

**TRITERPENE PRODUCTION IN
CENTELLA ASIATICA (L.) URBAN (PEGAGA)
CALLUS AND CELL SUSPENSION CULTURES**

By

ANNA LING PICK KIONG

**Thesis Submitted to the School of Graduate Studies,
Universiti Putra Malaysia, in Fulfilment of the Requirements for the
Degree of Doctor of Philosophy**

January 2004

Especially dedicated to:

My parents: Joseph Ling and Teresa Lau

Brothers and Sisters: Martin, Peter, Catherine,
Angela, Cecilia & Paul

Nieces and Nephews: Teresa, Anna, Grace, James,
John, Emi, Henry, Stephen, Austin and Justin

To all the **Fathers** and **Sisters, my love ones** and all
those who has sacrificed and supported me throughout
my studies

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

TRITERPENE PRODUCTION IN *CENTELLA ASIATICA* (L.) URBAN (PEGAGA) CALLUS AND CELL SUSPENSION CULTURES

By

ANNA LING PICK KIONG

January 2004

Chairman: Professor Maziah Mahmood, Ph.D.

Faculty: Science and Environmental Studies

Centella asiatica or locally known as ‘Pegaga’ is one of the most common medicinal plants used by diverse ancient cultures and tribal groups. Its medicinal values are mainly attributed to the presence of the triterpene constituents. As there is still no information available on the triterpene production in cultured tissues, studies were carried out in determining the triterpene distribution particularly asiatic acid, madecassic acid, asiaticoside and madecassoside in intact plants of the twelve accessions of *C. asiatica* collected throughout Malaysia as well as in the callus and cell suspension cultures.

Results obtained from the studies revealed that twelve accessions of *C. asiatica* differed both in their morphologies and their triterpene contents. The triterpenes constituents were detected at a range of 0.134 to 1.655 mg/g dry weight in the whole plant intact tissues. Triterpenes were also successfully detected in the callus (0.014 to 0.773 mg/g dry weight) and cell suspension cultures (0.005 to 0.084 mg/g dry weight), the amount that were lower than that produced in the intact

tissues. However, manipulating the physical culture conditions, feeding of precursor, elicitation as well as amino acid addition managed to increase the triterpenes content in cultured tissues. Studies on the effects of the medium composition show that full strength of the basal Murashige and Skoog medium supplemented with B5 vitamins and sucrose (3-4%) increased the triterpenes content in both callus and cell suspension cultures. An interaction of auxin-cytokinin has observed being important for both callus and cell suspension cultures in enhancing triterpenes production. Higher triterpenes content was obtained in callus treated with 2,4-D and kinetin while the combination of kinetin and dicamba enhanced the triterpenes production in cell suspension cultures. The precursor-feeding studies revealed that lower concentrations of squalene (0.16 mg/L in callus and 0.8 mg/L in cells) were preferred for triterpenes production. Squalene at 0.16 mg/L had successfully triggered the production of madecassoside, asiaticoside and madecassic acid in callus cultures while asiatic acid and madecassic acid content was increased in cells treated with 0.8 mg/L squalene. The elicitor studies exhibited that the different elicitors showed distinctive effects on triterpenes production. Nevertheless, supplementation of succinic acid at 3 and 4 mg/L was found the best in increasing the triterpenes production in callus and cell suspension cultures, respectively. Addition of amino acid into the culture media was also found to promote the triterpenes production in *in vitro* cultures. The study further concluded that the combinations of the optimized factors namely medium composition, precursor feeding, elicitation and amino acid addition is a very useful strategy in enhancing the triterpenes

production particularly the asiatic acid and madecassic acid in *in vitro* cultures of *C. asiatica*.

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

**PENGHASILAN TRITERPENA DALAM KULTUR KALUS DAN SEL
AMPAIAN *CENTELLA ASIATICA* (L.) URBAN (PEGAGA)**

Oleh

ANNA LING PICK KIONG

Januari 2004

Pengerusi : Profesor Maziah Mahmood, Ph.D.

Fakulti : Sains dan Pengajian Alam Sekitar

Centella asiatica atau dikenali sebagai pegaga oleh masyarakat tempatan adalah antara tumbuhan ubatan yang biasa digunakan oleh pelbagai suku kaum. Nilai perubatannya adalah disebabkan oleh kehadiran unsur-unsur triterpena. Memandangkan masih tidak terdapat informasi tentang penghasilan triterpena dalam tisu yang dikulturkan, kajian ini dijalankan untuk mengenalpasti taburan triterpena terutamanya asid asiatik, asid medikasik, asiatikosida dan medikasosida dalam 12 aksesori pokok induk *C. asiatica* yang telah dikumpul dari seluruh Malaysia serta dalam kultur kalus dan sel ampaiian.

