



UNIVERSITI PUTRA MALAYSIA

**PHYSALINS (13,14-SECO-16,24-CYCLOSTEROIDS) PRODUCTION
IN *PHYSALIS MINIMA* (LINN.)**

JUALANG GANSAU

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**PHYSALINS (13,14-SECO-16,24-CYCLOSTEROIDS) PRODUCTION
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By

JUALANG GANSAU

**Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of
Philosophy in the Faculty of Science and Environmental Studies
Universiti Putra Malaysia**

January 2001



**'IN THE NAME OF ALLAH, MOST GRACIOUS, MOST
MERCIFUL'**

Dedicated To:

My Parents

Father and mother: Mr. Gassu Dabigaa and Ms. Rullian Lunastun

Brothers and sisters: Ms. Nalays, Ms. Nora, Mr. Dlaton @ Maglaa,

Ms. Jubaya, Ms. Ejiasys, Ms. Norpiak,

Mr. Geavol and Mr. Mozli

My Wife: Loria @ Mariah Amirah Amari Abdullah

My Son: A'fil Iykharruddin

To all my Teachers and Lecturers

Abstract of thesis presented to Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Doctor of Philosophy.

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January 2001

Chairman : Professor Hj. Marziah Mahmood, Ph.D.

Faculty : Science and Environmental Studies

Physalis minima produces physalins, and these C₂₈-steroidal lactone compounds have great potentials in pharmaceutical industry. However, no detail information on the biosynthetic background of physalins in either intact plants or in cultured plant tissues. Therefore, this study was carried out to determine the physalins distribution in intact plants and in cultured plant tissues: callus, cell suspensions and hairy roots. Factors that control the growth and physalins production in plant tissue culture levels such as medium compositions, physical factors and precursors were also elucidated to improve the physalins productivity. The results showed that physalins accumulation in specific plant tissues of intact plants varied between 0.07 to 5.76 mg g⁻¹ DW. Physalin contents increased two folds as the plant matured. Physalin A accumulated mostly in young fruits (3.82 mg g⁻¹ DW), physalin B in young leaves (1.56-3.20 mg g⁻¹ DW) and flower buds (2.88-3.60 mg g⁻¹ DW), physalin D in flower buds (4.65-5.83 mg g⁻¹ DW), physalin F in older leaves (4.51-9.89 mg g⁻¹ DW), physalin J in immature and ripe fruit calyx (2.14-3.96 mg g⁻¹ DW), and physalin N in young and old leaves (2.68-4.48 mg g⁻¹ DW). In addition, the accumulation level of physalins in specific tissues was different among plants collected from different locations. In cultured plant tissues, the content of physalin B and F in hairy roots were

found to be higher (1.95-17.01 mg g⁻¹ DW) than that in intact plants, but lower in callus (1.51-1.91 mg g⁻¹ DW) and cell suspension (0.67-1.95 mg g⁻¹ DW) cultures. Higher physalins production in callus and suspension cultures were obtained in cells derived from leaves followed by root and stem explants. Cell suspension and hairy root cultures were also capable of excreting physalins at lower concentration into culture medium. The study on the effect of medium compositions has shown that higher physalins production in callus, cell suspension and hairy root cultures were obtained in 1/2MS (half strength), MS (full strength) and B5 (full strength) basal media, each supplemented with 2.5, 3.5 and 3.5% (w/v) sucrose, respectively. An auxin-cytokinin interaction was observed to be important for callus cultures, as these two classes of phytohormones are required for higher growth and physalins production. Higher physalins production in callus culture was obtained in medium supplemented with a combination of 2,4-D and kinetin (9.0:4.5 µM). However, the addition of cytokinin in cell suspension culture appeared to stimulate irregular compact globular cells and growth of many root-like structures in the cell clumps. Higher physalins production in cell suspension was obtained in cultures supplemented with 9.0-18.0 µM NAA or 18.0 µM IAA. Meanwhile, in hairy root cultures, phytohormones often caused a growth disorganisation. The addition of 3-4 µM NAA increased the physalins production. Further investigations on hairy root cultures have shown that physalins accumulated mainly in mature part of root tissues. Inoculum of different root morphology did not significantly influence growth and physalins production. Meanwhile, the increase in number of inoculum root tips and medium volume resulted in changes of certain growth parameters. Hairy root cultures were capable to grow in pH values between 4.0-9.0, and higher physalins production was obtained at pH 5.0-7.0. Physalin productions in hairy roots also increased up to 1.2.1 folds when cultured under dark conditions supplemented with alanine, leucine and valine.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah.

