

ACB - 6856  
INVC.....

CHEMICAL AND BIOCHEMICAL EVALUATION OF  
SELECTED *CITRUS* SPECIES  
INCLUDING SOME TISSUE CULTURE STUDIES

**CLOSED STACKS**

BY  
**NOR SA'ADAH BTE MOHD. NASOHAH**  
DEPARTMENT OF BOTANY  
FACULTY OF SCIENCE  
UNIVERSITY OF MALAYA

PERPUSTAKAAN UNIVERSITI MALAYA

DISSERTATION PRESENTED FOR THE  
DEGREE OF MASTER OF SCIENCE  
UNIVERSITY OF MALAYA  
KUALA LUMPUR  
(1996)

Perpustakaan Universiti Malaya



A506426264

Dimikrofikan pada ..... 15 . 03 . 2000 ..  
No. Mikrofis ..... 14538  
Jumlah Mikrofis ..... 3

HAMSIAH BT. MOHAMAD ZAHARI  
UPR UNIT REPROGRAFI  
PERPUSTAKAAN UTAMA  
UNIVERSITI MALAYA

## CONTENTS

	PAGE
ACKNOWLEDGEMENT .....	i
ABSTRACT .....	ii
ABSTRAK .....	iii
LIST OF TABLES .....	iv
LIST OF FIGURES .....	vi
LIST OF PLATES .....	vii
ABBREVIATIONS .....	viii
CHAPTER 1 : INTRODUCTION .....	1
1.1 : The genus <i>Citrus</i> .....	1
1.2 : <i>Citrus</i> in Malaysia .....	5
1.3 : Economic importance of <i>Citrus</i> .....	8
1.4 : <i>Citrus</i> essential oil .....	9
1.5 : Isoenzymes of <i>Citrus</i> .....	24
1.6 : Browning of young shoot homogenates of <i>Citrus</i> .....	26
1.7 : Tissue culture in <i>Citrus</i> .....	28
1.8 : Species description .....	37
1.9 : Objectives of present study .....	45
CHAPTER 2 : MATERIALS AND METHODS .....	46
2.1 : Plant materials .....	46
2.1.1 : Essential oils analysis .....	46

2.1.2	: Isoenzymes analysis .....	46
2.1.3	: Analysis of browning of young shoot extracts ....	47
2.1.4	: Tissue culture experiments .....	47
2.2	: Methods .....	48
2.2.1	: Extraction of the essential oils .....	48
2.2.1.1	: Preparation of essential oil extracts .....	48
2.2.1.2	: Identification of the essential oil components .....	48
	by GCMS	
2.2.1.3	: Infrared spectrometry .....	49
2.2.2	: Analysis of leaf isoenzymes .....	49
2.2.2.1	: Preparation of extracts .....	49
2.2.2.2	: Preparation of gel and electrophoresis .....	50
2.2.2.3	: Isoenzyme stainings .....	50
2.2.3	: Experiment on browning of young shoot extracts	51
2.2.3.1	: Preparation of shoot extracts .....	51
2.2.3.2	: Test on inhibition of browning .....	51
2.2.4	: Experiment on tissue culture of <i>Citrus</i> .....	52
2.2.4.1	: Preparation of explants .....	52
2.2.4.2	: Culture media .....	52
2.2.4.3	: Culture conditions .....	53
 CHAPTER 3	: RESULTS .....	54
3.1	: Essential oils extracted from leaves of .....	54
	selected <i>Citrus</i> species	
3.2	: Analysis of essential oil components by GCMS ....	55
3.3	: Isoenzymes analysis in leaves of selected .....	93
	<i>Citrus</i> species	
3.3.1	: Glutamate oxaloacetate transaminase .....	93
3.3.2	: Peroxidase .....	94

3.3.3	: Esterase .....	94
3.4	: Analysis of browning of young shoot extracts .....	101
	of selected <i>Citrus</i> species	
3.5	: Morphogenetic responses of explants of <i>C. hystrix</i> ..	109
3.5.1	: Leaf explants .....	109
3.5.2	: Stem explants .....	109
3.5.3	: Cotyledon explants .....	110
3.6	: Morphogenetic responses of explants .....	113
	of <i>C. madurensis</i>	
3.6.1	: Leaf explants .....	113
3.6.2	: Stem explants .....	114
3.6.3	: Cotyledon explants .....	114
3.7	: Morphogenetic responses of explants of .....	117
	<i>C. micrantha</i> var. <i>microcarpa</i>	
3.7.1	: Leaf explants .....	117
3.7.2	: Stem explants .....	117
3.7.3	: Cotyledon explants .....	118
3.8	: Morphogenetic responses of explants of .....	121
	citrumello ( <i>Citrus paradisi</i> X <i>Poncirus trifoliata</i> )	
3.8.1	: Leaf explants .....	121
3.8.2	: Stem explants .....	121
3.8.3	: Cotyledon explants .....	122
<b>CHAPTER 4</b>	<b>: DISCUSSIONS .....</b>	<b>129</b>
4.1	: Essential oils of selected <i>Citrus</i> species .....	129
4.2	: Determination of taxonomic relationships .....	132
	between selected <i>Citrus</i> species using isoenzymes	
4.3	: Classification of <i>Citrus</i> taxa based on the presence ..	134
	or absence of browning in young shoot extracts	

