsone have been shown to be present in *I. balsamina* plants.

However, based on the HPLC techniques developed in this study, it was found that the chemical composition of the root culture was considerably different from that of the leaves or the whole roots of *I. balsamina* plant. With respect to the naphthoquinones (Fig. 5A), the root cultures appeared to contain lawsone as the major constituent, while 2-methoxy-1,4-naphthoquinone seemed to be the major naphthoquinone in leaves and roots. Moreover, the root cultures produced bisnaphthoquinone which was not detected in the whole plant. For the coumarin derivatives, the HPLC chromatograms (Fig. 5B) showed that root culture could accumulate much higher amounts of the coumarin derivatives than leaves and the roots. Isofraxidin appeared to be the major coumarin derivative in the root cultures, whereas isofraxidin was detected only in trace amounts in leaves and the roots. Scopeletin, on the other hand, was found to be present in only small amounts in both the root culture and the intact plant. Biscoumarin 4,4'-biisofraxidin was also detected only in the root cultures not in the intact plant. Quantitatively, the content of each naphthoquinone and coumarin present in each type of tissue is summarized in Table 1. It is obvious that *I. basamina* root cultures are capable of producing various derivatives of naphthoquinone and coumarin groups, and are good material for biosynthetic studies of these compounds.

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LEGENDS

Fig. 1 The major secondary metabolites produced by *I. balsamina* root cultures.

Fig. 2 HPLC chromatogram of some authentic compounds (A) and a root culture extract of *I. balsamina* (B). The chromatograms were measured at 275 nm.

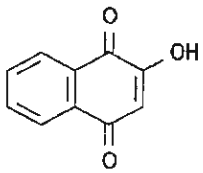
Fig. 3 The relationship between culture growth (A) and formation of naphthoquinones (B) and coumarins (C) in *I. balsamina* root cultures.

Fig. 4 Proposed biosynthetic pathways of of major secondary metabolites (bold structures) produced by *I. balsamina* root cultures.

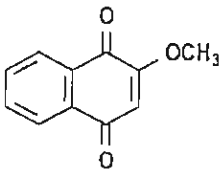
Fig. 5 HPLC Chromatograms detected with UV 275 nm (A) and UV 350 nm (B) of the methanolic extracts of leaves (a), root cultures (b) and whole roots (c) of *I. balsamina*. **1** : lawsone, **2** : 2-methoxy-1,4-naphthoquinone, **3** : methylene-3,3'-bilawsone, **4** : scopoletin, **5** : isofraxidine and **6** : 4,4'-biisofraxidin

Table 1 The content of various naphthoquinones an coumarins found in the root cultures (30 days old), the leaves and roots of *I. balsamina*.

Compound	Content (% dry weight)		
	Root culture	Leaves	Whole roots
Lawsone	0.082	0.025	<0.001
2-Methoxy-1,4-naphthoquinone	0.019	0.107	0.028
Methylene-3,3'-bilawsone	0.023	not detected	not detected
Scopoletin	0.010	0.070	0.010
Isofraxidin	0.015	<0.001	0.020
4,4'-Biisofraxidin	0.005	not detected	trace



Lawsone



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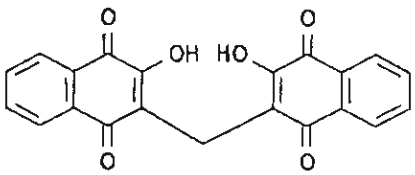
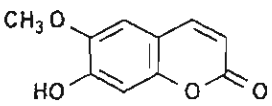
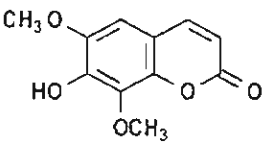