**PENGHASILAN FISALIN (13,14-SEKO-16,24-SIKLOSTEROID) DALAM
PHYSALIS MINIMA (LINN.)**

Oleh

JUALANG GANSAU

January 2001

Pengerusi : Profesor Hj. Marziah Mahmood, Ph.D.

Fakulti : Sains dan Pengajian Alam Sekitar

Pokok *P. minima* telah menghasilkan kompaun steroid lakton C_{28} yang dinamakan fisalin dan mempunyai potensi dalam industri farmaseutikal. Walau bagaimanapun, tiada kajian secara mendalam mengenai biosintesis fisalin dalam pokok induk atau dalam kultur tis. Oleh yang demikian, kajian telah dijalankan ke atas pokok induk dan kultur tis bagi kalus, sel ampai, dan akar transgenik untuk menentukan taburan penghasilan fisalin. Faktor yang mempengaruhi tumbesaran dan sintesis fisalin dalam kultur tis seperti komposisi medium, faktor fizikal dan bahan pemula juga dibincangkan untuk meningkatkan hasil fisalin. Keputusan kajian menunjukkan fisalin dikumpulkan dalam tis pokok induk dalam nisbah kepekatan tertentu ($0.07-5.76 \text{ mg g}^{-1} \text{ DW}$). Kandungan fisalin juga didapati meningkat dua kali ganda dengan perubahan pematangan tumbesaran pokok. Fisalin A kebanyakannya dikumpul dalam buah muda ($3.82 \text{ mg g}^{-1} \text{ DW}$), fisalin B dalam daun muda ($1.56-3.20 \text{ mg g}^{-1} \text{ DW}$) dan kudup bunga ($2.88-3.60 \text{ mg g}^{-1} \text{ DW}$), fisalin D dalam kudup bunga ($4.65-5.83 \text{ mg g}^{-1} \text{ DW}$), fisalin F dalam daun tua ($4.51-9.89 \text{ mg g}^{-1} \text{ DW}$), fisalin J dalam kalik buah masak dan matang ($2.14-3.96 \text{ mg g}^{-1} \text{ DW}$), dan fisalin N dalam daun muda dan tua ($2.68-4.48 \text{ mg g}^{-1} \text{ DW}$). Namun begitu, kandungan fisalin dalam tis tertentu didapati berbeza pada pokok yang diambil dari lokasi yang berlainan. Dalam

kultur tisu, penghasilan fisalin B dan F dalam akar transgenik didapati lebih tinggi ($1.95\text{-}17.01\text{ mg g}^{-1}\text{ DW}$) dari pokok induk, dan rendah dalam kalus ($1.51\text{-}1.91\text{ mg g}^{-1}\text{ DW}$) dan sel ampaian ($0.67\text{-}1.95\text{ mg g}^{-1}\text{ DW}$). Penghasilan tertinggi fisalin dalam kultur kalus dan sel ampaian telah diberikan oleh sel dari daun berbanding dengan sel dari batang atau akar. Kultur sel ampaian dan akar transgenik juga berupaya pada kepekatan rendah untuk membebaskan fisalin ke dalam media kultur. Kajian pada kesan komposisi medium menunjukkan penghasilan tertinggi fisalin pada kultur kalus, sel ampaian dan akar transgenik masing-masing diperolehi dalam media asas 1/2MS, MS dan B5 yang ditambah dengan 2.5-3.5% (w/v) sukrosa. Interaksi auksin-sitokinin didapati penting bagi pertumbuhan kalus dan memberikan hasil fisalin yang tinggi. Kandungan fisalin dalam kalus didapati tinggi dalam medium yang ditambah kombinasi 2,4-D-kinetin. Penambahan sitokinin dalam kultur sel ampaian didapati mengaruh pembentukan sel bulat yang keras dan pertumbuhan struktur seperti akar pada sekeliling sel. Penghasilan tertinggi fisalin dalam kultur sel ampaian diperolehi dalam kultur yang ditambah dengan NAA atau IAA. Penambahan pengawal atur pertumbuhan pada kultur akar transgenik didapati merangsang ketidaktentuan pertumbuhan akar dan akhirnya menurunkan berat tumbesaran. Namun begitu, penambahan NAA berupaya meningkatkan penghasilan fisalin. Kajian lanjut pada kultur akar transgenik menunjukkan fisalin lebih banyak dikumpulkan pada bahagian akar yang telah matang. Morfologi akar yang berbeza yang digunakan sebagai pemula kultur didapati tidak berbeza dari segi keupayaan tumbesaran dan penghasilan fisalin. Perubahan bilangan akar dalam kultur pemula dan isipadu media yang digunakan juga tidak secara berkesan mempengaruhi sintesis fisalin, tetapi didapati mengubah beberapa parameter pertumbuhan. Akar transgenik juga berupaya untuk tumbuh pada julat pH media antara pH 4.0-9.0, dan hasil fisalin tertinggi didapati pada julat pH 5.0-7.0. Fisalin dalam akar transgenik juga boleh ditingkatkan sehingga 1.2-2.1 kaliganda bila ditumbuhkan dalam keadaan gelap dan dengan penambahan asid amino (alanine, leucine dan valine).