Keputusan yang diperolehi dalam kajian ini mendedahkan bahawa 12 aksesori *C. asiatica* adalah berbeza dari segi morfologi dan kandungan triterpena. Unsur triterpena telah dikesan pada julat di antara 0.134 ke 1.655 mg/g berat kering dalam keseluruhan pokok induk. Triterpena juga berjaya telah dikesan di dalam kultur kalus (0.014 ke 0.773 mg/g berat kering) dan sel ampaiian (0.005 ke 0.084 mg/g berat kering) di mana kuantiti ini adalah lebih rendah daripada pokok induk.

Namun begitu, dengan memanipulasikan keadaan pengkulturan fizikal, pembekalan prekursor, penggunaan elisitor serta penambahan asid amino telah berupaya meningkatkan kandungan triterpena dalam kultur tisu. Kajian tentang kesan komposisi media menunjukkan media basal Murashige dan Skoog yang telah dibekalkan dengan vitamin B5 dan sukrosa (3-4%) dapat meningkatkan kandungan triterpena dalam kedua-dua kultur kalus dan sel ampaiian. Interaksi auksin-sitokinin diperhatikan amat penting dalam meninggikan penghasilan triterpena dalam kedua-dua jenis kultur. Kandungan triterpena yang lebih tinggi diperolehi dalam kalus yang telah dirawat dengan 2,4-D dan kinetin manakala kombinasi kinetin dan dicamba membawa kepada penghasilan triterpena yang lebih tinggi dalam kultur sel ampaiian. Kajian pembekalan prekursor mendedahkan kepekatan skualen yang rendah (0.16 mg/L dalam kalus dan 0.8 mg/L dalam sel) cenderung dalam penghasilan triterpena. Skualen pada 0.16 mg/L berjaya mengaruh penghasilan medikasosida, asiatikosida dan asid mekasik dalam kultur kalus manakala kandungan asid asiatic dipertingkatkan dalam sel yang dirawat dengan 0.8 mg/L skualen. Kajian elisitor menunjukkan elisitor yang berbeza memberikan kesan yang jelas dalam penghasilan triterpena. Namun begitu, pembekalan asid suksinik masing-masing pada kepekatan 3 dan 4 mg/L adalah terbaik dalam meningkatkan penghasilan triterpena dalam kultur kalus dan sel ampaiian. Penambahan asid amino ke dalam media pengkulturan juga didapati menggalakkan penghasilan triterpena dalam kultur *in vitro*. Kajian ini seterusnya menyimpulkan bahawa kombinasi faktor optimum iaitu komposisi media, pembekalan prekursor, penggunaan elisitor dan penambahan asid amino

merupakan strategi yang amat berguna dalam meninggikan penghasilan triterpena terutama asid asiatik dan asis medikasik dalam kultur *in vitro* *C. asiatica*.

ACKNOWLEDGMENTS

Praise and thank the Almighty God for His willingness that made the completion of this study possible.

Thank the good opportunity that was granted by Prof. Dr. Maziah Mahmood for studying at the Unviersiti Putra Malaysia. The guidance, advice, suggestions and assistance of Prof. Dr. Marziah as well as Associate Prof. Dr. Siti Khalijah and Dr. Nor'aini Mohd Fadzillah, who served as the committee members had enabled me to come out with this thesis.

I would like to thank all the members of my family, dad, mum, Martin and family, Peter and family, Cat, Angela and family, Cecilia and family and Paul for their support both morally and financially, encouragement and sacrifices throughout the course of my stay away from home.

The appreciation and gratitude are also extended to the Malaysia government and Universiti Putra Malaysia for supporting the MMBPP project as well as offering me the Graduate Research Assistantship.

Special thanks should also go to my brother, Associate Professor Dr. Hoe I. Ling at Columbia University, NY, USA, and my sister, Catherine, for reading the draft and offered numerous suggestions leading to the improvement of this thesis. This

thesis could not have been accomplished without my brother, who has always been very generous in letting me access to the publications available at Columbia University besides spending his valuable time in getting all the related publications.