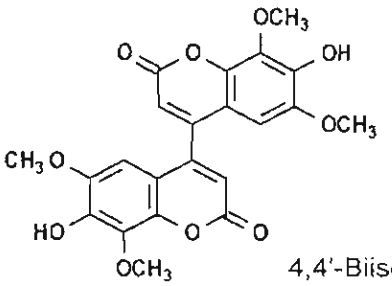
Methylene-3,3'-bilawsone



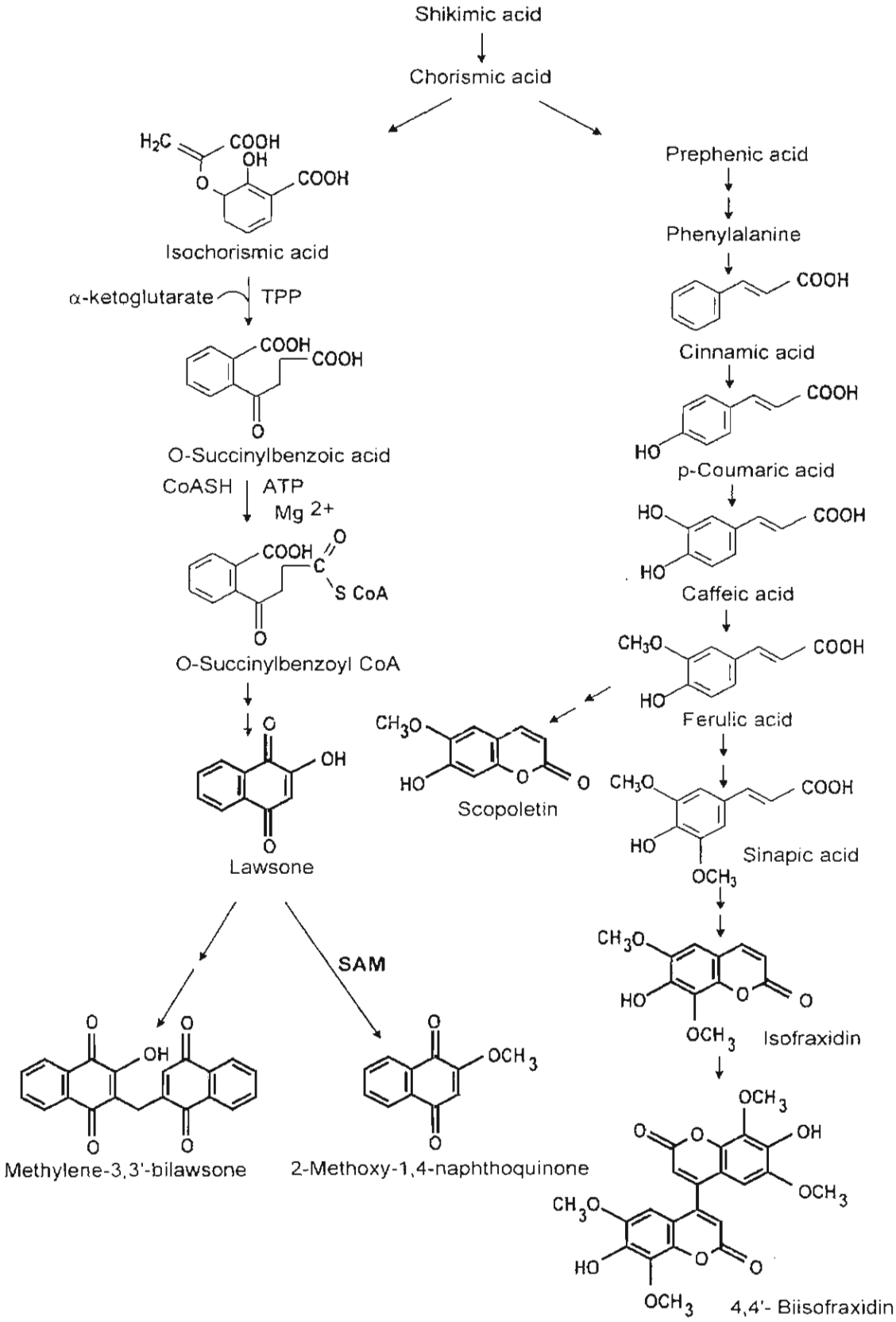
Scopoletin



Isofraxidin



4,4'-Biisofraxidin


Shikimic acid
Chorismic acid
Isochorismic acid
α-ketoglutarate
TPP
O-Succinylbenzoic acid
CoASH
ATP
Mg 2+
O-Succinylbenzoyl CoA
Lawsone
Prephenic acid
Phenylalanine
Cinnamic acid
p-Coumaric acid
Caffeic acid
Ferulic acid
Scopoletin
Sinapic acid
Isofraxidin
4,4'- Biisofraxidin
SAM
Methylene-3,3'-bilawsone
2-Methoxy-1,4-naphthoquinone