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TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	v
ACKNOWLEDGEMENTS	vii
APPROVAL SHEETS	viii
DECLARATION FORM	x
LIST OF TABLES	xv
LIST OF FIGURES	xvii
LIST OF PLATES	xx
LIST OF ABBREVIATIONS	xxi
 CHAPTER	
 1 INTRODUCTION	 1
 2 LITERATURE REVIEW	 7
2.1 Physalin Constituent of <i>Physalis sp.</i>	7
2.2 Antitumour Activity of Physalins	11
2.3 Biosynthetic Variation of Physalins	14
2.4 Genetic Transformation of <i>Physalis sp.</i>	15
2.5 <i>Agrobacterium rhizogenes</i> -mediated Transformation	15
2.6 Factors Affecting the Efficiency of <i>Agrobacterium</i> -mediated Transformation	17
2.6.1 Explant Types	17
2.6.2 Co-cultivation Medium	18
2.6.3 Co-cultivation Periods	19
2.6.4 Inoculation Techniques	19
2.7 Comparison of Hairy Roots With Other Type of Plant Cell Cultures	20
2.8 Factors Controlling Growth and Secondary Metabolites Product	21
2.8.1 Media Formulations	22
2.8.2 Physical Factors	26
2.8.3 Inoculum Density	29
2.8.4 Inoculum Morphology	30
2.9 Strategies in Enhancing the Production of Secondary Metabolites	31
2.9.1 Manipulation of Release Products	31
2.9.2 Elicitors	32
2.9.3 Precursors-Feeding	33

3	DISTRIBUTION OF PHYSALINS IN INTACT PLANTS	36
3.1	Introduction	36
3.2	Materials and Methods	37
3.2.1	Plant Sources	37
3.2.2	Extraction Procedures	38
3.2.3	TLC Isolation and Identification	39
3.2.4	Quantitative Analysis by HPLC	40
3.2.5	Standard Physalins	41
3.2.6	Statistical Analysis	41
3.3	Results and Discussion	44
3.3.1	Levels of Physalins in Different Plant Growth Developmental Stages	44
3.3.2	Physalin B and F Contents in Plant Collected from Different Locations	48
3.4	Conclusion	50
4	PHYSALIN B AND F PRODUCTION IN CALLUS CULTURES	51
4.1	Introduction	51
4.2	Materials and Methods	52
4.2.1	Establishment of <i>in vitro</i> Plant Cultures	52
4.2.2	Initiation of Callus	52
4.2.3	Initiation of Treatments	53
4.2.4	Growth Curves	54
4.2.5	Nutrient Formulation	54
4.2.6	Carbon sources	55
4.2.7	PGRs	55
4.2.8	Analytical Procedures	55
4.3	Results and Discussion	56
4.3.1	Establishment of Callus Cultures	56
4.3.2	Growth Curves	58
4.3.3	Nutrient Formulations	59
4.3.4	Carbon Sources	61
4.3.5	PGRs	64
4.4	Conclusion	76
5	PHYSALIN B AND F PRODUCTION IN CELL SUSPENSION CULTURES	77
5.1	Introduction	77
5.2	Materials and Methods	78
5.2.1	Initiation of Cell Suspension Cultures	78
5.2.2	Cell Suspension Cultures	79
5.2.3	Analytical Procedures	79