The visit of the scientists from Massachusetts Institute Technology, USA to Malaysia during the workshops and symposia, with their ideas, led to an improvement of this research work. Beside the work included in this thesis, I have a chance to gain better understanding in other fields of the biotechnology. Thanks!

I cannot leave this page without expressing my appreciation to Madam Indu Bala, Mr. Thiyagu and Vellu from MARDI for being so kind and helpful in providing me with the experimental plants as well as some useful information.

Lastly, thanks to all the postgraduate students, the research assistants in the Plant Biotechnology Laboratory and my friends, Dato', Sook Mun, Norhayati and Rozita for their cooperation, patience and support. Not forgetting, to ATAN, thanks for being my source of inspiration and I am grateful to all the care, encouragement and support.

I certify that an Examination Committee met on 19th January 2004 to conduct the final examination of Anna Ling Pick Kiong on her Doctor of Philosophy thesis entitled 'Triterpene production in *Centella asiatica* (L.) Urban (Pegaga) callus and cell suspension cultures' in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Dr. Mohd Arif Syed, Ph.D.

Associate Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Chairman)

Dr. Radzali Muse, Ph.D.

Associate Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

Dr. Mohd Puad Abdullah, Ph.D.

Lecturer
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

Normah Mohd Noor, Ph.D.

Professor
School of Bioscience and Biotechnology
Faculty of Science and Technology
Universiti Kebangsaan Malaysia,
(Independent Examiner)

GULAM RUSUL RAHMAT ALI, Ph.D.

Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis submitted to the Senate of University Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

Maziah Mahmood, Ph.D.

Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Chairman)

Siti Khalijah Daud, Ph.D.

Associate Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

Nor'aini Mohd Fadzillah, Ph.D.

Associate Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

AINI IDERIS, Ph.D.

Professor
School of Graduate Studies
Universiti Putra Malaysia

Date :

DECLARATIAON

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

ANNA LING PICK KIONG

Date:

TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vi
ACKNOWLEDGEMENTS	ix
APPROVAL	xi
DECLARATION	xiii
LIST OF TABLES	xviii
LIST OF FIGURES	xx
LIST OF PLATES	xlii
LIST OF ABBREVIATIONS	xliii
CHAPTER	
1 INTRODUCTION	1
1.1 Herbal Industry in Malaysia	2
1.2 Secondary Metabolites Production in Plant Cell Cultures	4
1.3 Plant Materials	7
1.4 Objectives of Research	11
2 LITERATURE REVIEW	12
2.1 <i>Centella asiatica</i> (L.) Urban	12
2.1.1 Morphological Description	13
2.1.2 Chemical Constituent	15
2.1.3 Therapeutic Applications	19
2.1.4 Cosmetic application	22
2.1.5 Other Applications	22
2.2 Triterpenes	23
2.2.1 Triterpenes in <i>Centella asiatica</i>	24
2.2.2 Pharmacological application of triterpenes	26
2.2.3 Triterpenes biosynthesis pathway	27
2.3 Plant cell cultures	31
2.3.1 Callus cultures	31
2.3.2 Cell suspension cultures	33
2.4 Plant Cell Cultures as A Source of Secondary Metabolite	35
2.5 Variations of Secondary Metabolite Profiles Between <i>In Vitro</i> Cultures and Whole Plant	38
2.5.1 Lack of differentiation and organization	41
2.5.2 Cell culture-induced variation	41

2.6	Yield improvement strategies	43
2.6.1	Screening and selection of highly productive cell lines	43
2.6.2	Medium manipulations	45
2.6.3	Precursors feeding	55
2.6.4	Elicitation	57
2.6.5	Amino acids addition	60
3	DIFFERENTIATION OF TWELVE ACCESSIONS OF <i>CENTELLA ASIATICA</i> BY MORPHOLOGICAL CHARACTERISTICS AND BIOCHEMICAL PROFILES	61
3.1	Introduction	61
3.2	Materials and Methods	62
3.2.1	Plant materials	62
3.2.2	Identification of morphological characteristics	64
3.2.3	Determination of triterpenes content	65
3.2.4	Total Soluble Protein	68
3.2.5	Chlorophyll content	69
3.2.5	Statistical analysis	69
3.3	Results and Discussions	70
3.3.1	Identification of morphological characteristics	70
3.3.2	Determination of triterpenes content	75
3.3.3	Total soluble protein content	90
3.3.4	Chlorophyll content	92
3.3.5	Dendogram	94
3.4	Conclusions	96
4	CALLUS INDUCTION, ESTABLISHMENT OF CELL SUSPENSION CULTURES AND TRITERPENES DISTRIBUTION IN <i>IN VITRO</i> CULTURES	98
4.1	Introduction	98
4.2	Materials and methods	99
4.2.1	Plant materials	99
4.2.2	Initiation of callus	99
4.2.3	Initiation of treatments	101
4.2.4	Growth curve of the callus culture	102
4.2.5	Establishment of cell suspension culture	102
4.2.6	<i>In vitro</i> shoot culture	104
4.2.7	Analysis of triterpenes content	104
4.2.8	Statistical analysis	104
4.3	Results and Discussions	105
4.3.1	Callus induction	105
4.3.2	Growth curve of callus culture	114
4.3.3	Growth and triterpenes content in leaf derived callus of twelve accessions	117