Formation of Napthoquinones by Cell Suspension Cultures of *Plumbago zeylanica*

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ABSTRACT

Cell suspension cultures of *Plumbago zeylanica* was successfully established from the leaf explants. The culture medium used for both callus induction and maintaining the cell suspension culture was LS containing 0.2 mg/l 2,4-D and 0.2 mg/l NAA. Studies on the effects of various basal media and plant growth regulators showed that the cell suspension culture produced more plumbagin in MS medium, in the presence of 2,4-D or BA as a growth regulator and manitol as a carbon source. The cell culture grown in such a medium produced 0.05 mg/g plumbagin and used for enzymological study.

INTRODUCTION

Plumbago zeylanica Linn. (Plumbaginaceae) is an important medicinal plant, distributed in the tropical regions of South Asia and Southeast Asia (Anomymous, 1989). It has been used in the treatment of dyspepsia, piles, anasarca, diarrhoea and skin disease (Dwarakanath, 1987). The roots of this plant are the main source of plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone. Some related derivatives of plumbagin have also been found, including 3-chlorophumbagin and 3,3'-biplumbagin (Sidhu and Sankaram, 1971 ; Padhye and Kulkarni, 1973). The root extract has been reported to contain a binaphthoquinone namely 3',6'-biplumbagin (Sankaram et al., 1976). Plumbagin is a naphthoquinone showing a broad range of pharmacological activities, such as antifertility (Bhargava, 1984), antifungal, antibacterial (Krishnaswamy and Purusottamain, 1980), antimalarial (Likhitwitayawuid et al., 1998), antitumor (Fujii et al., 1992). Plumabagin has also been reported to be an effective chitin-synthase inhibitor (Kubo et al., 1983) and therefore, it can be utilized for insecticidal in agriculture.

Biosynthetically, plumbagin and 7-methyljuglone are the first naphthoquinones in higher plants that were shown to be formed from the polyketide pathway (Manitto and Sammes, 1981). It has been demonstrated that the two naphthoquinones are biosynthesized by plants of the *Drosera* and *Plumbago* genera. Both arise from a hexaketide rather than the shikimic acid route as occurs for juglone and menadione (Durand and Zenk, 1971). Feeding experiments with [1-^{14}C, 2-^{14}C-acetate] and 2-^{14}C-malonate led to label both naphthoquinones heavily, suggesting that plumbagin is formed by the well-known polyketide pathway. Athough the enzyme polyketide synthase in *Plumbago* spp. has not yet been reported, the possible biosynthetic pathway of plumbagin has been proposed previously (Durand and Zenk, 1971).

Since information on the enzyme level of plumbagin biosynthesis has not been cleared and the plants of *plumbago zeylanica* are widely available in Thailand, we aim to search for the enzyme polyketide synthase in this plant. The plant parts are first used as explants for inducing the formation of callus and cell suspension. This is followed by detection of product formation in the established cell cultures. The producing cell culture will then be used for enzymological study.

MATERIALS AND METHODS

1. CHEMICALS

Standard plumbagin was purchased from Sigma (USA). Chemicals for culture media, all tissue culture grade, were also from Sigma (USA). Various plant growth regulators were purchased from Gibco Laboratories (New York, USA). Gelling agent (agar) was purchased from Difco Laboratories (Michigan, USA). Organic solvents used for phytochemical study were all analytical grade (Labscan). TLC plates of silica gel 60 F254 0.2 mm thick on aluminium sheet were obtained from Merck (Damstadt, Germany).

2. PLANT MATERIAL

The whole plants of *Plumbago zeylanica* Linn. were obtained from Amphur Minburi, Bangkok. Three mature plants with similar sizes were separated into flowers, leaves, stems and roots. The leaf and stem parts were further separated into smaller portions in which their positions in the whole plants were recorded. The leaves of *P. zeylanica* used for the tissue culture work were collected from the plant grown in the Botanical garden of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

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3. PLANT TISSUE CULTURE TECHNIQUES

3.1 Nutrient media

Standard basal medium used in this study was LS (Linsmaier and Skoog, 1965). For plant growth regulators, various concentrations of auxins : indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α -napthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), and of cytokinins : 6-benzylaminopurine (BA) and 6-furfurylaminepurine (kinetin). For solid media, 0.8% (w/v) agar was added into the nutrient solutions.

