UNIVERSITI PUTRA MALAYSIA

IMPACT OF CO2 ENRICHMENT AND ITS INTERACTION WITH NITROGEN ON GROWTH, PHYSIOLOGY AND SECONDARY METABOLITES OF *Labisia pumila* Benth

MOHD HAFIZ BIN IBRAHIM

FP 2012 6
IMPACT OF CO₂ ENRICHMENT AND ITS INTERACTION WITH NITROGEN ON GROWTH, PHYSIOLOGY AND SECONDARY METABOLITES OF *Labisia pumila* Benth

By

MOHD HAFIZ BIN IBRAHIM

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

September 2012
DEDICATION

For my beloved mother Sharifah bte Hassan, for all of your sacrifices and hardships in caring and teaching me as your son, you have raised me excellently. And for my future wife Nurul Amalina bte Mohd Zain who believed in me, without you there would be no excuses for me to stand still and work hard to achieve my dreams. My heartfelt gratitude for all love, encouragement and support through the years of my quest for knowledge. May this achievement shall be our stepping stone towards living our dreams and ambitions……………..

The vegetation of a good land comes forth (easily) by the Permission of its Lord; and that which is bad, brings forth nothing but (a little) with difficulty. Thus do We explain variously the Ayât (proofs, evidences, verses, lessons, signs, revelations, etc.) for a people who give thanks”.

“[Al-A’râf 7 : 58]
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Doctor of Philosophy

IMPACT OF CO₂ ENRICHMENT AND ITS INTERACTION WITH NITROGEN ON GROWTH, PHYSIOLOGY AND SECONDARY METABOLITES OF Labisia pumila Benth

