# INDUCTION OF HAIRY ROOT CULTURES FROM DIFFICULT-TO-TRANSFORM *Eurycoma longifolia* (TONGKAT ALI) USING WILD STRAINS OF *Agrobacterium rhizogenes* WITH ANTIBACTERIAL STUDIES

**MONICA DANIAL**

**UNIVERSITI SAINS MALAYSIA**

**2011**

# INDUCTION OF HAIRY ROOT CULTURES FROM DIFFICULT-TO-TRANSFORM *Eurycoma longifolia* (TONGKAT ALI) USING WILD STRAINS OF *Agrobacterium rhizogenes* WITH ANTIBACTERIAL STUDIES

**by**

**MONICA DANIAL**

**Thesis submitted in fulfillment of the requirements for the degree of**

**Master of Science**

**December 2011**

Specially Dedicated to:

*My mother*

*Saseliammah Ammiatham*

*My guru*

*Senior Assoc. Prof. Dr. Xavier Rathinam*

*My husband*

*Johnson Stephen Muriel*

## ACKNOWLEDGEMENT

This is a work that started as personal wish during my degree years at the university but which could not have been done without the help, support and dedication of those whom I met along the journey. I would like to personally thank my supervisor Dr. Sreeramanan Subramaniam and co-supervisors Prof. Chan Lai Keng and Dr. Syarifah Shahrul Rabiah Syed Alwee (FELDA) for granting this project and having the trust in me in the completion of this research. I am undoubtedly grateful for the continuous guidance of Dr. Sreeramanan Subramaniam without whom the thesis would not have been possible and for his invaluable contribution he has made to this work, which has been indeed the result of shared effort.

I wish to extend my appreciation to National Science Fellowship (NSF) and Felda for its contribution to the financial support of this work, and to those who collaborated with technical assistance and supply of research material: researchers from MAFF Institute of Agrobiological Sciences (Japan), Prof. Motoyasu Otani, Prof. David Tepfer, Dr. Sofiman, Asso. Prof. Sudesh Kumar, Mr. Then Kek Hoe, Puan Afida, Mr.Soma, Ms.Shantini, Ms. Suganti Applasamy, Ms. Geetha Saagal and staffs at the microscope unit USM. To everyone at Plant Biotechnology lab 310, thanks for your help and friendship: Pavallekoodi, Jessica, Safiah, Safrina, Jeevan, Arulvilee, Advina, Ranjetta, Bhavani and Rosli.

Lastly, I want to thank as well the members of the jury, for their corrections and suggestions in improving the quality of this thesis.

## TABLE OF CONTENTS


| | Page |
|---|---|
| Acknowledgement | ii |
| Table of Contents | iii |
| List of Figures | vii |
| List of Plates | ix |
| List of acronyms and abbreviations | xii |
| Abstrak | xiii |
| Abstract | xv |
| **CHAPTER 1.0-GENERAL INTRODUCTION** | 1 |
| 1.1 Research objectives | 7 |
| **CHAPTER 2.0-LITERATURE REVIEW** | 8 |
| 2.1 Introduction | 8 |
| 2.1.1 Locality | 8 |
| 2.1.2 Classification | 8 |
| 2.2 Tree Morphology | 11 |
| 2.3 Ethnobotany Uses | 11 |
| 2.4 Elucidations on *Eurycoma longifolia* compounds and its function | 12 |
| 2.5 Pharmacological properties of compounds isolated from *Eurycoma longifolia* | 13 |
| 2.5.1 Aphrodisiac activities | 14 |
| 2.5.2 Antimalarial and anticancer activity | 14 |
| 2.5.3 Anti-diabetic properties | 16 |
| 2.5.4 Antimicrobial activities | 17 |
| 2.6 Current propagation method of *Eurycoma longifolia* | 17 |
| 2.7 *Agrobacterium rhizogenes* | 20 |
| 2.7.1 *Agrobacterium rhizogenes* mediated transformation | 21 |
| 2.7.2 Nucleotide sequence of plasmid pRi1724 | 22 |
| 2.7.2.1 Features and Sequence of pRi1724 plasmid | 25 |
| 2.7.2.2 Gene organization and distribution | 25 |
| 2.7.2.3 Mikimopine catabolism by plasmid pRi1724 | 26 |
| 2.8 Hairy root cultures | 29 |
| 2.8.1 Beneficial attributes of hairy root cultures system | 29 |
| 2.8.1.1 Phytochemicals production from hairy root cultures | 30 |
| 2.8.1.2 Therapeutic protein production from hairy root cultures | 32 |
| 2.8.1.3 Elucidation of biochemical pathway | 34 |

2.8.1.4 Plant secondary metabolites production through hairy root cultures 36
2.8.1.5 Gene identification and its function using hairy root Cultures 37
2.8.1.6 Metabolic engineering using hairy root cultures 40
2.8.1.7 Hairy root cultures as bioreactors 42
2.8.1.8 *Ex-situ* conservation 43

**CHAPTER 3.0- MORPHOLOGY AND GROWTH OF A*grobacterium rhizogenes*** 44

3.1 Introduction 44
3.1.1 Objectives 46
3.2 Materials and Methods 46
3.2.1 Bacteria strains 46
3.2.2 Scanning Electron Microscope (SEM) preparation for viewing the bacteria 47
3.3 Results and Discussion 49
3.4 Conclusion 64

**CHAPTER 4.0-*Eurycoma longifolia* GERMINATED SEEDS BENEFICIAL ATTRIBUTES THAT MAKES IT A SUITABLE EXPLANT CHOICE FOR TRANSFORMATION** 65

4.1 Introduction 65
4.1.1 Objectives 66
4.2 Materials and Methods 67
4.2.1 Plant materials 67
4.2.2 Histology method 67
4.3 Results and Discussion 68
4.4 Conclusion 80