3.2 Culture conditions

The *in vitro* cultures of *P. zeylanica* were maintained in a culture room with 16-hour photoperiod of illumination at 2,000 lux and 8-hour dark. The incubation temperature was maintained at 25 ± 2 °C. The pH value of all the media used in this study was set to 5.6 before autoclaving.

3.3 Preparation of leaf explants

The leaves of *P. zeylanica* were first cleaned with running tap water and dipped in 70% ethanol for a few minutes. The leaves were then surface sterilized in 10% Clorox© solution containing a few drops of Tween 80 for 30 mins in a shaker, followed by washing three times with sterile distilled water. Each leaf was dissected into pieces (approx. 0.5 x 0.5 cm^2) and were used as explants.

3.4 Establishment of callus cultures

The surface-sterilized leaf explants were placed on semisolid basal LS medium (Linsmaier and Skoog, 1965) supplemented with 0.2 mg/l 2,4-D and 0.2 mg/l NAA for callus induction. The cultures were maintained at 25 $\pm$ 2°C under controlled 16-hr photoperiod (2000 lux) conditions. The formation of callus was observed after 2 weeks. After a month of callus induction, the callus cultures were maintained by regular subculture at 4-week intervals on fresh medium with the same composition.

3.5 Establishment of cell suspension cultures

Cell suspension cultures of *P. zeylanica* were initiated from the established callus cultures. The callus tissues were placed in a 250 ml Erlenmeyer flask containing 50 ml of LS medium containing 30 g/l sucrose, 0.2 mg/l NAA and 0.2 mg/l 2,4-D on a rotary shaker at 120 rpm at 25$\pm$2°C. These cell suspensions were maintained as stock culture by subculturing on the same medium every 3 weeks by adding 15 ml of the old suspension culture to 50 ml of fresh medium with the same composition

3.6 Study on the effect of basal media

The effects of basal media on growth and plumbagin production were tested with 5 different media. These included LS (Linsmaier and Skoog, 1965), MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1970), N (Nitsch *et al.*, 1968) and SH (Schenk and Hildebrandt., 1972). Experimentally, 10 ml of the stock culture was transferred in triplicate onto 50 ml of each medium containing 30 g/l sucrose, 0.2 mg/l NAA and 0.2 mg/l 2,4-D. The cultures were maintained at 25$\pm$2°C under controlled 16-hr photoperiod. After 7 days, the cultures were harvested by suction filtration, and subjected to freeze drying using a lyophilizer. The resulting dry biomass of each cell culture was weighed and recorded. All cell cultures were then kept in a sealed container under 4°C for subsequent analysis of plumbagin content.

3.7 Study on the effect of plant growth regulators

The stock cultures of *P. zeylanica* (10 ml) were transferred onto 50 ml of liquid MS medium (Murashige and Skoog, 1962) supplemented with different concentrations (0.0, 0.01, 0.1, and 10.0 mg/l) of auxins (2,4-D, NAA, IAA or IBA) or cytokinins (BA or kinetin) for testing the effect of these plant growth regulators on culture growth and plumbagin production. After 7 days, the cultures grown in various conditions were harvested and prepared for dry weight and plumbagin determination as described in Sections 4.1 and 4.2. Each culture was analyzed triplicates for both parameters.

3.8 Study on the effect of carbon source

This was carried out by using LS medium containing 3% (w/v) of various sugars (sucrose, glucose, sorbitol and manitol), 0.2 mg/l NAA and 0.2 mg/l 2,4-D.

3.9 Study on plumbagin distribution in *P. zeylanica* plant

The plant of *P. zeylanica* was separated into serveral organ parts (roots, stems, leaves and flowers). The separation was done in such a way that the connection of each part in the whole plant is still known and recorded. After separation, each part was dried by freeze-drying and determined for plumbagin content by TLC-densitometric analysis (see Sections 4.1 and 4.2).

