OPTIMIZING Agrobacterium-MEDIATED TRANSFORMATION PARAMETERS
OF Melastoma decemfidum ROXB EX JACK

GOH SHEE YIN

UNIVERSITI TEKNOLOGI MALAYSIA
OPTIMIZING Agrobacterium-MEDIATED TRANSFORMATION
PARAMETERS OF Melastoma decemfidum ROXB EX JACK

GOH SHEE YIN

A dissertation submitted in partial fulfillment of the
requirements for the award of the degree of
Master of Science (Biotechnology)

Faculty of Biosciences and Medical Engineering
Universiti Teknologi Malaysia

AUGUST 2015
ACKNOWLEDGEMENT

I would like to express my earnest gratitude towards my supervisor, Dr. Azman Abd Samad for his scholastic guidance, constructive criticisms, advices, and untiring motivation in completion of this dissertation.

I would also like to show my appreciation to postgraduate students, Ms. Zahidah bte Ayob, Ms. Nor’ Ain Abdul Rahman and Ms. Syakila Mohd Arif for their kindness guidance, advices and sharing of their professional knowledge during the course of this project.

Furthermore, I would like to express my appreciation to all lab staffs and lab officers for their assistance in dealing laboratory materials and equipment supplies.

Last but not least, I would like to thank my parents and family for their encouragement, emotional support and unconditioned loves to me.
ABSTRACT

*Melastoma decemfidum* is a tropical ornamental plant belongs to family of Melastomataceae and widely used in traditional medicine. The plant was reported to contain bioactive flavonoids that exhibited antioxidant and anticancer effects. To date, there is no report on transformation system for *M. decemfidum*. In this study, the effects of transformation parameters on the transformation efficiency of *Agrobacterium*-mediated transformation of *M. decemfidum* were investigated. Parameters such as bacterial concentration, infection time and acetosyringone concentration were optimized using histochemical GUS assay. Results showed that the highest transformation efficiency (96.7%) for *M. decemfidum* with an average of 479.6 ± 10.2 blue spots per explant was obtained when leaf explants were treated with bacterial concentration of OD$_{600\text{nm}} = 0.6$. Forty-five minutes of infection time gave the highest percentage (96.7%) of positive transformants and an average of 316.9 ± 7.6 blue spots per explant. Addition of 100 µM acetosyringone was optimum (100%) for transforming *M. decemfidum* with an average of 509.8 ± 26.7 blue spots per explant. In conclusion, an efficient *Agrobacterium*-mediated transformation protocol for *M. decemfidum* was successfully established. Thus, the developed protocol will facilitate the delivery of desirable genes into *M. decemfidum* via *Agrobacterium*-mediated transformation.
ABSTRAK

*Melastoma decemfidum* merupakan sejenis tumbuhan daripada keluarga Melastomataceae di kawasan tropika. Tumbuhan ini digunakan secara meluas dalam perubatan tradisional. *M. decemfidum* telah dilaporkan mengandungi bioaktif flavonoid yang mempamerkan aktiviti antioksa dan antikanser. Sehingga kini, tidak ada laporan mengenai system pemindahan genetik untuk *M. decemfidum*. Dalam kajian ini, pemindahan genetik untuk *M. decemfidum* melalui kaedah *Agrobacterium tumefaciens* telah dilaksanakan. Parameters seperti kepekatan bakteria, jangka masa infeksi dan kepekatan asetosiringon telah dioptimumkan dengan menggunakan β-glucuronidase (GUS) sebagai penanda. Keputusan menunjukkan bahawa kecekapan yang tertinggi (96.7%) untuk pemindahan gen secara *Agrobacterium* ke dalam daun *M. decemfidum* dicapai oleh kepekatan bakteria pada OD600nm = 0.6 dengan menghasilkan 479.6 ± 10.2 titik biru per eksplan secara purata. Jangka masa infeksi yang 45 minit memberi peratusan yang paling tinggi (96.7%) kepada transforman positif untuk *M. decemfidum* dan sebanyak 316.9 ± 7.6 titik biru per eksplan. Penambahan asetosiringon 100 µM adalah optimum (100%) untuk pemindahan gen ke dalam *M. decemfidum* dengan memberikan jumlah purata 509.8 ± 26.7 titik biru per eksplan. Kesimpulannya, protokol pemindahan genetik yang optimum untuk *M. decemfidum* telah berjaya dihasilkan. Protokol yang dibangunkan akan memudahkan kemasukan gen yang diinginkan ke dalam *M. decemfidum* melalui kaedah *Agrobacterium*. 
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DECLARATION</td>
<td>ii</td>
</tr>
<tr>
<td></td>
<td>ACKNOWLEDGEMENT</td>
<td>iii</td>
</tr>
<tr>
<td></td>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td></td>
<td>ABSTRAK</td>
<td>v</td>
</tr>
<tr>
<td></td>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td></td>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td></td>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>LIST OF SYMBOLS</td>
<td>xii</td>
</tr>
<tr>
<td></td>
<td>LIST OF ABBREVIATIONS</td>
<td>xiii</td>
</tr>
<tr>
<td></td>
<td>LIST OF APPENDICES</td>
<td>xiv</td>
</tr>
<tr>
<td>1</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.1 Background of Study</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.2 Problem Statement</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1.3 Objectives of Study</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1.4 Scope of Study</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1.5 Research Significance</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>LITERATURE REVIEW</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>2.1 Melastomataceae</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>2.2 <em>Melastoma decemfidum</em></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>2.2.1 Ornamental Values</td>
<td>9</td>
</tr>
</tbody>
</table>
2.2.2 Medicinal Values
2.2.3 Bioactive compounds
2.3 Plant Genetic Transformation
2.4 Agrobacterium tumefaciens-Mediated Transformation
  2.4.1 Agrobacterium-Mediated Transformation Process
  2.4.2 Factors Influencing Agrobacterium-Mediated Transformation
  2.4.3 Agrobacterium-Mediated Transformation of Plant
2.5 Reporter Genes