**CHAPTER 5.0-CHEMOTAXIS MOVEMENT ASSAY OF WILD STRAINS OF *Agrobacterium rhizogenes* TOWARDS EXPLANTS OF *Eurycoma longifolia*** 81

5.1 Introduction 81
5.1.1 Objective 82
5.2 Materials and Methods 83
5.2.1 Plant materials 83
5.2.2 *Agrobacterium rhizogenes* strains 84
5.2.3 Chemotaxis assays 84
5.2.4 Chemotaxis assay media 85

5.2.5 Scanning Electron Microscope (SEM) viewing of bacterial attachment on the roots 85
5.3 Results and Discussion 86
5.4 Conclusion 109

**CHAPTER 6.0-HAIRY ROOT INDUCTION FROM VARIOUS EXPLANTS OF *Eurycoma longifolia* USING WILD STRAINS OF *Agrobacterium rhizogenes*** 110

6.1 Introduction 110
6.1.1 Objectives 112
6.2 Materials and Methods 112
6.2.1 Plant materials 112
6.2.1.1 *In vivo* leaves, petiole and rachis 113
6.2.1.2 *In vitro* leaves and root segments, three weeks grown seedlings, somatic embryos and embryo of *Eurycoma longifolia* 113
6.2.2 Wild strains of *Agrobacterium rhizogenes* 114
6.2.3 Hairy root induction 115
6.2.3.1 *In vivo* leaves, petiole, rachis and *in vitro* leaves, roots, embryo and somatic embryo of *Eurycoma longifolia* 115
6.2.3.2 Three weeks old germinated seedlings of *Eurycoma longifolia* 115
6.2.4 Establishment of the hairy root cultures in solid medium 116
6.2.5 Genomic DNA isolation and detection of the transformed *rol* genes by polymerase chain reaction (PCR) 116
6.2.6 Scanning Electron Microscope (SEM) analysis of the transformed hairy roots and the untransformed (control) roots 117
6.2.7 Transmission Electron Microscope (TEM) sample preparation with Spurr's resin 118
6.3 Results and Discussion 119
6.4 Conclusion 137

**CHAPTER 7.0- ANTIBACTERIAL STUDIES TOWARDS *IN VIVO* PLANT PARTS OF *Eurycoma longifolia*** 138

7.1 Introduction 138
7.1.1 Objective 140
7.2 Materials and Methods 141
7.2.1 Plant materials 141
7.2.2 Bacterial strains 143
7.2.3 Agar disc diffusion assay 143
7.2.4 Statistical analysis 144

7.3 Results and Discussion 145
7.4 Conclusion 172

**CHAPTER 8.0- GENERAL DISCUSSION** 173

**CHAPTER 9.0- GENERAL CONCLUSION** 175

9.1 Suggestions for further studies 176

**BIBILIOGRAPHY**

**APPENDIX**

**LIST OF PRESENTATIONS AND PUBLICATIONS**

# LIST OF FIGURES


| | | |
|---|---|---|
| Figure 2.1 | Gene map of pRi1724 plasmid in *Agrobacterium rhizogenes* MAFF 301724 | 24 |
| Figure 2.2 | The many beneficial uses of hairy root culture | 28 |
| Figure 3.1 | Profile for *Agrobacterium rhizogenes* at 600nm | 51 |
| Figure 5.1 | Intact and wounded roots and somatic embryos used in chemotaxis assay | 83 |
| Figure 5.2 | Chemotaxis assay of wild strains of *Agrobacterium rhizogenes* towards intact roots of *Eurycoma longifolia* after 24 hours | 87 |
| Figure 5.3 | Chemotaxis assay of wild strains of *Agrobacterium rhizogenes* towards intact roots of *Eurycoma longifolia* after 48 hours | 88 |
| Figure 5.4 | Chemotaxis assay of wild strains of *Agrobacterium rhizogenes* towards intact roots of *Eurycoma longifolia* after 72 hours | 89 |
| Figure 5.5 | Chemotaxis assay of wild strains of *Agrobacterium rhizogenes* towards wounded roots of *Eurycoma longifolia* after 24 hours | 92 |
| Figure 5.6 | Chemotaxis assay of wild strains of *Agrobacterium rhizogenes* towards wounded roots of *Eurycoma longifolia* after 48 hours | 93 |
| Figure 5.7 | Chemotaxis assay of wild strains of *Agrobacterium rhizogenes* towards wounded roots of *Eurycoma longifolia* after 72 hours | 94 |
| Figure 5.8 | Chemotaxis assay of wild strains of *Agrobacterium rhizogenes* towards intact somatic embryos of *Eurycoma longifolia* after 24 hours | 97 |
| Figure 5.9 | Chemotaxis assay of wild strains of *Agrobacterium rhizogenes* towards intact somatic embryos of *Eurycoma longifolia* after 48 hours | 98 |
| Figure 5.10 | Chemotaxis assay of wild strains of *Agrobacterium rhizogenes* towards intact somatic embryos of *Eurycoma longifolia* after 72 hours | 99 |