5.3	Results and Discussion	80
5.3.1	Initiation of Cell Suspension Cultures	80
5.3.2	Growth Curves	81
5.3.3	Nutrient Formulations	83
5.3.4	Carbon Sources	84
5.3.5	PGRs	86
5.3.6	Secretion of Physalins into the Culture Medium	89
5.4	Conclusion	101
6	ESTABLISHMENT OF HAIRY ROOT CULTURES	102
6.1	Introduction	102
6.2	Materials and Methods	104
6.2.1	<i>In vitro</i> Plant Cultures	104
6.2.2	<i>Agrobacterium rhizogenes</i>	104
6.2.3	Colony Screening and Selection	104
6.2.4	Gene Transfer Techniques to Promote Hairy Root Formation	105
6.2.5	Decontamination from Bacterium	106
6.2.6	Kanamycin Resistance Test	107
6.2.7	GUS Histochemical Assay	107
6.2.8	Plasmid Mini-Prep of pBI121	108
6.2.9	Isolation of Genomic DNA by CTAB Method	109
6.2.10	Agarose Gel Electrophoresis	110
6.2.11	Southern Blot Analysis	111
6.3	Results and Discussion	114
6.3.1	<i>Agrobacterium</i> -mediated Transformation	114
6.3.2	Decontamination from Bacterium	116
6.3.3	Kanamycin Resistance Test	117
6.3.4	Analysis of Transgenic Nature	118
6.4	Conclusion	126
7	GROWTH CHARACTERISTIC OF HAIRY ROOTS IN SHAKEN FLASK CULTURES	127
7.1	Introduction	127
7.2	Materials and Methods	129
7.2.1	Hairy Root Cultures	129
7.2.2	Inoculum Root Morphology	130
7.2.3	Localisation of Physalin Accumulation in Hairy Roots	131
7.2.4	Inoculum Size and Medium Volumes	131
7.2.5	Kinetics Growth Analysis	131
7.2.6	Analytical Procedures	132

7.3	Results and Discussion	132
7.3.1	Effects of Inoculum Roots Morphology on Culture Performance	132
7.3.2	Localisation of Physalin Accumulation in Hairy Roots	135
7.3.3	Effects of Inoculum Size and Medium Volume on Culture Performance	137
7.4	Conclusion	148
8	PHYSALIN B AND F PRODUCTION IN HAIRY ROOT CULTURE	150
8.1	Introduction	150
8.2	Materials and Methods	151
8.2.1	Growth Curves and Light	151
8.2.2	Nutrient Formulations	152
8.2.3	Carbon sources	152
8.2.4	PH Medium	152
8.2.5	Amino Acids	153
8.2.6	PGRs	153
8.2.7	Analytical Procedures	154
8.3	Results and Discussion	154
8.3.1	Growth Curves and Light	154
8.3.2	Nutrient Formulations	157
8.3.3	Carbon Sources	158
8.3.4	PH Medium	161
8.3.5	Amino Acids	163
8.3.6	PGRs	165
8.3.7	Secretion of Physalins into the Culture Medium	169
8.4	Conclusion	181
9	GENERAL DISCUSSION AND CONCLUSION	182
	REFERENCES	194
	APPENDICES	227
	BIODATA OF THE AUTHOR	235

LIST OF TABLES

Table	Page
2.1 List of plant secondary metabolites derived from <i>Physalis sp.</i>	9
2.2 Cytotoxicity index (CI) for various human leukaemia cells exposed to physalin B and F confirmed by trypan blue dye exclusion assay (DEA).	12
2.3 ID_{50} ($\mu\text{g mL}^{-1}$) for various human leukaemia cells exposed to physalin B and F confirmed by DEA assay.	13
2.4 Anti-leukaemia effects of physalin B and F at concentration $10 \mu\text{g mL}^{-1}$ by counting of viable cells.	13
2.5 Cytotoxicity index (CI) and inhibition dose (ID_{50}) ($\mu\text{g mL}^{-1}$) for various cancer cell lines exposed to physalin B and F confirmed by DEA assay	13
2.6 Properties of <i>in vitro</i> cultures	21
2.7 The strategies for achieving the release of secondary metabolites in the culture medium of plant cells.	32
3.1 Plant source materials in different stages of developmental growth were collected from vegetable farm of Universiti Putra Malaysia, Serdang, Malaysia.	39
3.2 Individual physalin contents in different aerial parts of <i>P. minima</i> at different stages of the growth developmental.	45
3. Accumulation of physalin B and F in different parts of <i>P. minima</i> plant collected from different locations.	49
4.1 Effect of 2,4-D and kinetin on callus induction in different explants of <i>P. minima</i>	67
4. Growth and physalin productions of <i>P. minima</i> callus cultured in different sucrose concentrations.	68
4.3 Growth and physalin productions of <i>P. minima</i> callus cultured in the same carbon ration of sucrose and its monomers.	68