By

MOHD HAFIZ BIN IBRAHIM

September 2012

Chairman: Associate Professor Hawa ZE Jaafar, PhD

Faculty: Agriculture

The demand for Labisia pumila (Kacip Fatimah) keeps increasing annually as this plant is believed to have high phytoestrogen activity that widely used in nutraceutical industries. However the plant is relatively difficult slow to grow, and takes about 16 to 36 months before it is ready for use in medicinal preparation. Carbon dioxide (CO₂) enrichment technique is known to be able to enhance slow growing plants and also to improve and alter plant bioactive compounds. Enriching L. pumila with elevated CO₂ may extend these benefit. Therefore, a project was conducted with the objectives ; (i) to determine and establish the most suitable microclimate for CO₂ enrichment of L. pumila under growth house based on ventilation per floor rate area and shading levels (ii) to investigate the effects of different CO₂ concentration on the growth, leaf gas exchange and secondary metabolites of three varieties of L. pumila (iii) to determine the interaction between CO₂ and nitrogen levels on growth, leaf gas exchange, primary and secondary metabolites of L. pumila and (iv) to establish and understand the biochemical regulation of secondary metabolites in L. pumila seedlings under interaction effects between CO₂ and nitrogen levels. There were four experiments
conducted to fulfill the objectives. In the first experiment, two levels of shade (0%, and 90%) and five different ventilation to floor area (v/f) ratio (0 = close; 0.375, =open-up; 0.450 = open-doors; 0.750 = open-bottom; 1.35 = open-all) were conducted to determine the best combination of these factors for establishment of the suitable microclimate for *L. pumila* under growth house for CO₂ enrichment. Result showed that shade+open-all give the best microclimate inside the growth chamber. Analysis of variance revealed that means of temperature and vapor pressure deficit (VPD) were significantly (P≤0.01) influenced by v/f ratio. Mean relative humidity (RH) inside the chamber was significantly (P≤0.01) enhanced by the shade used. Under shade+open-all, the stomatal conductance (gs) of *L. pumila* was found to be very sensitive to VPD. At VPD, temperature and RH of 1.41 kpa, 33°C and 68% respectively, gs of *L. pumila* started to decrease from 1100 am onwards suggesting that CO₂ enrichment should be carried out before this time to optimize benefit from CO₂ exposure. In the second experiment, three varieties of *L. pumila* seedlings (*alata*, *pumila* and *lanceolata*) were exposed to three levels of CO₂ (400, 800,1200 µmol mol⁻¹). In this experiment, the manipulation of CO₂ enrichment on *L. pumila* seedling seemed to be able to reduce the nursery period through growth enhancement, that was solely contributed by CO₂ enrichment. Neither varietal differences nor its interaction effects with CO₂ were observed. Increasing CO₂ from 400 to 1200 µmol mol⁻¹ had significantly improved growth, net photosynthesis (A), water use efficiency, maximum efficiency of photosystem II, carbohydrate, total phenolics and total flavonoids. However, chlorophyll content, specific leaf area, and net assimilation rate were found to decrease by end of the experiment. From this experiment, it was found that the production of secondary metabolites was negatively correlated with chlorophyll content implying that nitrogen levels might be playing an important role
in the production of secondary metabolites in *L. pumila*. In the third experiment, three varieties of *L. pumila* seedlings were exposed to four levels of nitrogen (0, 90, 180, 270 kg N ha\(^{-1}\)) under 1200 µmol mol\(^{-1}\) CO\(_2\) in a split plot design. Absolute control at 400 µmol mol\(^{-1}\) CO\(_2\) and 180 kg N ha\(^{-1}\) (Standard) were also included. Under CO\(_2\) enrichment, application of low nitrogen (0 and 90 kg N ha\(^{-1}\)) managed to enhance the production of secondary metabolites significantly regardless of differences in varieties. As levels of nitrogen increased, plant growth, photosynthesis, photosynthesis nitrogen use efficiency (PNUE) and chlorophyll content were enhanced. However, carbohydrate, total phenolics and flavonoids were reduced with increase in nitrogen fertilization. Increase in the production of secondary metabolites was found to be positively correlated with increase in the carbon to nitrogen ratio (C/N) and PNUE. In the last experiment, two CO\(_2\) levels (400 and 1200 µmol mol\(^{-1}\)) and three nitrogen levels (0, 90, 270 kg N ha\(^{-1}\)) were used to establish the mechanism of enhancement of secondary metabolites under varied CO\(_2\) levels and nitrogen fertilization. It was found that the highest production of secondary metabolites was obtained at 1200 µmol mol\(^{-1}\) + 0 kg N ha\(^{-1}\) and lowest at 400 µmol mol\(^{-1}\) + 270 kg N ha\(^{-1}\). The interaction effect between CO\(_2\) and nitrogen was found to influence the production of secondary metabolites, fructose, PAL activity and protein in *L. pumila*. Generally, these findings support the protein competition model that suggest the increase in production of secondary metabolites under high CO\(_2\) and low nitrogen levels was due to increased availability of phenylalanine (precursor for phenolics and flavonoids) due low sink ratio which increases the availability for production of secondary metabolites. From the project, it can be concluded that CO\(_2\) enrichment onto *L. pumila* was able to enhance growth and medicinal properties especially at low nitrogen fertilization.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGARUH PERKAYAAN CO₂ DAN INTERAKSI DENGAN NITROGEN TERHADAP PERTUMBUHAN, FISIOLOGI DAN METABOLIT SEKUNDER Labisia pumila Benth