| | | |
|---|---|---|
| Figure 5.11 | Chemotaxis assay of wild strains of *Agrobacterium rhizogenes* towards wounded somatic embryos of *Eurycoma longifolia* after 24 hours | 102 |
| Figure 5.12 | Chemotaxis assay of wild strains of *Agrobacterium rhizogenes* towards wounded somatic embryos of *Eurycoma longifolia* after 48 hours | 103 |
| Figure 5.13 | Chemotaxis assay of wild strains of *Agrobacterium rhizogenes* towards wounded somatic embryos of *Eurycoma longifolia* after 72 hours | 104 |
| Figure 7.1 | The placement of the disc containing the crude plant extracts for the antibacterial assay | 144 |
| Figure 7.2 | Inhibition zone (mm) for *Shigella flexneri* when tested with extract from various parts of *in vivo Eurycoma longifolia* | 147 |
| Figure 7.3 | Inhibition zone (mm) for *Escherichia coli* when tested with extract from various parts of *in vivo Eurycoma longifolia* | 151 |
| Figure 7.4 | Inhibition zone (mm) for *Pseudomonas aeruginosa* when tested with extract from various parts of *in vivo Eurycoma longifolia* | 155 |
| Figure 7.5 | Inhibition zone (mm) for *Staphylococcus aureus* when tested with extract from various parts of *in vivo Eurycoma longifolia* | 159 |
| Figure 7.6 | Inhibition zone (mm) for *Bacillus cereus* when tested with extract from various parts of *in vivo Eurycoma longifolia.* | 163 |
| Figure 7.7 | Inhibition zone (mm) for *Bacillus subtilis* when tested with extract from various parts of *in vivo Eurycoma longifolia* | 167 |

# LIST OF PLATES


| | | |
|---|---|---|
| Plate 2.1 | *Eurycoma longifolia* tree that grows in Universiti Sains Malaysia | 10 |
| Plate 3.1 | The morphology of wild strain of *Agrobacterium rhizogenes* MAFF 106590 using light microscope | 54 |
| Plate 3.2 | The morphology of wild strain of *Agrobacterium rhizogenes* MAFF 106591 using light microscope | 54 |
| Plate 3.3 | The morphology of wild strain of *Agrobacterium rhizogenes* MAFF 210265 using light microscope | 55 |
| Plate 3.4 | The morphology of wild strain of *Agrobacterium rhizogenes* MAFF 301726 using light microscope | 55 |
| Plate 3.5 | The morphology of wild strain of *Agrobacterium rhizogenes* MAFF 720002 using light microscope | 56 |
| Plate 3.6 | The morphology of wild strain of *Agrobacterium rhizogenes* MAFF 106590 using scanning electron microscope (SEM) | 57 |
| Plate 3.7 | The morphology of wild strain of *Agrobacterium rhizogenes* MAFF 106591 using scanning electron microscope (SEM) | 57 |
| Plate 3.8 | The morphology of wild strain of *Agrobacterium rhizogenes* MAFF 210265 using scanning electron microscope (SEM) | 58 |
| Plate 3.9 | The morphology of wild strain of *Agrobacterium rhizogenes* MAFF 301726 using scanning electron microscope (SEM) | 58 |
| Plate 3.10 | The morphology of wild strain of *Agrobacterium rhizogenes* MAFF 720002 using scanning electron microscope (SEM) | 59 |
| Plate 3.11 | The morphology of adhesion of wild strains of *Agrobacterium rhizogenes* MAFF 106591 using scanning electron microscope (SEM) | 63 |
| Plate 3.12 | The morphology of adhesion of wild strains of *Agrobacterium rhizogenes* MAFF 106590 using scanning electron microscope (SEM) | 63 |
| Plate 4.1 | Histological observations of transverse sections of cotyledon of *Eurycoma longifolia* | 70 |

| | | |
|---|---|---|
| Plate 4.2 | Histological observations of transverse sections of embryo of *Eurycoma longifolia* | 72 |
| Plate 4.3 | Histological observations of transverse sections of embryo of *Eurycoma longifolia* that reveals the vascular system development | 74 |
| Plate 4.4 | Histological observations of transverse sections of embryo of *Eurycoma longifolia* that reveals the vascular system development | 75 |
| Plate 5.1 | *Agrobacterium rhizogenes* MAFF 210265 attachment photos on the root explant | 95 |
| Plate 5.2 | Chemotaxis assay for roots and somatic embryos of *Eurycoma longifolia* using swarm agar plate method | 100 |
| Plate 6.1 | Observation on 3 weeks old germinated seedling of *Eurycoma longifolia* after subjected to transformation at its hypocotyl region with *Agrobacterium rhizogenes* MAFF 210265 | 122 |
| Plate 6.2 | Observation on 3 weeks old germinated seedling of *Eurycoma longifolia* after subjected to transformation at its hypocotyl region with *Agrobacterium rhizogenes* MAFF 210265 | 123 |
| Plate 6.3 | Observation on 3 weeks old germinated seedling of *Eurycoma longifolia* after subjected to transformation at its hypocotyl region with *Agrobacterium rhizogenes* MAFF 301726 | 124 |
| Plate 6.4 | Observation on 3 weeks old germinated seedling of *Eurycoma longifolia* after subjected to transformation at its hypocotyl region with *Agrobacterium rhizogenes* MAFF 720002 | 125 |
| Plate 6.5 | Observation of the untransformed and transformed hairy roots using scanning electron microscope (SEM) | 127 |
| Plate 6.6 | *Agrobacterium rhizogenes* MAFF 210265 attachment photos on hypocotyl region. | 128 |
| Plate 6.7 | *Agrobacterium rhizogenes* MAFF 210265 integration into the genome of *Eurycoma longifolia* observed using transmission electron microscope (TEM) | 129 |
| Plate 6.8 | Genome of the untransformed and transformed roots of *Eurycoma longifolia* | 132 |

| | | |
|---|---|---|
| Plate 6.9 | Detection of the integration of the *rol* genes sequence | 133 |
| Plate 7.1 | *Eurycoma longifolia* plant used in antibacterial assay | 142 |
| Plate 7.2 | Inhibition zone (mm) for *Shigella flexneri* when tested with extract from various parts of *in vivo Eurycoma longifolia* | 148 |
| Plate 7.3 | Inhibition zone (mm) for *Escherichia coli* when tested with extract from various parts of *in vivo Eurycoma longifolia* | 152 |
| Plate 7.4 | Inhibition zone (mm) for *Pseudomonas aeruginosa* when tested with extract from various parts of *in vivo Eurycoma longifolia* | 156 |
| Plate 7.5 | Inhibition zone (mm) for *Staphylococcus aureus* when tested with extract from various parts of *in vivo Eurycoma longifolia* | 160 |
| Plate 7.6 | Inhibition zone (mm) for *Bacillus cereus* when tested with extract from various parts of *in vivo Eurycoma longifolia* | 164 |
| Plate 7.7 | Inhibition zone (mm) for *Bacillus subtilis* when tested with extract from various parts of *in vivo Eurycoma longifolia* | 168 |