3 MATERIALS AND METHODS
  3.1 Source of Plant Materials and Agrobacterium
  3.2 Preparation of Glassware
  3.3 Preparation of Culture Media and Chemicals
  3.4 Axenic Shoot Culture
  3.5 Agrobacterium Culture and Maintenance
  3.6 Preparation of Agrobacterium Suspension
  3.7 Agrobacterium tumefaciens-Mediated Transformation of M. decemfidum
  3.8 Histochemical GUS Assay
  3.9 Data Collection
  3.10 Statistical Analysis

4 RESULTS AND DISCUSSION
  4.1 Transient GUS Expression
  4.2 Effect of Bacterial Concentration on Transformation Efficiency of M. decemfidum
  4.3 Effect of Infection Time on Transformation Efficiency of M. decemfidum
  4.4 Effects of Acetosyringone Concentration on Transformation Efficiency of M. decemfidum
CONCLUSION AND FUTURE WORK

5.1 Conclusion

5.2 Future Work
### LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE NO.</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Taxonomic hierarchy of <em>M. decemfidum</em></td>
<td>9</td>
</tr>
<tr>
<td>2.2</td>
<td>Factors influencing <em>Agrobacterium</em>-mediated plant transformation</td>
<td>17</td>
</tr>
<tr>
<td>2.3</td>
<td><em>Agrobacterium</em>-Mediated transformation of some plant species</td>
<td>20</td>
</tr>
<tr>
<td>3.1</td>
<td>Composition of GUS buffer and staining solution used in histochemical GUS assay</td>
<td>29</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE NO.</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td><em>M. decemfidum</em> plant</td>
<td>8</td>
</tr>
<tr>
<td>2.2</td>
<td>A model for the <em>Agrobacterium</em>-mediated genetic transformation</td>
<td>16</td>
</tr>
<tr>
<td>3.1</td>
<td>Axenic shoot culture of <em>M. decemfidum</em></td>
<td>26</td>
</tr>
<tr>
<td>3.2</td>
<td>A schematic representation of the binary vector of ( \text{pCAMBIA1305.2} )</td>
<td>27</td>
</tr>
<tr>
<td>4.1</td>
<td>Effect of bacterial concentration on transformation efficiency of <em>M. decemfidum</em> leaves</td>
<td>32</td>
</tr>
<tr>
<td>4.2</td>
<td>The number of blue spots per explant for <em>M. decemfidum</em> in response to different bacterial concentration</td>
<td>33</td>
</tr>
<tr>
<td>4.3</td>
<td>GUS expression (blue spots) on leaf explants of <em>M. decemfidum</em> transiently transformed with bacterial concentration of ( \text{OD}_{600\text{nm}} = 0.6 )</td>
<td>34</td>
</tr>
<tr>
<td>4.4</td>
<td>Effect of infection time on transformation efficiency of <em>M. decemfidum</em> leaves</td>
<td>36</td>
</tr>
<tr>
<td>4.5</td>
<td>The number of blue spots per explant for <em>M. decemfidum</em> in response to different infection time</td>
<td>37</td>
</tr>
<tr>
<td>4.6</td>
<td>GUS expression (blue spots) on leaf explants of <em>M. decemfidum</em> transiently transformed for 45 min</td>
<td>38</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>4.7</td>
<td>Effect of acetosyringone concentration on transformation efficiency of <em>M. decemfidum</em> leaves</td>
<td></td>
</tr>
<tr>
<td>4.8</td>
<td>The number of blue spots per explant for <em>M. decemfidum</em> in response to different infection time</td>
<td></td>
</tr>
<tr>
<td>4.9</td>
<td>GUS expression (blue spots) on leaf explants of <em>M. decemfidum</em> transiently transformed in the presence of 100 μM acetosyringone</td>
<td></td>
</tr>
</tbody>
</table>
LIST OF SYMBOLS