Oleh

MOHD HAFIZ IBRAHIM

September 2012

Pengerusi : Profesor Madya Hawa ZE Jaafar, PhD
Fakulti Pertanian

Permintaan terhadap Labisia pumila (Kacip Fatimah) terus meningkat setiap tahun kerana tanaman ini diyakini memiliki kandungan fitoestrogen tinggi yang banyak digunakan dalam industri neutraseutikal. Walaubagaimanapun, pertumbuhan relatif pokok ini lambat, dan memerlukan waktu sekitar 16 - 36 bulan untuk boleh digunakan untuk pernyediaan ubat. Perkayaan karbon dioksida pada L. pumila mungkin berpotensi untuk meningkatkan pertumbuhan L. pumila serta sebatian bioaktifnya. Perkayaan dengan karbon dioksida mungkin melanjutkan faedah tersebut. Disebabkan itu satu penyelidikan telah dijalankan dengan objektif; (i) untuk menentukan mikroiklim yang sesuai untuk perkayaan CO₂ pada L.pumila berdasarkan nisbah ventilasi per luas lantai dan naungan (ii) untuk mengenalpasti kesan pelbagai kepekatan CO₂ pada pertumbuhan, pertukaran gas daun dan metabolit sekunder pada tiga varieti L.pumila (iii) untuk mengkaji kesan interaksi perkaryaan CO₂ dan nitrogen pada pertumbuhan, pertukaran gas daun dan metabolit primer dan sekunder dan (iv) untuk memahami regulasi biokimia metabolit sekunder pada interaksi antara
CO₂ dan nitrogen di dalam *L. pumila*. Empat eksperimen telah dijalankan untuk memenuhi objektif tersebut. Pada eksperimen pertama, dua tahap naungan (0%, 90%) dan lima nisbah ventilasi kepada luas lantai (v/f) (0, tertutup sepenuhnya; 0.375, terbuka-atas; 0.450, terbuka-pintu; 0.750, terbuka-bawah; 1.35, terbuka-penuh) telah dijalankan untuk menentukan kombinasi terbaik faktor tersebut untuk pewujudan mikroklimat yang sesuai untuk *L. pumila* di bawah rumah perkayaan CO₂. Keputusan menunjukkan naungan+ terbuka-penuh memberikan mikroklimat sesuai di dalam rumah perkayaan CO₂. Analisis varians menunjukkan yang purata suhu, defisit tekanan vapor (DTV) secara signifikan (P≤0.01) mempengaruhi kadar v/f. Purata kelembapan bandingan (KB) secara signifikannya (P≤0.01) mempengaruhi naungan yang digunakan. Di bawah terbuka-penuh kekonduktasi stomata (gs) *L. pumila* didapati sangat sensitif terhadap defisit tekanan Vapor (DTV). Pada DTV, suhu dan KB 1.41 kPa, 33°C dan 68% masing-masing, gs *L. pumila* mulai menurun bermula dari pukul 1100. Ini menunjukkan bahawa perkaryaan CO₂ harus dilakukan sebelum waktu ini untuk mendapat manfaat optima dari perkayaan. Dalam eksperimen kedua, tiga varieti *L. pumila* (*alata, pumila* dan *lanceolata*) didedahkan pada tiga kepekatan CO₂ (400 800 dan 1200 µmol mol⁻¹). Dalam kajian ini, didapati perkaryaan boleh mengurangkan tempoh di dalam tapak semaian melalui peningkatan pertumbuhan yang secara tunggalnya dipengaruhi oleh perkaryaan CO₂. Sepanjang eksperimen tiada kesan variati dan interaksi didapati. Didapati bahawa peningkatan CO₂ dari 400 - 1200 µmol mol⁻¹ secara signifikan meningkatkan pertumbuhan, fotosintesis bersih (A), kecekapan penggunaan air (WUE), kecekapan maksimum fotosistem II (*f₆*/*f₅*), karbohidrat, total fenol dan flavonoid. Namun, kandungan klorofil, luas daun spesifik (SLA), dan kadar assimilasi bersih (NAR) telah didapati menurun pada akhir eksperimen. Dari kajian ini, didapati bahawa pengeluaran metabolit sekunder
berkorelasi negatif dengan kandungan klorofil dan boleh disimpulkan bahawa nitrogen mungkin memainkan peranan langsung dalam pengeluaran metabolit sekunder di dalam *L. pumila*. Pada eksperimen ketiga, tiga variati *L. pumila* telah didedahkan pada empat aras nitrogen yang berbeza (0, 90, 180 dan 270 kg N ha\(^{-1}\)) di bawah 1200 µmol mol\(^{-1}\) CO\(_2\) di dalam rekabentuk split plot. Kawalan mutlak pada 400 µmol mol\(^{-1}\) CO\(_2\) dan 180 kg N ha\(^{-1}\) juga disertakan. Dalam perkayaan CO\(_2\), pembajaan nitrogen yang rendah (0 dan 90 kg N ha\(^{-1}\)) telah didapati berjaya meningkatkan pengeluaran metabolit sekunder walaupun tiada kesan variati didapati. Pada pembajaan nitrogen yang tinggi didapati pertumbuhan tanaman, fotosintesis, photosynthesis nitrogen use efficiency (PNUE) dan kandungan klorofil telah meningkat. Namun, kandungan karbohidrat, fenol dan flavonoid berkurang dengan peningkatan pembajaan nitrogen. Peningkatan pengeluaran metabolit sekunder didapati berkorelasi positif dengan peningkatan nisbah karbon kepada nitrogen (C/N) dan PNUE. Pada eksperimen terakhir, dua tahap CO\(_2\) (400 dan 1200 µmol mol\(^{-1}\)) dan tiga tahap nitrogen (0, 90 dan 270 kg N Ha\(^{-1}\)) telah digunakan untuk mengkaji mekanisme peningkatan metabolit sekunder di bawah perkaryaan CO\(_2\) dan pembajaan nitrogen. Didapati pengeluaran tertinggi metabolit sekunder diperolehi pada 1200 µmol mol\(^{-1}\) + 0 kg N Ha\(^{-1}\) dan terendah di 400 µmol mol\(^{-1}\) + 270 kg N Ha\(^{-1}\). Kesan interaksi antara CO\(_2\) dan nitrogen didapati mempengaruhi pengeluaran metabolit sekunder, fruktosa, aktiviti phenyll alanine lyase (PAL) dan kandungan protein di dalam *L. pumila*. Secara umumnya, penemuan ini menyokong model protein competition model (PCM) yang menunjukkan peningkatan pengeluaran metabolit sekunder di bawah perkaryaan CO\(_2\) dan pembajaan nitrogen rendah disebabkan peningkatan phenylalanine (prekursor untuk fenol dan flavonoid) kerana “keupayaan sinki rendah” yang meningkatkan ketersediaan untuk pengeluaran metabolit sekunder.
Dari projek ini, boleh disimpulkan perkayaan CO$_2$ pada *L. pumila* mampu meningkatkan pertumbuhan dan khasiat perubatan terutamanya pada pembajaan nitrogen yang rendah.
ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and gratitude to Assoc. Prof. Dr. Hawa Z.E Jaafar, chairman of my supervisory committee, for her attentive supervision, unfailing guidance, consistent encouragement, patience, useful discussions and devotion during the course of this study. Her constructive criticisms and valuable comments during the preparation of this manuscript are highly valued. Besides her guidance and kindness, she help to fulfill all the necessary facilities that made my study successful and fruitful. Thank you for believing me and change my life with your tutelage.