## LIST OF ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| bp | base pairs |
| h | Hour |
| kb | kilo base |
| KB | human cervical carcinoma cell line |
| LB | Luria Bertani |
| MS | Murashige and Skoog |
| OD | optical density |
| PCR | polymerase chain reaction |
| Ri | root inducing plasmid of *Agrobacterium rhizogenes* |
| T-DNA | transfer DNA |
| v/v | volume per volume |
| *vir* | virulence (gene, operons) of *Agrobacterium rhizogenes* |
| w/v | weight per volume |

**PENGHASILAN AKAR RERAMBUT DARI POKOK SUKAR-DI-TRANSFORMASI *Eurycoma longifolia* (TONGKAT ALI) MENGGUNAKAN STRAIN LIAR *Agrobacterium rhizogenes* DENGAN KAJIAN ANTIBAKTERIA**

## ABSTRAK

Penghasilan akar rerambut dari pokok herba penting yang sukar-di-transformasi iaitu *Eurycoma longifolia* akan memastikan bekalan yang berterusan untuk metabolit sekunder dan seterusnya mengurangkan penuaian pokok herba ini dari liar. Analisis morfologi dan histologi untuk biji *Eurycoma longifolia* menggunakan mikroskop cahaya menunjukkan struktur biji pokok herba penting *Eurycoma longifolia* pada peringkat pertumbuhan yang berbeza. Fasa pertumbuhan biji dan perkembangan sistem vaskular semasa percambahan biji memberikan informasi yang betul dan tepat tentang perkembangan kotiledon *Eurycoma longifolia.* Biji merupakan organ penyimpan yang membantu dalam penghasilan akar rerambut, kerana mempunyai sistem pergerakan air atau saluran trakea yang merupakan lokasi utama untuk jangkitan oleh *Agrobacterium rhizogenes*. Ujian pergerakkan kemo menggunakan kaedah 'swarm agar plate' memulakan proses penjakitan bakteria terhadap sel tumbuhan dan seterusnya menyalurkan ciri baik kepada perumah. Pergerakkan kemo yang kuat dan positif dilihat pada semua bakteria yang diuji terhadap akar yang ditumbuh di makmal dan pada sel somatik embrio. Akar rerambut berjaya dihasilkan menggunakan strain liar *Agrobacterium rhizogenes* MAFF 210265, 301726 dan 720002 pada lokasi hipokotil biji Eurycoma *longifolia.* Amplikasi gen *rol* pada 1100bp menggunakan analisis PCR mengesahkan intergrasi T-DNA dari Ri plamid di akar rerambut. Pada masa yang sama, ujian anti bakteria dilakukan menggunakan beberapa bahagian pokok *Eurycoma longifolia* menggunakan kaedah penyerapan cakera menunjukkan bahawa ekstrak dari

akar *Eurycoma longifolia* menunjukkan aktiviti anti bakteria yang tinggi dan berkesan pada semua bakteria patogenik yang diuji seperti *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, *Shigella flexneri* ATCC 12022 dan *Bacillus subtilis* (CDR). *Eurycoma longifolia* memberi banyak kebaikkan maka ia sesuai dijadikan sebagai tumbuhan ideal untuk kajian. Penjanaan akar rerambut dari *Eurycoma longifolia* akan menguntungkan industri ubatan kerana metabolit sekunder dikaitkan dengan pembezaan akar menggunakan bahan permulaan yang rendah.

**INDUCTION OF HAIRY ROOT CULTURES FROM DIFFICULT-TO-TRANSFORM *Eurycoma longifolia* (TONGKAT ALI) USING WILD STRAINS OF *Agrobacterium rhizogenes* WITH ANTIBACTERIAL STUDIES**

## ABSTRACT

Production of hairy roots from the difficult-to-transform medicinally important plant *Eurycoma longifolia* will ensure continuous supply of secondary metabolites and thus minimizes the harvesting of this plant from the wild. Seed morphology and histology analysis of *Eurycoma longifolia* by light microscopes revealed seeds structures of this important medicinal plant at different growing stages. The seed development phases and the development of the vascular system on the progression of germination provide the insight of the actual and accurate information on the cotyledon development period. Seeds being the storage organ may facilitate the generation of the hairy roots, as it evidently has the essential features like tracheas, which are the main site of infection for *Agrobacterium rhizogenes*. Chemotaxis using the swarm agar plate method initiates the process of bacterial infection towards the plant cells and thus conferring beneficial attributes to the host. Strong positive chemotactic response was observed in most of the tested bacteria strains towards the *in vitro* root and somatic embryos. Hairy roots were successfully initiated using three wild strains of *Agrobacterium rhizogenes* namely MAFF 210265, MAFF 301726 and MAFF 720002 at the hypocotyls region of *Eurycoma longifolia*. Amplification of the *rol* gene at 1100bp by PCR analysis confirmed the T-DNA integration of the Ri plasmid in the hairy roots. In addition, the antibacterial assay conducted for the *in vivo* plant parts of *Eurycoma longifolia* using disc diffusion method reveals that the crude extract obtained from the roots, possesses the highest amount of the antibacterial substance that works

well with the most of the tested pathogenic bacteria like *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, *Shigella flexneri* ATCC 12022 and *Bacillus subtilis* (CDR). Thus, the many beneficial properties of *Eurycoma longifolia* makes it an ideal plant to be researched and studied extensively. Generating hairy roots in *Eurycoma longifolia* will be highly beneficial to the pharmaceutical industry with valuable secondary metabolites, which is directly linked to its root differentiation at a low biomass starting material.