	4.3.4	Triterpenes distribution in callus derived from different explants of accession CA01	121
	4.3.5	Triterpenes production profile in callus culture	123
	4.3.6	Establishment of cell suspension cultures	126
	4.3.7	Growth curve of cell suspension culture	128
	4.3.8	Triterpenes content in cell suspension culture	131
	4.3.9	Comparison between triterpenes content in intact plant and <i>in vitro</i> cultures of <i>C. asiatica</i> accession CA01	133
	4.4	Conclusions	136
5		EFFECTS OF DIFFERENT CULTURE CONDITIONS ON THE BIOMASS AND TRITERPENES PRODUCTION IN <i>IN VITRO</i> CULTURES	138
	5.1	Introduction	138
	5.2	Materials and Methods	139
	5.2.1	Initiation of treatments	139
	5.2.2	Culture conditions studied	140
	5.2.3	Extraction and HPLC analysis	142
	5.3	Results and Discussions	143
	5.3.1	Callus cultures	143
	5.3.2	Cell suspension cultures	177
	5.4	Conclusions	207
6		EFFECTS OF TRITERPENES PRECURSORS FEEDING ON THE BIOMASS AND TRITERPENES PRODUCTION IN <i>IN VITRO</i> CULTURES	209
	6.1	Introduction	209
	6.2	Materials and Methods	210
	6.2.1	<i>In vitro</i> cultures and culture conditions	210
	6.2.2	Preparation of triterpenes precursors	211
	6.2.3	Extraction and analysis	212
	6.3	Results and Discussions	212
	6.3.1	Callus culture	212
	6.3.2	Cell suspension culture	227
	6.4	Conclusions	241
7		EFFECTS OF DIFFERENT ELICITORS ON THE BIOMASS AND TRITERPENES PRODUCTION IN <i>IN VITRO</i> CULTURES	242
	7.1	Introduction	242
	7.2	Materials and Methods	243
	7.2.1	<i>In vitro</i> cultures and culture conditions	243
	7.2.2	Preparation of elicitors	243
	7.2.3	Extraction and analysis	245

	7.3 Results and Discussions	245
	7.3.1 Callus cultures	245
	7.3.2 Cell suspension cultures	271
	7.4 Conclusions	297
8	EFFECTS OF DIFFERENT AMINO ACIDS ON THE BIOMASS AND TRITERPENES PRODUCTION IN <i>IN VITRO</i> CULTURES	299
	8.1 Introduction	299
	8.2 Materials and Methods	300
	8.2.1 <i>In vitro</i> cultures and culture conditions	300
	8.2.2 Preparation of amino acids	301
	8.2.3 Extraction and analysis	301
	8.3 Results and Discussions	302
	8.3.1 Callus culture	302
	8.3.2 Cell suspension culture	314
	8.4 Conclusions	324
9	EFFECTS OF OPTIMIZED CULTURE CONDITIONS, PRECURSOR, ELICITORS AND AMINO ACID IN COMBINATION ON THE TRITERPENES PRODUCTION IN <i>IN VITRO</i> CULTURES	326
	9.1 Introduction	326
	9.2 Materials and Methods	327
	9.2.1 <i>In vitro</i> cultures and culture conditions	327
	9.2.2 Preparation of precursor, elicitor and amino acid	328
	9.2.3 Extraction and analysis	328
	9.3 Results and Discussions	328
	9.3.1 Callus cultures	328
	9.3.2 Cell suspension cultures	335
	9.4 Conclusions	341
10	SUMMARY, GENERAL DISCUSSIONS AND CONCLUSION	343
	REFERENCES	351
	APPENDICES	383
	BIODATA OF THE AUTHOR	389