4.4	Growth and physalins production of callus cultures derived from leaf in different auxins and kinetin combinations.	69
5.1	Growth and physalin productions of <i>P. minima</i> cell suspensions cultured in different initial sucrose concentrations.	91
5.2	Growth and physalin productions of <i>P. minima</i> cell suspensions cultures in the same carbon ratio of sucrose and its monomers.	91
6.1	Transformation efficiency of different explants of <i>P. minima</i> using different techniques of <i>A. rhizogenes</i> strain LBA9402 infection.	119
6.2	Efficiency of hairy roots formation of <i>P. minima</i> depending upon co-cultivation time for <i>A. rhizogenes</i> strain LBA9402.	120
6.3	Effects of different antibiotics on efficiency of removing the <i>A. rhizogenes</i> from <i>P. minima</i> hairy root cultures.	120
7.1	Effects of different inoculum morphologies on growth characteristics of <i>P. minima</i> hairy root cultures.	142
7.2	Effects of medium volume and inoculum size on growth characteristics of <i>P. minima</i> hairy root cultures.	142
8.1	Growth and physalin productions of <i>P. minima</i> hairy roots cultured in different initial sucrose concentration.	171
8.2	Growth and physalin productions of <i>P. minima</i> hairy roots cultured in same carbon ration of sucrose and its monomer.	171

LIST OF FIGURES

Figure	Page
2.1 Chemical structures of physalins belong to <i>Physalis sp.</i>	8
2.2 Possible metabolic function of amino acids catabolism to enter the MVA-shunt and MVA pathway for terpenoids, steroids and sterol biosynthesis.	35
3.1 Physalins spectra at 220 nm analysed by isocratic-reverse phase HPLC technique in the mixtures of MeOH-H ₂ O (65:35, v/v) solvent systems.	43
4.1 Growth and physalins production of <i>P. minima</i> callus cultures.	70
4.2 Growth and physalins productions of <i>P. minima</i> callus cultured in different	71
4.3 Growth and physalins production of <i>P. minima</i> callus cultured in different MS nutrient strength.	72
4.4 Growth and physalins production of <i>P. minima</i> callus cultured in different carbon sources.	73
4.5 Effect of different PGRs concentrations on growth and physalins production of <i>P. minima</i> callus cultures.	74
5.1 Effect of 2,4-D and kinetin on initiation of <i>P. minima</i> cell suspension cultures.	92
5.2 Growth and physalins production of <i>P. minima</i> cell suspension cultures.	93
5.3 Growth and physalins productions of <i>P. minima</i> cell suspension cultured in different basal medium.	94
5.4 Growth and physalins production of <i>P. minima</i> cell suspension cultured in different	95
5.5 Growth and physalins production of <i>P. minima</i> cell suspension cultured in different	96
5.6 Effect <i>minima</i> cell suspension cultures.	97

