CHAPTER III

MATERIALS AND METHODS

1. CHEMICALS

Standard lawsone was purchased from Sigma, standard 2-methoxy-1,4-naphthoquinone was a gift from Dr. E. Saifah and standard scopoletin was a gift from Dr. R. Bavovada. Both are at the Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The culture media (MS, B5) and various plant hormones were from Gibco Laboratories. All other chemicals were reagent grade or better, as available. Solutions were prepared in water obtained from triple distillation. TLC plate (Silica gel 60 F_{254}) and HPLC-organic solvents were from Merck. The plant *Impatiens balsamina* Linn. were cultivated in the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2. PLANT TISSUE CULTURE TECHNIQUES

2.1 Preparation of *I. balsamina* Leave Explants

Callus cultures of *Impatiens balsamina* were initiated in February 1991 using young leaves of the mature plants as starting material. Before initiating the callus, the surface of the explants were sterilized as describes below.

young leaves of mature plants

↓

cleaned with running tap water

↓

dipped into 15% Clorox® solution for 15 minutes

↓

washed with sterile distilled water 2 times

↓

excised with a sharp scalpel

↓

transferred to solid nutrient medium

2.2 Medium Preparation

The culture media of B5 and MS, as shown in Table 5, were prepared from either stock solutions of nutrients and hormones or commercially prepared media.

Table 5. Inorganic Salt and Vitamin Compositions of Plant Tissue Culture Media

Constituent	Concentration (mg/liter) MS	B5
Macronutrients :		
$MgSO_4 . 7H_2O$	370	250
KH_2PO_4	170	-
$NaH_2PO_4 . H_2O$	-	150
KNO_3	1900	2500
NH_4NO_3	1650	-
$CaCl_2 . 2H_2O$	440	150
$(NH_4)_2 . SO_4$	-	134
Micronutrients:		
H_3BO_3	6.2	3
$MnSO_4 . H_2O$	15.6	10
$ZnSO_4 . 2H_2O$	8.6	2
$NaMoO_4 . 2H_2O$	0.25	0.25
$CuSO_4 . 5H_2O$	0.025	0.025
$CoCl_2 . 6H_2O$	0.025	0.025
KI	0.83	0.75
$FeSO_4 . 7H_2O$	27.8	-
Na_2EDTA	37.3	-
EDTA Na Ferric	-	40
Sucrose (g)	30	30
Vitamins :		
Thiamine HCl	0.5	10
Pyridoxine HCl	0.5	1
Nicotinic acid	0.5	1
myo-Inositol	100	100
pH	5.8	5.5

2.2.1 Preparation of MS and B5 culture media from stock solutions.

Various stock solutions of B5 and MS and plant hormones were prepared at the concentrations shown in Table 6.

Table 6. Preparation of Stock Solution of B5 and MS Media

B5		MS		Remarks
Stock 1 (Macronutrients)	g/1000 ml	**Stock 1** (Macronutrients)	g/1000 ml	store in refrigerator
$NaH_2PO_4 . H_2O$	3.00	NH_4No_3	33.0	
$(NH_4)_2SO_4$	2.68	KNO_3	38.0	
$MgSO_4 . 7H_2O$	5.00	$MgSO_4 . 7H_2O$	7.4	
KNO_3	50.0	KH_2PO_4	3.4	
Stock 2 (Micronutrients)	mg/100 ml	**Stock 2** (Micronutrients)	mg/100 ml	store in refrigerator
$MnSO_4 . H_2O$	1000	H_3BO_3	620	
H_3BO_3	300	$MnSO_4 . H_2O$	1690	
$ZnSO_4 . 7H_2O$	200	$ZnSO_4 . 7H_2O$	860	
$Na_2MoO_4 . 2H_2O$	25	$Na_2MoO_4 . 2H_2O$	25	
$CuSO_4 . 5H_2O$	2.5	$CuSO_4 . 5H_2O$	2.5	
$CoCI_2 . 6H_2O$	2.5	$CoCI_2 . 6H_2O$	2.5	
Stock 3 (Ca Stock)	g/100 ml	**Stock 3** the same as for B5		store in refrigerator
$CaCI_2 . 2H_2O$	15			
Stock 4 (KI stock)	mg/100 ml	**Stock 4** the same as for B5		store in amber bottle in referator
KI	75			
Stock 5 (Vitamins)	mg/100 ml	**Stock 5** the same as for B5		store in freezer (10-ml fraction)
Nicotinic acid	100			
Thiamine. HCI	1000			
Pyridoxine. HCI	100			
Myo-Inositol	10,000			
Stock 6 (Fe-EDTA stock)	g/500 ml	**Stock 6** the same as for B5		store in refrigerator
Na_2EDTA	3.73			
$FeSO_4 . 7H_2O$	2.78			
2,4-D stock solution (100 mg/l)	mg/100 ml	**2,4-D stock solution** the same as for B5		dissolve 2,4-D in 5 ml ethanol ; heat slightly and gradually dilute to 100 ml with water
2,4-D	10			
NAA stock solution (100 mg/l)		**NAA stock solution** the same as for B5		
NAA	10			
Kinetin stock solution (100 mg/l)		**Kinetin stock solution** the same as for B5		dissolve kinetin in a small volume of 0.5 N HCI by heating slightly and gradually dilute to 100 ml with distilled water. Store in refrigerator.
Kinetin	10			