# CHAPTER 1.0
# GENERAL INTRODUCTION

In recent years there has been much hype on the benefits attributed by medicinal plants. Many researchers validate scientifically the claims of the indigenous people on the use of locally found and indigenously belonging medicinal plants, which have been used for generations for treating disease and as beneficial health supplements (Rajeev and Karim, 2010). There are also seems to be growing interest by the mainly pharmaceutical and some other companies worldwide to commercial the parts or the whole plant of the medicinally valuable plant. The commercializations are often in the forms of pills, supplements, condiment, in food preparations, ingredient of health supplements or a health drink. The success of these companies attributed with the growing interest of people in asserting a good health and also for disease prevention. Therefore to meet the ever increasing demands, there have been massive up rooting of many valuable plants from nature to meet the demands (Sobri et al., 2005a).

The Global Industry Analyst Incorporation has released the comprehensive global report on Herbal Supplements and Remedies market which reported that the global herbal supplements and remedies market is forecast to reach US$93.15 billion by the year 2015. The increase in the global herbal market is spurred by increasing incidence of aging population and consumer awareness about general health and well being (Global Industry Analysts, 2011). The East Coast Economic Region (ECER) proposed and being implemented in Malaysia intends to position itself as a major player in the herbal industry that aims to draw in investments for upstream, midstream and

downstream activities. In a statement, Nor Mohamed (2011) said the region had been earmarked as a key implementer to develop the herbal industry under the New Economic Model's Entry Point Projects (EPP), in which some RM110mill would be invested in four herbal plantations named in the EPP. It was also estimated that once completed, the ECER Herbal Plantation project is expected to generate a gross national income (GNI) amounting to USD1.016 billion or RM3.25 billion by 2020, while creating over 2,500 new jobs and 530 contract farmers (Nor Mohamed, 2011). In addition, it was also reported that, the use of herbal plants and their related products had become increasingly important worldwide over the past two decades. Global trade of natural plant products is projected to triple by 2020, with the herbal medicine market expected to grow by 10 and 20 percent. On the domestic front, the herbal industry in Malaysia is estimated to grow at the rate of 15 percent per annum, with the market value rising from 7 billion ringgit in 2010 to some 29 billion Ringgit in 2020 (Nor Mohamed, 2011).

In 2001, the Global Diversity Outlook recognized Malaysia as one of the 12 mega-diversity centers of the world (Steven, 2009). As reviewed by Steven (2009) in the 1$^{ST}$ edition of Burkill et al.(1935) on Dictionary of the Economic Products of the Malay Peninsula noted that there are about 1,200-1,300 medicinal plants of Malaysia (Burkill, 1966). Out of more than 20,000 species of vascular plants, about 10%, or approximately 2,000 species, have documented to have medicinal properties (Ananymous, 2000). Raw materials for herbal medicine are often imported from China, India, Indonesia, Hong Kong, Taiwan, and the United States. Even with market increases, Malaysia is thought to produce between only 5-10% of herbal medicines used

by Malaysians, with the rest imported from other countries (Batugal et al., 2004; Chang and Ali, 2004; Steven, 2009).

*Eurycoma longifolia* Jack (Tongkat Ali) has emerged as one of the most intriguing medicinal plants of Malaysia (Steven, 2009). Currently, the most common method of propagating *Eurycoma longifolia* plant is through seeds. However, being a recalcitrant plant, the seeds have a low precentage of germination and it takes a long time to germinate due to extremely immature state of zygotic embryo at the time of dispersal. The gradual dissapearance of this plant due to the indiscriminate collection of taproot from the forest to be used as the raw material for many health related and supplement preparation industries. As conclusion, the plant needed to be rapidly mass-multiplied on a commercial scale to comply with the need of the herbal and pharmaceutical industry (Sobri et al., 2005a). Therefore, *in vitro* techniques can be an important alternative approach to produce useful plant chemical products from *Eurycoma longifolia* (Luthfi and Chan, 2002; Sobri et al., 2005 a,b).

The limited supply of plant materials from *Eurycoma longifolia* for the recovery of active compounds has stipulated to the development of alternative *in vitro* methods for the production of active compounds. One such method is the generation of hairy roots cultures. The *in vitro* culture of hairy roots appears as an interesting biotechnological system for naturally producing secondary metabolites which are in the case of *Eurycoma longifolia* the secrection of many desired compounds and secondary metabolites are associated with its roots.

Hairy roots cultures offers numerous advantages, due to primarily of their fast growth rate in the phytohormone-free medium. The greatest advantage of hairy roots is that hairy root cultures can accumulate phytochemicals more or equal than the parent plants and are usually stable in their biosynthetic capacity (Ono and Tian, 2011). In addition, when hairy root cultures are grown optimally in liquid cultures, hairy roots can be grown in industrial scale bioreactors and thus provides convenient, abundant and sustainable source of phytochemicals for utilization (Ono and Tian, 2011). Furthermore, many valuable secondary metabolites are synthesized in roots *in vivo* and often synthesis is linked to root differentiation (Mukundan et al., 1998). Hairy roots would be the best choice for metabolic engineering of the secondary metabolite pathways to enhance the accumulation and secretion of high value metabolites (Kim et al., 2002). Thus this attribute makes hairy roots a better choice compared to propagation through tissue culture method, which is a very laborious process (Monica et al., 2011a). Moreover, hairy root system is more advantageous because the tissue culture process must be done under sterile laboratory conditions using sterile media on the other hand the hairy root system can be induced and grown without the laboratory condition through the generation of composit plants (Christopher et al., 2006; Monica et al., 2011b), which not only saves the production cost but also increases many fold the yield of the secondary metabolite production in a much simpler way. Furthermore, the secondary metabolites can be harvested directly with few extraction steps compared to the conventional tissue culture techniques (Monica et al., 2011b).