I am also indebted to Prof. Dr. Asmah Rahmat, my supervisory committee from Faculty of Medicine, for her attentive supervision, unfailing guidance, and provided me laboratory facilities to do my job far more effectively. Besides her advice was most valuable and made me more passionate in my research works.

I also would like to thank Prof. Dr. Zaharah Abdul Rahman, for her helpful guidance and discussions. I am also grateful to the laboratory and field staffs/personnel of the Crop Physiology group, UPM, for their help and co-operation during laboratory analysis and field work.I will not forget the patience of my mother Sharifah bte Hassan, my late father Ibrahim bin Kadir, my brother Mohd Ikhbar and Mohd Khairon Hambali, and also my friends for their frequent communication, moral support and constant encouragement, which made my life easy throughout my study. Thank you very much.
I certify that an Examination Committee has met on 21/09/2012 to conduct the final examination of Mohd Hafiz Bin Ibrahim on his Doctor of Philosophy thesis entitled “IMPACT OF CO₂ ENRICHMENT AND ITS INTERACTION WITH NITROGEN ON GROWTH, PHYSIOLOGY AND SECONDARY METABOLITES OF Labisia pumila Benth”, 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. Member of the Examination Committee are as follows:

Mohd Ridwan Abd Halim, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Abdul Shukor Juraimi, PhD
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Puteri Edaroyati Megat Wahab, PhD
Senior Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Fitzgerald Booker, PhD
Professor
United States Agricultural Research Services
Plant Science Research Unit
Raleigh, USA
(External Examiner)

HASANAH MOHD GHAZALI, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia
This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

**Hawa ZE Jaafar, PhD**  
Associate Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Chairman)

**Asmah Rahmat, PhD**  
Professor  
Faculty of Medicine  
Universiti Putra Malaysia  
(Member)

**Zaharah Abdul Rahman, PhD**  
Professor  
Faculty of Agriculture  
Universiti Putra Malaysia  
(Member)

---

**BUJANG BIN KIM HUAT, PhD**  
Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date:
DECLARATION

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

______________________________
MOHD HAFIZ BIN IBRAHIM

Date:
### TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>vi</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>x</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>xi</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xxi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS AND SYMBOLS</td>
<td>xxxi</td>
</tr>
</tbody>
</table>