The culture media were then prepared by mixing the stock solutions and added 3% sucrose as described in Table 7. The pH of each medium was adjusted to its desired value with 1N potassium hydroxide or 1N hydrochloric acid. The media were heated on water bath and 0.8% w/v agar was added to make solid media. The media were then sterilized by autoclaving using the conditions of 121°C, 15 lb/in^2 for 15 minutes. Liquid media were also prepared similarly but the agar was omitted.

Table 7. Preparation of B5 and MS Media

B5		MS	
Distilled Water	1000 ml	Distilled Water	1000 ml
Stock 1	50 ml	Stock 1	50 ml
Stock 2	1.0 ml	Stock 2	1.0 ml
Stock 3	1.0 ml	Stock 3	2.9 ml
Stock 4	1.0 ml	Stock 4	1.0 ml
Stock 5	1.0 ml	Stock 5	1.0 ml
Stock 6	5.0 ml	Stock 6	5.0 ml
Sucrose	30 g	Sucrose	30 g
Agar (solid medium)	8 g	Agar (solid medium)	8 g
Auxin (100 mg/l)	as needed	Auxin (100 mg/l)	as needed
Cytokinin (100 mg/l)	as needed	Cytokinin (100 mg/l)	as needed
Final pH adjust to	pH 5.5	Final pH adjust to	pH 5.8

2.2.2 Preparation of B5 and MS culture media from commecially prepared media

a) Preparation of B5 medium from Gibco Laboratories

1. Measure out 20% less deionized, distilled water (approx. 800 ml) than desired total volume of medium (one liter).

2. While stirring, dust in powder gradually.
3. Rinse out inside of container to remove all traces of powder.
4. For liquid culture, check and adjust pH if necessary to 5.0 for an agar culture, check and adjust pH if necessary to 5.7, then add agar.
5. Dilute to final volume of one liter.
6. For agar based media, heat gently, with continuous mixing until the solution clears.
7. Dispense desired amount of medium into culture ressels.
8. Autoclave for 15 minutes at 15 lb/in^2, 121°C

b) Preparation of MS medium from Gibco Laboratories

1. Measure out 20% less deionized, distilled water (800 ml) than desired total volume of medium (one liter).
2. While stirring, dust in powder gradually.
3. Rinse out inside of container to remove all trace of powder.
4. The content of this packge have been adjusted to pH 5.7 $\pm$ 0.1. No further adjustment necessary.
5. Dilute to final volume of one liter.
6. For agar base media, heat gently, with continuous mixing until the solution clears.
7. Dispense desired amount of medium into culture vessels.
8. Autoclave for 15 minutes at 15 lb/in^2, 121°C.

2.3 Study on the Effect of Hormonal Factors on Callus Formation

Various types and concentrations of hormones in B5 and MS medium were varied to study their effect on callus formation of *I. balsamina* leaf explants. Three auxins (NAA, 2,4-D and IAA) were used at the concentration between 0.1 and 1.0 mg/l combined with two cytokinins (kinetin and BA) at the concentration 1.0 mg/l (Table 8) and sucrose

was used at the concentration 3% (w/v). The callus formation in each medium was observed periodically and the results were recorded.