Generating the hairy roots using the wild type *Agrobacterium rhizogenes*, which is a natural genetic transformants will be highly beneficial. This is because the wild type of *Agrobacterium rhizogenes* present naturally in the wild. Due to this attribute, wild type *Agrobacterium rhizogenes* are free from the legal controls of Genetically Modified Organisms (GMO) (Watanabe et al., 2004). In addition, genetic transformation mediated by *Agrobacterium* are affected by factors like explant genotype, structure and type, bacterial strains and cell density of *Agrobacterium* used during inoculation, inoculation period, co-culture medium, surfactants in the inoculation medium and induction agents like acetosyringone, which is added in the inoculation and co-culture media (Ozawa, 2009; Karthikeyan et al., 2011). Furthermore, Khanna and Daggard (2003) and Tyagi et al. (2007) reported that the use of super binary vectors carrying additional *vir* genes, supplemented with 100 to 250 µM acetosyringone to the inoculation and co-cultivation media, and the modification of the polyamine ratio in the regeneration medium greatly improved the final transformation efficiency of 22.2% in rice and 3.9% in wheat respectively (Karthikeyan et al., 2011). The acetosyringone was reported to interact at specific temperature and an acidic environment to promote the expression of *Agrobacterium vir* genes (Terada et al., 2004).

Benefits that we can obtain from the research includes providing new informations and tools for the optimization of induction and production of *Eurycoma longifolia* hairy roots cultures from the genetic transformation technology. This information is vital as it enable us to formulate the optimum medium and conditions for the optimal production of the hairy roots. This work is the first report on the *in vitro* induction of *Eurycoma longifolia* in generating hairy root cultures.

In future studies, after the optimization of the growth parameters, quantitative measurements of many important secondary metabolites associated with the roots of *Eurycoma longifolia* will be done. One such compound is the 9-methoxycanthin-6-one alkaloid compound. The 9-methoxycanthin-6-one alkaloid compound from the hairy roots of *Eurycoma longifolia* demonstrated significant *in vitro* cytotoxicity against human lung cancer (A-549) and human breast cancer (MCF-7) cell lines (Kuo et al., 2003). In addition, the 9-methoxycanthin-6-one compound in *Eurycoma longifolia* was observed to possibly have anti-proferative effect in ovarian cancer cells and also antimalarial activity (Nurhanan et al., 2002). A study was carried out by Rosli et al. (2009) on the improvement of 9-methoxycanthin-6-one compound productivity from callus cultures of *Eurycoma longifolia* concluded that factors that affects 9-methoxycanthin-6-one accumulation in callus cultures are like media compositions and physical factors. In addition, Luthfi (2000) and Rosli et al. (2009) reported that, the thin layer chromatographic (TLC) method can be utilized in determining the content of 9-methoxycanthin-6-one compound in callus and cell suspension cultures of *Eurycoma longifolia*.

Thus, the present study serves as the fundamental works which will pave the way for the development of standardized commercial *Eurycoma longifolia* (Tongkat Ali) formulations as herbal remedies. Production of pill and capsules containing *Eurycoma longifolia* compound will be materialised in Universiti Sains Malaysia (USM) in near future.

## 1.1 Objectives

**This research was carried out with the following objectives:**

a. To study the growth phase and determine the suitable time for all the wild strains of *Agrobacterium rhizogenes* to be used in generating successful transformation event,

b. To investigate the development phases of the seeds of *Eurycoma longifolia* and to determine the suitable physiological stage to be used as starting material during the induction of hairy roots,

c. To conduct chemotaxis assay for all the wild strains of *Agrobacterium rhizogenes* towards the explants of root and somatic embryo of *Eurycoma longifolia*

d. To induce hairy root cultures of *Eurycoma longifolia* using wild strains of *Agrobacterium rhizogenes,*

e. To carry out molecular analysis using polymerase chain reaction method (PCR) as to confirm the integration of the *rol* genes from *Agrobacterium rhizogenes* into the transformed roots,

f. To conduct antibacterial assay on *in vivo Eurycoma longifolia* explants using pathogenic bacteria.

# CHAPTER 2.0

# LITERATURE REVIEW

## 2.1 Introduction

### 2.1.1 Locality

Universiti Sains Malaysia (USM) main campus, Minden, Penang has an area of approximately 238 hectare. The 144 hectare is the original east side and the extension 94 hectare is on the west side. The west side located on steep hill slopes 300m above sea level (Lim et al., 2008). There are about 108 species from 40 families of trees in the main campus which includes families of Simaroubaceae, Leguminosae and Sapindaceae (Nurhanis, 2011).

*Eurycoma longifolia* can be found mainly around the behind gate of School of Biological Sciences, USM. As reported by Nurhanis (2011), the most commonly found *Eurycoma longifolia* plant in the main campus of Universiti Sains Malaysia is *Eurycoma longifolia* Jack and the plant is deposited in herbarium with the ascension number of 11050/SD0001.