3.10 Study on growth and plumbagin production in cell suspension cultures.

Duplicate 10 ml samples of a 7-day-old suspension were inoculated in liquid MS medium supplemented with 0.1 mg/l 2,4-D, 0.01 mg/l BA, and 3% (w/v) manitol. The cell suspension cultures were harvested every day for 10 days by suction filtration and then harvested every other day until day 16. The dry weight was recorded after freeze drying for 18 hours. The amount of plumbagin was examined as described in Sections 4.1 and 4.3 and calculated in units of both percent dry weight and total content. These data were then plotted to obtain growth and plumbagin production curves.

4. PHYTOCHEMICAL TECHNIQUES

4.1 Sample preparation for plumbagin analysis

The dried samples of either cell suspension cultures or various plant parts of *P. zeylanica* were ground to fine powder in a grinder. Five hundred milligrams of each powdered sample was extracted with 20 ml methanol under reflux for one hour in a 20 x 25 cm tube connected with a 15 cm condenser. The crude extracts were then filtered (Whatman no. 1) and evaporated *in vacuo* to dryness. One hundred microliters of petroleum ether were then added to each tube to dissolve the residue to obtain a solution for plumbagin analysis.

4.2 TLC-densitometric analysis of plumbagin

Five or Ten-microlitre aliquot of the above-mentioned solutions was each spotted on a TLC plate (silica gel 60 F254, 0.2 mm thickness). The plate was developed ascendingly for 10 cm by using the mobile phase of toluene : formic acid (9.9:0.1). The TLC plate was scanned by Shimadsu Dual-Wavelength TLC-Scanner (Model CS-930) using the wavelength of 270 nm. Areas under the peaks of plumbagin were used for determinating plumbagin content based on standard curve of the authentic compound. The standard curve showed linearity of the relationship between the values of absorbance and plumbagin content from 0.125 to 2.5 µg. The identity of plumbagin was evidenced by UV absorption spectrum which was identical with the spectrum of the authentic compound. The accuracy of this analytical technique was confirmed by HPLC. The HPLC was performed by the instrument model of Shimadzu C-R6A using Merck 50943 Li Chrospher 100 RP-18 columm. The mobile phase was methanol : 4% acetic acid (60:40, v/v), flow rate 1.0 ml/min and the detector was set at the wavelength of 270 nm. Similarly, the standard curve of plumbagin obtained by HPLC analysis showed linearity (0.078 to 2.5 mg) but was slightly more sensitive than the TLC-densitometric method.

RESULTS

1. TLC separation of plumbagin in the crude extracts of various *P. zeylanica* parts

In this study, the development of TLC-densitometry for plumbagin determination was started with finding a solvent system suitable for separation of various compounds in the crude methanolic extracts. It was found that the solvent system of toluene : formic acid, 9.9:0.1 could separate a yellow spot (which was co-chromatographed with authentic plumbagin) form other components present in the crude methanolic extracts of various *P. zeylanica* parts. These included the plant parts of flowers, leaves, stems and roots. No significant interference from other components in these extracts was observed under this TLC condition. When the TLC plate was scanned at the wavelength of 254 nm by a TLC-densitometer, various chromatograms of the crude methanolic extracts were obtained. As shown in Fig. 4, all plant parts of *P. zeylanica* appeared to contain plumbagin which showed its peak well separated from others under the established conditions.

2. Plumbagin content in various parts of *P. zeylanica* plant

The results of plumbagin content in *P. zeylanica* are summerized Fig. 7. It can be seen that various parts of *P. zeylanica* contained highly variable plumbagin content ranging from 0.002 to 0.266% (w/w) dry weight. The content of plumbagin in the roots appeared to be the highest (0.226%) followed by the stems, mature leaves (0.02-0.03%), young leaves (0.015%) and flowers (0.002%), respectively. The distribution of plumbagin along the stem and leaves showed that the content decreased rapidly along the stem up to the shoot. For the leaf parts, those at the middle of the stem seemed to have higher plumbagin content than at the top or the bottom of the plants (Fig. 7).