4.4	: Tissue culture studies in selected <i>Citrus</i> species ....	137
CHAPTER 5	: CONCLUSION .....	144
REFERENCES	.....	145
APPENDIX 1	.....	165
APPENDIX 2	.....	171
APPENDIX 3	.....	172
APPENDIX 4	.....	173

## **ACKNOWLEDGEMENT**

*I would like to express my sincere gratitude to my supervisors Prof. Madya Muhamad Zakaria and Dr. Nur Zulaani Khalid for their useful guidance and advice throughout completing this thesis.*

*Special thanks to Prof. Madya Dr. Halijah Ibrahim and Prof. Madya Dr. Tung Heng Fong for their advice in the isoenzyme work.*

*I would also like to thank Mr. Michael Arokiam for helping me with the photograph of isoenzyme.*

*I wish to express my thanks to all my friends who have helped me during the course of my study. To those whom I failed to mention but have helped me in one way or another may Allah blessed them.*

*Last but not least, a special appreciation to my beloved family for their constant encouragement during my university years.*

## **ABSTRACT**

Chemical and biochemical evaluation to determine the relationships between species were carried out. The species studied were *Citrus halimii*, *C. hystrix*, *C. madurensis*, *C. micrantha* var. *microcarpa* and citrumello.

The chemical characteristic used in this study was the component of essential oil extracted from the leaves. The biochemical characteristics were the banding patterns from three isoenzyme systems (glutamate oxaloacetate transaminase, peroxidase and esterase) and the occurrence or absence of enzymatic browning in young shoot extracts. However all the characteristics described above did not show a clear relationship between all the species.

Tissue culture study was carried out to determine the best explant and regeneration medium for propagation. It was found that cotyledon was the best explant for *C. hystrix* and *C. micrantha* var. *microcarpa*. MS medium supplemented with 4 mg/l 6-BAP and 2 mg/l IAA was the best medium for *C. hystrix*, whereas for *C. micrantha* var. *microcarpa* the best medium was the medium incorporated with 5 mg/l 6-BAP and 1 mg/l NAA. The best regeneration medium for citrumello was the medium supplemented with combination of 4 mg/l 6-BAP, 2 mg/l IAA and 1 mg/l GA<sub>3</sub>, whilst the best explant was stem. *C. madurensis* was not very responsive in shoot regeneration.

## **ABSTRAK**

Penilaian secara kimia dan biokimia telah dijalankan untuk menentukan perkaitan di antara spesies. Spesies-spesies yang telah dikaji ialah *Citrus halimii*, *C. hystrix*, *C. madurensis*, *C. micrantha* var. *microcarpa* dan citrumello.

Komponen minyak pati yang diekstrak dari daun adalah ciri kimia yang dikaji. Ciri-ciri biokimia pula ialah corak-corak jaluran dari tiga sistem isoenzim (glutamat oksaloasetat transaminase, peroksidase dan esterase) dan kehadiran atau ketidakhadiran warna coklat yang disebabkan oleh enzim pada ekstrak pucuk yang muda. Bagaimanapun berdasarkan kepada ciri-ciri yang di terangkan di atas tiada perkaitan yang jelas ditunjukkan di antara kesemua spesies.

Kajian kultur tisu telah dijalankan untuk menentukan eksplan dan medium regenerasi yang terbaik untuk propagasi. Kotiledon merupakan eksplan yang terbaik untuk *C. hystrix* dan *C. micrantha* var. *microcarpa*. Penambahan medium MS dengan 4 mg/l 6-BAP dan 2 mg/l IAA menghasilkan medium yang terbaik untuk *C. hystrix*, manakala medium yang ditambahkan dengan 5 mg/l 6-BAP dan 1 mg/l NAA adalah medium yang terbaik untuk *C. micrantha* var. *microcarpa*. Medium yang terbaik untuk regenerasi bagi citrumello ialah medium yang mengandungi kombinasi 4 mg/l 6-BAP, 2 mg/l IAA dan 1 mg/l GA<sub>3</sub>. *C. madurensis* tidak begitu memberi respons untuk propagasi.