5.7	Released product of physalin B and F by <i>P. minima</i> cell suspension cultured with function of culture time.	98
5.8	Released product of physalin B and F by <i>P. minima</i> cell suspension cultured in different basal media.	98
5.9	Effect of different carbon sources on released product of physalin B and F by <i>P. minima</i> cell suspension cultures.	99
5.10	Effect of different PGRs on released product of physalin B and F into culture medium by <i>P. minima</i> cell suspension cultures.	99
6.1	Diagram of pBI121 (kanamycin resistance) and GUS gene with CaMV 35S promoter.	120
6.2	Toxicity level of different antibiotics on <i>P. minima</i> hairy root cultures	121
6.3	Effect of kanamycin on growth of <i>P. minima</i> hairy and normal root cultures.	121
7.1	Different inoculum morphologies of <i>Physalis minima</i> hairy root cultures.	143
7.2	Effect of different inoculum morphologies on growth performance and physalins production of <i>P. minima</i> hairy root cultures.	144
7.3	Localisation and accumulation levels of physalin B and F in <i>P. minima</i> hairy roots after 15 d in shake flask cultures.	145
7.4	Effect of inoculum size on (a) growth performance (total root length, number of lateral root (LR) and length of LR), (b) root growth units, and (c) biomass DW and physalins productions of <i>P. minima</i> hairy root cultures.	146
7.5	Effect of initial medium volume on (a) growth and (b) physalin production in <i>P. minima</i> hairy root cultures.	147
8.1	Growth and physalins production of <i>P. minima</i> hairy roots (HR) and non-transformed roots (NT) cultures grown under dark and light conditions.	172
8.2	Growth and physalins production of <i>P. minima</i> hairy root cultures in different basal media.	173

8.3	Growth and physalins production of <i>P. minima</i> hairy root cultures in different nutrients strength of B5 basal medium.	173
8.4	Growth and physalins production of <i>P. minima</i> hairy root cultures in different carbon sources.	174
8.5	Changing of pH profile in medium culture with respect to growth and physalins production of <i>P. minima</i> hairy root cultures.	175
8.6	Growth and physalins production of <i>P. minima</i> hairy root cultures by addition of alanine, leucine and valine.	176
8.7	Effect of exogenous PGRs on growth and physalins production of <i>P. minima</i> hairy root cultures.	177
8.8	Released product of physalin B and by <i>P. minima</i> hairy root cultures with function of culture time.	178
8.9	Effect of different carbon sources on released product of physalin B and F by <i>P. minima</i> hairy root cultures.	178
8.10	Effect of various initial pH mediums on released product of physalin B and F by <i>P. minima</i> hairy root culture.	179
8.11	Effect of exogenous PGRs on released product of physalin B and F into culture medium by <i>P. minima</i> hairy root cultures.	179
8.12	Effect of amino acids on released product of physalin B and F into culture medium by <i>P. minima</i> cell suspension cultures.	180

LIST OF PLATES

Plate		Page
1.1	<i>P. minima</i> intact plant.	3
3.1	TLC spot profile of physalins on silica gel 60 F ₂₅₄ , plate and developed in benzene-ethyl acetate (3:7 v/v) eluents after sprayed with 50% (w/v) H ₂ SO ₄ .	42
4.1	Callus cultures of <i>P. minima</i> derived from (a) leaf, (b) stem and (c) root explants induced in MS basal medium supplemented with B5 vitamins, 3% (w/v) sucrose, 0.25% (w/v) Gelrite and PGRs (2,4-D: kinetin; 9.0:4.5 μ M).	75
5.1	(a) Cell suspension culture (1) on day 0, and (2) on day 17 commonly observed for all tested explants. (b) Micrograph of aggregate cells cultured in MS medium supplemented with 3% (w/v) sucrose and 18.0 μ M 2,4-D (400x magnification).	100
6.1	Different inoculation methods used to initiate <i>P. minima</i> hairy root cultures using <i>A. rhizogenes</i> strain LBA9402.	122
6.2	Establishment of <i>P. minima</i> hairy root cultures.	123
6.3	Histochemical GUS assay of <i>P. minima</i> hairy and normal root cultures.	124
6.4	Southern Blot analyses.	125

LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
IAA	1 mg L ⁻¹ indole-3-acetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-benzylaminopurine
CHCl ₃	Chloroform
cm	Centimetre
CoA	Co-enzyme A
conc.	Concentration
ctrl	Control
cul.	Culture
d	Day
dH ₂ O	Distilled water
Dicamba or Dic	3,6-Dichloro-o-anisic acid
DMAPP	3,3-Dimethylallyl pyrophosphate
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid (ferric sodium salt)
e.g.	Example
EtOH	Ethanol
fruc	Fructose
FW	Fresh weight
FPP	Farnesyl pyrophosphate
g	Gram
gluc	Glucose
GPP	Geranyl pyrophosphate
h	Hours
H ₂ SO ₄	Sulphuric acid
HMG-CoA	3-Hydroxy-3-methylglutaryl-Coenzyme A
HR	Hairy root
i.e.	That is
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IPP	Isopentenyl pyrophosphate
αKIA	α-Kitoisocaproate
Kinetin or Kin	6-furfurylaminopurine
L	Litre
LR	Lateral branches
MC-CoA	3-methylcrotonyl-coenzyme A
MCCase	3-methylcrotonyl-coenzyme A carboxylase
MeOH	Methanol
mg	Milligram