3. Tissue cultures of *P. zeylanica*

3.1 Establishment of callus cultures

Callus formation of *P. zeylanica* was induced successfully on LS medium supplemented with the combination of 30 g/l sucrose, 0.2 mg/l NAA, 0.2 mg/l 2,4-D and 0.8% (w/v) agar. As shown in Fig. 8A, the callus tissue was initiated at the top edge of leaf segments before enlarging to the bottom and formed as friable tissue with pale gray color. These callus cultures could be maintained by using the

same medium with regular subculturing for every three weeks. In this medium, the callus had a high growth rate resulting in the formation of friable, soft and grayish tissues (Fig. 8B)

3.2 Establishment of cell suspension cultures

Cell suspension cultures of *P. zeylanica* were obtained from the friable grayish callus which was maintained by a regular subculturing as described earlier. The callus tissues were separated into small aggregates before transferring into LS liquid medium containing 0.2 mg/l NAA and 0.2 mg/l 2,4-D and rotated at 120 r.p.m. on a rotary shaker. Under these conditions, the suspension cultures appeared to have high growth rate and formed small grayish aggregates (Fig. 9). After the cell suspension cultures had a suitable cell density, it was maintained in the same medium by subculturing for every two weeks.

3.3 Time-courses of culture growth and plumbagin content in *P. zeylanica* cell suspension

Cell suspension cultures of *P. zeylanica* were regularly maintained in LS medium containing 0.2 mg/l 2,4-D, 0.2 mg/l NAA. Monitoring of the growth and plumbagin content of the suspension culture under these conditions was performed. As shown in Fig. 10, it was found that after subculturing, *P. zeylanica* cell cultures (0.35 g/flask) had a short lag phase of only one day. After that the culture started to growth and increased in dry weight rapidly until day 9 when the maximum cell mass was observed (1.27 g/flask). Subsequently, the growth appeared to decline continuously until day 16 (0.7 g/flask) of the culture cycle. Thus, the cell suspension showed a ca. 4-fold increase in dry biomass during the culture cycle.

For the formation of plumbagin, the cell culture also appeared to start producing plumbagin after one day of subculturing. The content also increased rapidly until day 6 or 7. After that the level of plumbagin was declined also rapidly until the end (day 16) of the culture cycle.

3.4 The effect of basal media on plumbagin production in cell suspension cultures

The effect of various culture media on growth and plumbagin content was examined after a culture period of 7 days. The media tested were LS, MS, B5, N and SH. The cell suspension cultures showed their fastest growth in MS medium, followed by SH, LS, NH and B5 media, respectively (Table 1). Plumbagin was detected in the cell suspension culture grown in all the culture media. The highest yield was found in MS medium (130 μg/g DW), and the lowest was found in B5 and NH media (ca. 70 μg/g DW). Therefore, MS medium was effective in promoting both cell growth and plumbagin content. Thus, MS medium was chosen as the medium for the whole course of this study.

Table 1 Dry weight and plumbagin content obtained from various culture media

Medium	dry weight (g/flask)	plumbagin (μg/g)
LS	1.090 ± 0.045	19.6 ± 0.4
MS	1.200 ± 0.037	26.0 ± 1.0
B5	0.883 ± 0.048	13.4 ± 0.6
NH	0.907 ± 0.015	13.8 ± 0.2
SH	1.203 ± 0.029	15.0 ± 0.8

3.5 Effects of auxins

This experiment aimed to study 4 types of auxins, namely IAA, NAA, 2,4-D and IBA on the growth and plumbagin formation in *P. zylanica* cell cultures. These auxins were examined at various concentrations (0.01-10.0 mg/l) in MS medium. As shown in Fig. 11, NAA and IBA at a wide range of concentrations gave higher culture growth than IAA and 2,4-D. The formers, on the other hand, resulted to low contents of plumbagin at all concentrations tested whereas both 2,4-D and IAA appeared to induce plumbagin formation with slightly superior for 2,4-D. The concentrations of 2,4-D from 0.01 to 1.0 showed no difference on the product formation although at 10.0 mg/l 2,4-D, plumbagin content was reduced significantly.

3.6 Effects of cytokins

The effects of cykinins on culture growth and plumbagin formation were also investigated. The two cytokinins chosen for this study were the commonly used BA and kinetin. The concentration range from 0.01 to 10 mg/l was also used. It was found that both cytokinins had no difference on inducing culture growth. For the plumbagin formation, BA showed slightly better although its concentrations gave variable values (Fig. 12). However, comparing to the auxin supplement, it was found that the degree of plumbagin induction by BA was about 10 fold lower than 2,4-D.