### 2.1.2 Classification

To date there are only four different spesies of *Eurycoma* that have been reported, which includes *Eurycoma longifolia, Entomophthora apiculata, Polyathia bullata* and *Goniothalamus* sp. (Kamarudin and Latiff, 2002; Aziz et al., 2003). Among them, the most popularly sought after for its beneficial attributes is *Eurycoma longifolia*. Botanical classification of *Eurycoma longifolia* classifies it to the division of

Magnoliophyta and further classified under dicotyledonous plant which belongs to the order of Geraniales, family of Simaroubaceae, genus *Eurycoma* and species *longifolia.*

This tropical herbal plant can be found extensively in forests of South-East Asia countries like Malaysia, Indonesia, Thailand, Myanmar, Vietnam and Cambodia. Depending on the places and countries that this plant is found the local names given are such as Long Jack, Malaysian Ginseng, Local Ginseng, Natural Viagra, Pasak Bumi, Payung Ali, Penawar Pahit, Setunjang Bumi, Bedara Pahit, Tongkat Baginda, Pokok Syurga, Tongkat Ali Hitam, Pokok Jelas, Cay ba binh, Ian-don, and Jelaih, ae phan chan, plaalai phuenk, phiak, tho nan (Rajeev and Karim, 2010).


**Plate 2.1: *Eurycoma longifolia* tree that grows in Universiti Sains Malaysia.** The whole trees can grow up to few meters high (A). Plant parts of *Eurycoma longifolia* which consist of (B) seeds, (C) leaves, (D) branches, (E) roots and (F) stem.

## 2.2 Tree Morphology

*Eurycoma longifolia* has thin stem (Plate 2.1[A and F]) that can grow up to 15m tall. Each of the leaves (Plate 2.1[C]) is about 20-40 cm long with 13-41 leaflets and the leaves are spirally arranged (Plate 2.1[D]). The flowers found on *Eurycoma longifolia* are dioecious. It means that the male and female flowers are found on different trees. The flowers are produced in large panicles. Each flower of the flowers has 5-6 minute petals. The fruit of *Eurycoma longifolia* is yellow to light green and becomes red to blackish red when ripe (Nurhanis, 2011). The size of each of the fruit is about 1-2 cm long and 0.5-1 cm broad (Plate 2.1[B]).

## 2.3 Ethnobotany Uses

For many years, almost all parts like bark, roots, seeds and stem core (Plate 2.1) of *Eurycoma longifolia* (Tongkat Ali) plant have been used traditionally for medicinal purposes and taken as supplements. In traditional practices, the roots (Plate 2.1[E]) being the most valuable part of the plant was believed to posses healing attribute were boiled to obtain its extract and will be either consumed directly or mixed with other herbs or provisions. For many generations, the various plant parts are believed to posses antimalarial, aphrodisiac and anti-pyretic activities, treatment for aches, persistent fever, malaria, sexual insufficiency, dysentery, glandular swelling, ulcers, prevention of gum diseases and for the treatment of sexually transmitted diseases like syphilis and gonorrhea (Rajeev and Karim, 2010) and as health supplements (Perry, 1980; Darise et al., 1982; Darise et al., 1983; Kuo et al., 2003) for restoring energy, vitality, enhancing

blood flow and functioning as a herbal ingredient for women after child birth (Ismail et al., 1991; Yus et al., 2011).

### 2.4 Elucidations on *Eurycoma longifolia* compounds and its function

At present, there are many chemical compounds that have been isolated and characterized from *Eurycoma longifolia* with majority of them are from its roots. Chemical compounds that have been identified so far from *Eurycoma longifolia* are like canthin-6-one alkaloids, β-carboline alkaloids, quassinoids, quassinoid diterpenoids, eurycomaoside, tirucallane-type triterpenes, squalene derivatives, biphenylneolignans, eurycolactone, laurycolactone and eurycomalactone (Kardono et al., 1991; Itokawa et al., 1993; Morita et al., 1993a; Morita et al., 1993b; Ang et al., 2002; Bedir et al., 2003).

Interestingly many novel compounds are still being isolated from *Eurycoma longifolia* with specific importance given to its roots. Kuo et al. (2004) reported the isolation and charterisation of nearly sixty-five compounds from the roots of *Eurycoma longifolia*. Among these isolates, for the first time four quassinoids diterpenoids were identified namely eurycomalide A, eurycomalide B, 13β, 21-dihydroxyeurycomanol, and 5α, 14β, 15β-trihydroxyklaineanone. Chan et al. (1989) isolated quassinoid glycoside which is eurycomanol-2-O-β-Dglycopyranoside and eurycomanol which possess antimalarial activity. Tada et al. (1991) separated four quassinoids, pasak bumin-A, pasak bumin -B, pasak bumin -C and pasak bumin -D and discovered that pasak bumin-A and pasak bumin-B to exhibit potent antiulcer activity. Morita et al.

(1992) isolated two novel isomeric 2,2′-dimethoxy-4-(3-hydroxy-1-propenyl)-4′-(1,2,3-trihydroxypropyl) diphenyl ethers and two novel biphenyls, 2-hydroxy-3,2′,6′-trimethoxy-4′-(2,3-epoxy-1-hydroxypropyl)-5-(3-hydroxy-1-ropenyl)-biphenyl and 2-hydroxy-3,2′-dimethoxy-4′-(2,3-epoxy-1-hydroxypropyl)-5-(3-hydroxy-1-propenyl)-biphenyl from the stem of *Eurycoma longifolia*. In addition, Mitsunaga et al. (1994) isolated from the bark and wood five new canthin-6-one alkaloids (9, 10-dimethoxycanthin-6-one, 10-hydroxy-9-methoxycanthin-6-one, 11-hydroxy-10-methoxycanthin-6-one, 5, 9 dimethoxycanthin - 6- one and 9 -methoxy-3-methylcanthin-5, 6-Dione). From the leaves, seven types of quassinoids were isolated like lonilactone, 6-dehydrolonilactone, 11-dehydroklaineanone, 12-epi-dehydroklaineanone, 15β-hydroxyklaineanone, 14, 15 β-dihydroxyklaineanone and 15-β-O-acetyl-14-hydroxyklaineanone and these isolated compounds have been reported to possess anti-tumor promoting and anti-parasitic activities (Jiwajinda et al., 2001).