### CHAPTER

1. **INTRODUCTION** 1

2. **LITERATURE REVIEW** 6
   2.1 Myrsinaceae 6
   2.2 *Labisia Pumila* Benth 6
   2.3 Uses and economic importance of *Labisia pumila* Benth 8
   2.4 Plant secondary metabolites 9
   2.5 Phenolics compounds 10
   2.6 Flavonoids compounds 12
   2.7 Biosynthesis of Secondary metabolites in Plants 14
   2.8 Carbon dioxide and plant responses 15
   2.9 Carbon dioxide enrichment 19
   2.10 Carbon dioxide enrichment in Malaysia 20
   2.11 The use of ventilation/ floor rate area for greenhouse in the tropics 21
   2.12 Plants adaptation to increases in CO₂ levels 23
      2.12.1 Effects of CO₂ on biomass and growth 26
      2.12.2 Plant leaf gas exchange effects under elevated CO₂ 27
      2.12.3 Maximal photochemical efficiency ($f_{v}/f_{m}$) under CO₂ enrichment 28
      2.12.4 Carbohydrates increases under elevated CO₂ 28
      2.12.5 Plant secondary metabolites and elevated CO₂ 30
   2.13 Interaction between elevated CO₂ and nutrient 33
   2.14 Interaction between CO₂ and nitrogen on primary and secondary metabolites 34
INFLUENCE OF SHADE AND VENTILATION TO FLOOR AREA RATE RATIO ON STOMATA CONDUCTANCE OF *LABISIA PUMILA* BENTH.: IMPLICATION ON THE TIMING OF CO₂ ENRICHMENT FOR DOMESTICATION OF DIFFICULT-TO-GROW MEDICINAL HERB

3.1 Introduction

3.2 Material and Methods
   3.2.1 Experimental site and greenhouse description
   3.2.2 Instrumentation for microclimate measurement
   3.2.3 Vapor pressure deficit calculation
   3.2.4 Leaf gas exchange measurement
   3.2.5 Experimental design
   3.2.6 Data collection and analysis

3.3 Results
   3.3.1 Solar radiation (W m⁻²)
   3.3.2 Photosynthetic photon flux density, PPFD (µmol m⁻² s⁻¹)
   3.3.3 Wind speed (m s⁻¹)
   3.3.4 Temperature
   3.3.5 Relative humidity
   3.3.6 Vapor pressure deficit
   3.3.7 Vent opening to floor area ratio and air exchange rates
   3.3.8 Relationships among stomatal conductance, vapor pressure deficit, relative humidity and temperature
   3.3.9 Correlation analysis

3.4 Discussion

3.5 Conclusion

4 GROWTH, GAS EXCHANGE, PRIMARY AND SECONDARY METABOLITES RESPONSE TO SHORT-TERM ELEVATED CO₂ CONCENTRATION ON *LABISIA PUMILA* BENTH

4.1 Introduction

4.2 Material and methods
   4.2.1 Experimental design and experiment treatment
   4.2.2 Carbon dioxide fumigation chamber structure
   4.2.3 Carbon dioxide exposure methods
   4.2.4 Fertilization and Watering
   4.2.5 Weeding
   4.2.6 Control of pest and diseases
   4.2.7 Data collection
4.2.7.1 Growth measurement
  Plant height (cm/plant)  77
  Leaf area per seedling (cm²/plant)  78
  Total dry biomass  78
  Specific leaf area (SLA; cm²/g)  78
  Shoot to root ratio (S/R)  78
  Net assimilation rate  79
4.2.7.2 Leaf gas exchange  80
4.2.7.3 Light response curve  82
4.2.7.4 Chlorophyll fluorescence measurements (fᵣ/fₘ)  83
4.2.7.5 Total chlorophyll content (mg g⁻¹ fresh weight)  84
4.2.7.6 Total Phenolics and Total Flavonoids Quantification  86
4.2.7.7 Carbohydrate Determination  86
4.2.7.8 Data analysis  87
4.3 Results  88
  4.3.1 Growth and development  88
    Plant total biomass  88
    Plant height  89
    Total leaf area  89
    Shoot to root ratio  92
    Specific leaf area (SLA)  92
    Net assimilation rate (NAR)  93
    Total chlorophyll content  95
  4.3.2 Leaf gas exchange  96
    Net photosynthesis (A)  96
    Stomata conductance (gs)  96
    Transpiration rate  98
    Instantaneous water use efficiency (WUE)  98
    Intercellular CO₂ (Ci)  100
    Maximum efficiency of photosystem II (fᵣ/fₘ)  101
    Light response curve  102
  4.3.3 Carbohydrate content  104
  4.3.4 Secondary metabolites  105
    Total phenolics  105
    Total flavonoids  106
  4.3.5 Correlation analysis  107
4.4 Discussion  113
4.5 Conclusion  122