% - Percent
= - Equal to
< - Less than
≤ - Less than or equal to
°C - Degree Celsius
α - Alpha
β - Beta
µmol/m²/s - Micromoles of light per square meter per second
µM - Micromolar
cm - Centimeter
g - Gram
h - Hour
L - Liter
m - Meter
M - Molar
mg - Milligram
min - Minute
mL - Milliliter
mM - Millimolar
nm - Nanometer
pH - Power of Hydrogen
v/v - Volume per volume
w/v - Weight per volume
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BAP</td>
<td>6-Benzyaminopurine</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>dH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GUS</td>
<td>Beta-glucuronidase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LUC</td>
<td>Luciferase</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Michigan Cancer Foundation-7</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>spp</td>
<td>Species</td>
</tr>
<tr>
<td>T-DNA</td>
<td>Transfer-deoxyribonucleic acid</td>
</tr>
</tbody>
</table>
# LIST OF APPENDICES

<table>
<thead>
<tr>
<th>APPENDIX</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Preparation of Stock Solution for MS Medium and Chemicals</td>
<td>53</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Background of Study

In Malaysia, Melastomataceae spp., or locally known as ‘Senduduk’, are identified as potentially important flowering ornamentals and they are used for commercial purposes (Yong et al., 2006). The leaves and roots of these plants can be used for the treatment of various ailments such as diarrhea, dysentery (Wiart, 2006), gastric ulcers, epilepsy, arthritis, rheumatism (Lohezic-Le et al., 2002) and jaundice (Libman et al., 2006). Melastoma decemfidum, or formerly known as white petal Melastoma malabathricum (locally known as ‘Senduduk Putih’), belongs to the family Melastomataceae and they usually grow in open fields of lowlands and mountain forest (Sarju et al., 2012). It has been reported that leaf extracts of M. malabathricum contain characterized hydrolysable tannin. Several tannins isolated from dry leaves of light pink-magenta petal M. malabathricum were oligomers such as nobotannin B, nobotannins G, nobotannins H, nobotannins J, malabathrins B, malabathrins C and malabathrins D, and monomers such as 1,4,6-tri-O-galloyl-ß-D-glucose, 1,2,4,6-tetra-O-galloyl-ß-D-glucose, strictinin, casuarictin, pedunculagin, nobotanin D, pterocarmin (Yoshida et al., 1992). Whereas leaves of white petal M. malabathricum contains four flavonoids, including naringenin, kaempferol, kaempferol-3-O-D-glucoside and
kaempferol-3-O-[2’,6’-di-O-p-trans-coumaroyl] glucoside (Susanti et al., 2007). Among these flavonoids, naringenin and kaempferol-3-O-[2’,6’-di-O-p-trans-coumaroyl] glucoside were found to exhibit anti-proliferative effect against MCF-7, a human breast cancer cell line.

To improve the quality of plants and develop new varieties, genetic transformation is recommended for introduction of useful genes into a variety of plants because it has opened new avenues to the modification of characteristics, such as flower color, fragrant, longevity, shape and size (Yong et al., 2010). There are several approaches available for transferring desired genes into plant genome, such as Agrobacterium-mediated method, microprojectile bombardment and electroporation. Among these approaches, Agrobacterium-mediated is the most commonly used method as no costly equipment is required and the simplicity of the plant transformation protocols (Yong et al., 2006; Yong et al., 2008). This method involves the transferring of well-defined DNA from the Agrobacterium tumor-inducing (Ti) plasmid to the host-cell genome (Tzfira and Citovsky, 2006).