Table 8. The Combination of Auxins and Cytokinins in Tested Culture Media

Concentration of Cytokinins	Concentration of Auxins (mg/ml)		
	NAA	2,4-D	IAA
BA 1.0 mg/l	0.1	-	-
	0.5	-	-
	1.0	-	-
	-	0.1	-
	-	0.5	-
	-	1.0	-
	-	-	0.1
	-	-	0.5
	-	-	1.0
Kinetin 1.0 mg/l	0.1	-	-
	0.5	-	-
	1.0	-	-
	-	0.1	-
	-	0.5	-
	-	1.0	-
	-	-	0.1
	-	-	0.5
	-	-	1.0

2.4 Establishment of Cell Suspension Cultures

Cell suspension cultures of *I. balsamina* were initiated by using the callus cultures maintained in B5 medium containing 0.1 mg/l 2,4-D and 1.0 mg/l kinetin. The suspension were incubated in rotary shaken Erlenmeyer flask (50 ml medium in 250 ml flask) at 120 r.p.m. at 25°C. After obtaining stable *I. balsamina* cell cultures, the cell suspension were maintained under the same conditions and subcultured (1:5 dilution) every 3 weeks in a modified B5 medium (2 x calcium chloride concentration) supplemented with 0.1 mg/l 2,4-D, 1.0 mg/l BA and 1% (w/v) sucrose.

2.5 Establishment of Root Cultures

Root cultures of *I. balsamina* were also initiated from the same callus cultures described in the cell suspension cultures. The callus was suspended in liquid B5 medium

supplemented with 0.1 mg/l NAA, 0.1 mg/l kinetin, 1.0 mg/l BA and 3% (w/v) sucrose. The cultures were maintained as described above and the roots were formed on the first week after the first passage. The root cultures were maintained in the same medium by subculturing every 3 weeks (2 g. of root per 50 ml medium).

2.6 Study on Growth and Lawsone Production in the Root Cultures

Two grams of the root cultures, after cutting into pieces (1 cm long), were inoculated in liquid B5 medium supplemented with 0.1 mg/l NAA, 0.1 mg/l kinetin, 1.0 mg/l BA and 3 %(w/v) sucrose. The roots were harvested every day for 12 days by filtration and then harvested every other day until day 20. The dry weight were recorded after drying at 50°C for 24 hours. The amount of lawsone was examined as described in the sections of 3.1 and 3.3 and calculated in the units of both percentages of dry weight and total content. These data were then plotted to obtain growth and lawsone production curves.

3. PHYTOCHEMICAL TECHNIQUES

3.1 Preparation of Crude Extracts of Various *I. balsamina* Cultures and Plant Parts

The dry plants or tissue cultues of *I. balsamina* (*ca* 1.5 g) were ground and extracted under reflux with 40 ml chloroform and ethanol (1:1) for 1 hour and filtered. After filtering, the filtrate was evaporated to dryness and the residue was then dissolved in the same solvent and readjusted to 1 ml. The resulted crude extracts were used for qualitative and quantitative analysis of lawsone and 2-methoxy-1,4-naphthoquinone.

3.2 Identification of Lawsone and 2-Methoxy-1,4-naphthoquinone

Thin layer chromatography (TLC) was used as the method for identification of lawsone and its methyl ether in the crude extracts obtained from both the *in vitro* cultures

and intact plants of *I. balsamina*. The TLC conditions were described below. The identity of lawsone and its methyl ether on the TLC was also confirmed by another TLC system using chloroform as a mobile phase.

TLC conditions

Technique	:	one way, ascending, double development
Absorbent	:	aluminium sheet silica gel 60 F254 (precoated, Merck)
Plate size	:	10 x 15 cm
Layer thickness	:	0.2 mm
Solvent system	:	a) first development - cholroform : petroleum ether (8 : 2) b) second develpment in the same dimension - benzene : acetic acid (9.8 : 0.2)
Sample size	:	5 µl
Distance	:	10 cm
Temperature	:	25 - 30°C
Detection	:	Ultraviolet light at 254 nm

3.3 Quantitative Analysis of Lawsone and Its Methyl Ether

The TLC plates after subjected to the second solvent system described above were used for producing chromatogram by TLC densitometer. The conditions of the equipment were described below. The areas under the peaks of lawsone and its methyl ether were recorded and converted to concentration by using their standard curves. The standard curves were established from the standard lawsone and 2-methoxy-1,4-naphthoquinone at the concentration range of 0.02-0.4 mg/ml.

3.3.1 TLC densitometer conditions

Model	:	Shimadsu Dual Wavelength Model CS-930

Wave length	:	280 nm
Scan width	:	"X" width 6 mm
		"Y" width 0.2 mm
Slit width	:	1.2 mm
Sensitivity	:	medium

The accuracy of TLC densitometric method was confirmed by high performance liquid chromatography (HPLC). The condition of HPLC was described below.

3.3.2 HPLC conditions

Chromatographic column	:	SP-C18-5 column, 4.0 mm x 15 cm
Guard column	:	Micro Pak SP-C18-5 column, 4.0 mm x 4 cm
Mobile phase	:	methanol : water (65 : 35)
Flow rate	:	1 ml/min
Detector	:	UV 275 nm
Injection volume	:	20 µl