MgCl ₂	Magnesium chloride
MG-CoA	3-Methylglutaryl-CoA
min	Minute
mL	Millilitre
MVA	Mevalonic acid
Na ₂ EDTA	EDTA disodium salt
Na ₂ EDTA-2H ₂ O	Na ₂ EDTA dihydrate
NAA	α-Naphthaleneacetic acid
NaOH	Sodium hydroxide
NBT	Nitro tetrazolium blue
nd	Not determine
NT	Normal root or non-transformed root
O ₂	Oxygen
ORF(s)	Open reading frame(s)
PGR(s)	Plant growth regulator(s)
Picloram or Pic	4-Amino-3,5,6-trichloropicolinic acid
R _f	Distance of the substance over distance of the solvent movement
<i>rol</i>	Rooting locus
rpm	Revolution per minute
R _T	Retention time (min)
SDS	Sodium dodecyl sulphate
suc	Sucrose
<i>t_d</i>	Doubling time (d)
v/v	Volume for volume
w/v	Weight for volume
<i>N</i>	Normality
μ	Specific growth rate (d)
μg	Microgram
μM	Micromolar

CHAPTER 1

INTRODUCTION

Plants are widely known as superb synthesisers of 'natural products'. These compounds, also called as 'secondary plant products', which are low molecular weight and often restricted to special plant families or even genera. They are not important for the primary metabolism of the plant, but in many cases of great importance for the plants to survive in its environment (Farnsworth, 1985; Alfermann and Petersen, 1995). Plant secondary products are used extensively in commerce and trade especially between countries, particularly in the food additives, nutraceutical and pharmaceutical industries. The use of plant-based medicines either as natural drugs or herbal remedies varies greatly among countries. Recent surveys estimate that over 80% of the population in parts of the developing world still rely on plant-derived medicines for their primary health cares and food supplements (Simmonds and Grayer, 1999). Meanwhile, total world trade of medicinal plants in 1980 only, was in excess of US\$ 551 million (Phillipson, 1990). The trade in plants used within Europe for non-conventional medicines is increasing by 15-20% a year, with an import value of US\$3.6 billion in 1995 (Simmonds and Grayer, 1999). Thus, plants are still as the main immediate source of medicine available to the majority of people in the world. It has been estimated that 20-30% of the world's flora of 250,000-500,000 species have been subjected to phytochemical and pharmacological investigations (Simmonds and Grayer, 1999). Herbal and medicinal plants have still to be collected from the wild and

some sources are locally planted or cultivated (e.g. garlic, ginger, and ginseng) (Phillipson, 1990).

The majority of plant natural products used medicinally are terpenoids (mono-, sesqui-, di-, tri-, steroids, cardenolides), quinones, ligans, flavonoids, alkaloids and saponins. Some of these compounds cannot be synthesised in laboratories. Compounds that possess interesting bio-pharmacological or other biological properties and consumed in large quantities required more detailed investigations. Parent plant materials are not always available and some are endangered due to severe over collection. Therefore, plant tissue culture techniques offer an alternative source for the production of such compounds. *In vitro* cultures lead to the possibility of harvesting the desired natural products everywhere in the world without contamination of pesticides, herbicides or insecticides, and also to overcome the natural heterogeneity in plant material and variations in product content (Taticek *et al.*, 1991). There have been a number of reports on using plant tissue and organ cultures to produce a wide range of secondary compounds (Zenk, 1977; Staba, 1980; Rhodes *et al.*, 1990,1997). However, plant callus, cell suspension and hairy root cultures are the common plant tissue culture systems that have been adopted by many researchers as compared to other cell or organ cultures (Su, 1995; Hamill and Lidgett, 1997). Additionally, those plant culture systems (cell suspension and hairy root cultures) can potentially grow in the bioreactor, which is much quicker than from plants grown in the field (Alfermann and Petersen, 1995).