3.7 Effects of carbon sources

The effect of different carbon sources at 3% concentration on growth and plumbagin formation in *P. zeylanica* is shown in Table 2. Among the 4 types tested of sucrose, glucose, sorbitol and manitol, sucrose was found to give good growth followed by glucose, sorbitol and manitol. On the other hand, manitol affected the cell culture to form highest plumbagin content. Glucose showed slightly lower plumbagin content than manitol.

Table 2 The effect of carbon source on growth and plumbagin production in *P. zeylanica*

Carbon source (3%)	Dry weight (g/flask)	plumbagin (μg/g dry weight)
Sucrose	1.45 ± 0.09	10 ± 3
Glucose	1.26 ± 0.15	20 ± 1
Sorbitol	0.96 ± 0.02	8 ± 0
Manitol	0.96 ± 0.13	31 ± 2

DISCUSSION

The major aim of the establishment of *P. zeylanica* cell cultures is to study its potential in producing plumbagin. Plumbagin is a plant naphthoquinone presumably biosynthesized by the polyketide pathway. So far, very little is known about the enzyme polyketide synthase in this plant. Therefore, a successful induction of *P. zeylanica* cell culture to form plumbagin would lead to a possibility to study such an enzyme and to understand the biosynthetic pathway of plumbagin eventually.

Generally, several plant cell culture media widely used to support growth are normally not optimum for product formation. This has been demonstrated by the successful use of a production medium (Fujita *et al.*, 1981) or induction medium (Sasse *et al.*, 1982), to optimize the yields of a desired product. Such investigations, as well as other numerous reports concerning effects of the nutritional environment on secondary product formation (for review, see Dougall, 1980) have clearly shown that hormonal and nutritional factors in the medium can exert an influence on the expression of secondary metabolism.

For this case of *P. zeylanica* cell cultures, we have shown that both hormonal and nutritional factors have an effect on plumbagin formation. The culture grows well and produce plumbagin in MS medium. On the other hand, NAA appears to clearly stimulate growth but diminish product formation whereas 2,4-D seems to have opposite effect. For the cytokinins, the effect of BA and kinetin also seems to have different degrees on plumagin formation although the product level is much lower than the 2,4-D containing medium. Therefore, as shown in many cases, the effects of these hormonal factors are usually unpredictable and cannot be generalized.

Comparing with the whole plant of *P. zeylanica*, it is obvious that plumbagin content in the cell culture is still much lower than that found in the plant. Based on this work, plumbagin can be found in every part of the plant. The root part contains highest level which is up to 2.26 mg/g (or 0.26% dry weight).

Although the cell cultures of *P. zeylanica* produces plumbagin in relatively low amount, the biosynthetic pathway of plumbagin is still expressed. This allows us to use the cell culture to study the biosynthetic enzyme of this pathway. Our preliminary study has shown that the presence of acetyl CoA, malonyl CoA and NADPH can lead to a formation of a compound that might be an intermediate of the plumbagin pathway. This is due to the possible condensation of malonyl CoA with acetyl CoA to form a polyketide chain. This enzymatic reaction proceeds very slowly in the absence of NADPH. Therefore, it is likely that the cell culture of *P. zeylanica* producing plumbagin can be used for enzymological study of its biosynthetic pathway. This aspect of study is still going on in our laboratory.

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LEGENDS

Fig. 1 TLC chromatograms of methanolic extracts obtained from various plant parts of *P. zeylanica* scanned under the wavelength of 270 nm. Sovent system : toluene : formic acid (9.9:0.1).

Fig. 2 Distribution of plumbagin in the mature *P. zeylanica* plant.

Fig. 3 A) Induction of callus from the leaf explants of *P.zeylanica* on LS agar medium containing 0.2 mg/l NAA and 0.2 mg/l 2,4-D.
B) The apparent friable, soft and grayish callus cultures in LS agar medium containing 0.2 mg/l NAA and 0.2 mg/l 2,4-D.

Fig. 4 Cell suspension cultures of *P.zeylanica* maintained in LS liquid medium containing 0.2 mg/l NAA and 0.2 mg/l 2,4-D.

Fig. 5 Time-courses of culture growht and plumbagin centent in *P.zeylanica* cell suspension cutures.

Fig. 6 The effect of auxins un plumbagin production in cell suspension cultures.

Fig. 7 The effect of cytokins on plumbagin production in cell suspension celtures.