In this study, Agrobacterium tumefaciens-mediated transformation system was established for M. decemfidum by using β-glucuronidase (GUS) gene as a marker. To date, there was no report of genetic transformation carried out on this plant species. The effects of parameters such as bacterial concentration, infection time and acetylsyringone concentration on transformation efficiency were investigated.

1.2 Problem Statement

M. decemfidum has been shown to contain flavonoids which exhibit anticancer effect (Susanti et al., 2007). This beneficial effect of flavonoids for
human health may increase their demand in dietary supplements and drug discovery. Therefore, improvement of flavonoid productivity in *M. decemfidum* is necessary. Some strategies such as introduction of useful genes into *M. decemfidum* can be adopted to increase the flavonoid synthesis of the plants.

*Agrobacterium tumefaciens*-mediated transformation is an evolved process involving genetic determinants for both the bacteria and the host plant cells. The gene transfer and its integration into the plant genome are governed by various *Agrobacterium* and plant tissue-specific factors, such as plant genotype, type of explant, plasmid vector, bacterial strain, bacterial concentration, composition of culture medium, wounding types, co-cultivation period and selection markers (Ziemienowicz, 2013). Although *Agrobacterium*-mediated transformation protocols are now available for some *Melastoma* species such as *M. malabathricum*, the protocols are applicable within each species to only a few genotypes. The transformation protocol for *M. malabathricum* may not be successful in *M. decemfidum* transformation. Therefore, the main focus of this research is to study the effects of transformation factors on *M. decemfidum* with regard to bacterial concentration, infection time and acetosyringone concentration in order to establish a transformation protocol for *M. decemfidum*. Optimization of transformation parameters are needed in order to produce an efficient *A. tumefaciens*-mediated transformation protocol for *M. decemfidum*.

### 1.3 Objectives of Study

The objectives of this study were:

1. To determine the effect of bacterial concentration on transformation efficiency of *M. decemfidum* using histochemical GUS assay.
1. To determine the effect of infection time on transformation efficiency of *M. decemfidum* using histochemical GUS assay.

2. To determine the effect of acetosyringone concentration on transformation efficiency of *M. decemfidum* using histochemical GUS assay.

1.4 Scope of Study

This study was mainly focused on the optimization of *Agrobacterium*-mediated transformation parameters for *M. decemfidum*. Three parameters assessed were *Agrobacterium* concentration, infection time and acetosyringone concentration which are known to influence the transformation efficiency in various plant species. *M. decemfidum* was used as the source of explant in this study. *In vitro* tissue culture was carried out for the maintenance of *M. decemfidum* and preparation of explants. *A. tumefaciens* strain LBA4404 carrying GUS reporter gene was used for *Agrobacterium*-mediated transformation. Histochemical GUS assay was performed to determine transient GUS expression. The transformation efficiency was determined based on the percentage of positive transformed explants as well as the number of blue spots per explant.

1.5 Research Significance

*M. decemfidum* has been widely used as herbal for treatment of various ailments. Besides, it was found to contain flavonoids which exhibit cytotoxic effects. These flavonoids can be isolated from *M. decemfidum* and potentially used in pharmaceutical industry. However, the mechanism underlying cytotoxic activity of the flavonoids is still unknown. Thus, establishment of a genetic transformation
protocol for *M. decemfidum* might be necessary. With an efficient transformation protocol, the desirable genes can be efficiently introduced into the plants and the useful genetically engineered characteristics can be expressed. The mechanisms of action of bioactive compounds in *M. decemfidum* can, therefore, be investigated. Increased production of flavonoids might also be achieved by transforming *M. decemfidum* with related genes. Moreover, the functions of other genes in *M. decemfidum* can be discovered.
REFERENCES


Vellmont, E., Dubois, F., Sangwan, R. S., Vasseur, G., Bourgeois, Y., and Sangwan-Norreel, B. S. (1997). Role of the host cell cycle in the Agrobacterium-


Yong, W. T. L., Henry, E. S., and Abdullah, J. O. (2010). Enhancers of *Agrobacterium*-mediated transformation of *Tibouchina semicandra* selected