5 IMPACT OF ELEVATED CO₂ AND NITROGEN AVAILABILITY ON GROWTH, LEAF GAS EXCHANGE, CARBOHYDRATES AND SECONDARY METABOLITES OF LABISIA PUMILA BENTH.
5.1 Introduction 124
5.2 Materials and methods 127
  5.2.1 Experimental location, plant materials and treatments 127
  5.2.2 Growth house microclimate and CO\textsubscript{2} enrichment treatment 128
  5.2.3 Growth analysis 130
  5.2.4 Chlorophyll content 130
  5.2.5 Leaf gas exchange measurement 131
  5.2.6 Carbohydrate and Total phenolics and total flavonoids quantification 132
  5.2.7 Total carbon, nitrogen and C:N ratio 132
  5.2.8 Data analysis 133
5.3 Results 134
  5.3.1 Growth and development 134
    Total plant biomass 134
    Total leaf area 137
    Shoot to root 138
    Specific leaf area 139
    Total chlorophyll content 140
    Relative growth rate 142
  5.3.2 Net photosynthesis (A) 143
  5.3.3 Photosynthesis nitrogen use efficiency (PNUE) 144
  5.3.4 Leaf nitrogen 145
  5.3.5 Leaf C/N ratio 146
  5.3.6 A/C\textsubscript{i} curves analyses 147
  5.3.7 Carbohydrate and profiling 149
  5.3.8 Total phenolics and profiling 151
  5.3.9 Total flavonoids and profiling 153
5.4 Discussion 157
5.5 Conclusion 166

6 BIOCHEMICAL REGULATION PRODUCTION OF PLANT SECONDARY METABOLITES UNDER HIGH CO\textsubscript{2} AND DIFFERENT NITROGEN LEVELS OF LABISIA PUMILA BENTH
6.1 Introduction 167
6.2 Material and Methods 169
  6.2.1 Experimental Location, Plant Materials and Treatments 169
  6.2.2 Growth House Microclimate and CO\textsubscript{2} Enrichment Treatment 171
  6.2.3 Total Phenolics and Total Flavonoid Quantification 171
  6.2.4 Glucose determination 172
  6.2.5 Sucrose determination 172
  6.2.6 Fructose determination 173
  6.2.7 Starch Determination 173
6.2.8 Total soluble sugar and Total Non Structural Carbohydrate (TNC) 174
6.2.9 Total carbon, Nitrogen and C/N Ratio 174
6.2.10 Phenylalanine-ammonia-lyase (PAL) 175
6.2.11 Protein determination 175
6.2.12 Statistical analysis 176

6.3 Results 177
6.3.1 Sucrose 177
6.3.2 Glucose 177
6.3.3 Fructose 179
6.3.4 Total soluble sugar (TSS) 180
6.3.5 Starch 182
6.3.6 Total non structural carbohydrate (TNC) 182
6.3.7 Carbohydrate partitioning 185
6.3.8 Carbon 186
6.3.9 Nitrogen 187
6.3.10 C/N ratio 188
6.3.11 Total phenolics 189
6.3.12 Total flavonoid 191
6.3.13 Phenyll alanine lyase, PAL activity 193
6.3.14 Soluble protein content 194
6.3.15 Sucrose/starch ratio 195
6.3.16 The relationship between secondary metabolites production with fructose, PAL activity, soluble protein and sucrose/ starch ratio 196

6.4 Discussion 199
6.5 Conclusion 206

7 GENERAL CONCLUSION AND DISCUSSION 207

8 RECOMMENDATIONS 211

REFERENCES 212
APPENDICES 238
BIO DATA OF THE STUDENT 266
PUBLICATIONS 267