## LIST OF TABLES

	PAGE
1.1 : <i>Citrus</i> in Malaysia (Jones, 1984) .....	7
1.2 : Compounds identified in <i>Citrus</i> oils.....	13
1.3 : Summary of results obtained from tissue culture of .....	31
<i>Citrus</i> species	
3.1 : Characteristics of the essential oils obtained from leaves .....	54
of selected <i>Citrus</i> species	
3.2 : Essential oil composition of <i>Citrus halimii</i> .....	56
3.3 : Essential oil composition of <i>Citrus hystrix</i> .....	61
3.4 : Essential oil composition of <i>Citrus madurensis</i> .....	65
3.5 : Essential oil composition of <i>Citrus micrantha</i> var. <i>microcarpa</i> .....	69
3.6 : Essential oil composition of citrumello .....	74
3.7 : Compounds identified in essential leaf oils of five selected .....	80
<i>Citrus</i> species	
3.8 : Comparison of the essential oils composition of five .....	81
selected <i>Citrus</i> species	
3.9 : Isoenzyme systems studied in selected <i>Citrus</i> species .....	95
3.10 : Distribution of enzymatic browning of young shoot .....	103
homogenates in selected <i>Citrus</i> species	
3.11 : Extract colour of shoot homogenates of <i>Citrus halimii</i> in .....	104
various inhibitors after centrifugation	
3.12 : Extract colour of shoot homogenates of <i>Citrus hystrix</i> in various .....	105
inhibitors after centrifugation	
3.13 : Extract colour of shoot homogenates of <i>Citrus madurensis</i> in .....	106
various inhibitors after centrifugation	
3.14 : Extract colour of shoot homogenates of <i>Citrus micrantha</i> .....	107

## LIST OF FIGURES

	PAGE
1.1 : Classification of the genus <i>Citrus</i> (Swingle, 1967) .....	4
1.2 : Some of <i>Citrus</i> essential oil compounds .....	23
3.1 : Total ion chromatogram of <i>Citrus halimii</i> leaf oil .....	83
3.2 : Total ion chromatogram of <i>Citrus hystrix</i> leaf oil .....	84
3.3 : Total ion chromatogram of <i>Citrus madurensis</i> leaf oil .....	85
3.4 : Total ion chromatogram of <i>Citrus micrantha</i> var. <i>microcarpa</i> .....	86
leaf oil	
3.5 : Total ion chromatogram of citrumello leaf oil .....	87
3.6 : Infrared spectrum of <i>C. halimii</i> .....	88
3.7 : Infrared spectrum of <i>C. hystrix</i> .....	89
3.8 : Infrared spectrum of <i>C. madurensis</i> .....	90
3.9 : Infrared spectrum of <i>C. micrantha</i> var. <i>microcarpa</i> .....	91
3.10 : Infrared spectrum of citrumello .....	92
3.11 : Schematic zymogram of glutamate oxaloacetate transaminase .....	98
in <i>Citrus</i> studied	
3.12 : Schematic zymogram of peroxidase in <i>Citrus</i> studied .....	99
3.13 : Schematic zymogram of esterase in <i>Citrus</i> studied .....	100

## LIST OF PLATES

	PAGE
Plate 1 : <i>Citrus halimii</i> .....	38
Plate 2 : <i>Citrus hystrix</i> .....	39
Plate 3 : <i>Citrus madurensis</i> .....	41
Plate 4 : <i>Citrus micrantha</i> var. <i>microcarpa</i> .....	42
Plate 5 : Citrumello ( <i>C. paradisi</i> X <i>P. trifoliata</i> ) .....	44
Plate 6 : Zymogram of glutamate oxaloacetate transaminase .....	96
of <i>Citrus</i> species studied	
Plate 7 : Zymogram of peroxidase of <i>Citrus</i> species studied .....	96
Plate 8 : Zymogram of esterase of <i>Citrus</i> species studied .....	96
Plate 9a : Plant regeneration from cotyledon explant of citrumello .....	125
Plate 9b : Callus from leaf explant of <i>C. madurensis</i> .....	125
Plate 9c : Regenerants from cotyledon explant of <i>C. hystrix</i> .....	125
Plate 9d : Regenerants from leaf explant of <i>C. madurensis</i> .....	125
Plate 9e : Regenerants from cotyledon explant of <i>C. micrantha</i> .....	125
var. <i>microcarpa</i>	

## ABBREVIATIONS

6-BAP	6-benzylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
GCMS	Gas Chromatograph Mass Spectrometry
GA <sub>3</sub>	gibberelic acid
IAA	indole acetic acid
IR	Infrared spectra
MS	Murashige and Skoog (1962)
MT	Murashige and Tucker (1969)
MW	molecular weight
MF	molecular formula
NAA	1-naphthalene acetic acid
RT	retention time