Even though various types of chemical compounds have been isolated and characterized from *Eurycoma longifolia*, reports on the bioactivity and the mechanism of action of the isolated compounds are limited (Rajeev and Karim, 2010).

### 2.5 Pharmacological properties of compounds isolated from *Eurycoma longifolia*

There are many studies done to scientifically validate and to support the evidence of the traditional uses of *Eurycoma longifolia*. Important works on the pharmacological properties have been grouped into four major areas like aphrodisiac

activities, antimalarial and anticancer activity, anti-diabetic properties, and antimicrobial activities.

### 2.5.1 Aphrodisiac activities

The root extracts of *Eurycoma longifolia* popularly well known for its exclusive feature of enhancing the virility and sexual prowess (Gimlette and Thomson, 1977). Ang and Sim (1997) concluded that *Eurycoma longifolia* is a potent stimulator of sexual arousal in sexually vigorous male rats with the absence of feedback from genital sensation. Furthermore, Ang and Ngai (2001) supports the use of the roots for aphrodisiac property. In addition, Ang and Lee (2002) provided evidence pertaining to changes in sexual behavior in specific to the orientation activities (anogenital sniffing, licking and mounting) among the middle-aged male rats after administering different fractions of the *Eurycoma longifolia* root extracts. Study conducted by Lin et al. (2001) reported that the crude ethanolic extract of the root of *Eurycoma longifolia* could decrease the basal release of testosterone, but increase the human chorionic gonadotropin (hcG)-induced production of testosterone by rat Leydig cells.

### 2.5.2 Antimalarial and anticancer activity

Many studies provided scientific base for the traditionally popular *Eurycoma longifolia* extracts against malaria. In their study, Chan et al. (1986) tested the extracts of *Eurycoma longifolia* for antiplasmodial activity against a multi-drug resistant Thailand strain (K-1) of *Plasmodium falciparum* under *in vitro* conditions. Furthermore, Ridzuan et al. (2007) reported the antimalaria properties of the root extracts of *Eurycoma longifolia* standardized extract (TA164) alone and in combination with

artemisinin *in vivo* and found that the combination treatment suppressed *Plasmodium yoelii* infection in the experimental mice. Chan et al. (2004) revealed that some of the quassinoids isolisolated like eurycomanone and 13,21- dihydroeurycomanone possessed potential antimalarial properties against *in vitro* culture of chloroquine-resistant *Plasmodium falciparum* and showed higher selectivity indices for cytotoxicity against human cervical carcinoma cell line (KB) cells.

In addition, Kardono et al. (1991) isolated and characterized five cytotoxic constituents from the roots of *Eurycoma longifolia* obtained from Kalimantan, Indonesia and found out that four of the alkaloids were found to possess cytotoxic effects against human cancer cell types like breast, colon, fibrosarcoma, lung, melanoma, human cervical carcinoma cell line (KB), KB-V1 (a multi-drug resistant cell line derived from KB) and murine lymphocytic leukemia (P-388). Jiwajinda et al. (2002) isolated six quassinoids from the leaves of this plant and studied their possible effects on anti-tumor promoting, antischistosomal and plasmodicidal activities by *in vitro* methods. Their results revealed that one of the tested compound 14, 15-β-dihydroxyklaineanone is a potent active compound for inhibition of tumor promoter-induced Epstein–Barr-virus activation (anti-tumor promotion).

Kuo et al. (2003) reported three new alkaloids from the roots of *Eurycoma longifolia*, which showed significant cytotoxicity against human lung cancer (A-549) and human breast cancer (MCF-7) cell lines. Furthermore, Kuo et al. (2004) isolated and identified nearly 65 compounds from the roots of *Eurycoma longifolia* and screened them for the potential cytotoxicity, anti-HIV and antimalarial activities by *in vitro*

assays. Among the compounds evaluated, nearly eight compounds demonstrated strong cytotoxicity toward human lung cancer (A-549) cell lines, while seven compounds exhibited strong cytoxicity towards human breast cancer (MCF-7) cell lines. Two of the compounds displayed potent antimalarial activity against the resistant *Plasmodium falciparum*.

Fractions of *Eurycoma longifolia* extract have also been reported to induce apoptosis in breast cancer cells (Tee and Azimahtol, 2005). Tee et al. (2007) elucidated the mode of action of F16 which is a plant derived pharmacologically active fraction, inhibited the proliferation of MCF-7 human breast cancer cells by inducing apoptotic cell death. In addition, Nurhanan et al. (2005) evaluated the extracts obtained from the root of *Eurycoma longifolia* have possible cytotoxic effect against KB, DU-145, RD, MCF-7, CaOV-3 and MDBK cell lines.

### 2.5.3 Anti-diabetic properties

Daily consumption of *Eurycoma longifolia* leaves and roots are believed to control blood sugar levels (Rajeev and Karim, 2010). Husen et al. (2004) reported about the possible hyperglycemic activity of *Eurycoma longifolia* in the rat model system. The purpose of their study was to determine the blood glucose lowering effect using *Eurycoma longifolia* extracts in normal glycemic and hyperglycemic rats. They concluded that, after the administration of *Eurycoma longifolia* extracts, positive results in hyperglycemic rats were obtained when 150 mg/kg of body weight of extract was used. Recently, Hussin et al. (2011) proposed that *Eurycoma longifolia* consumption does not take effect immediately. *Eurycoma longifolia* has to be consumed regularly

over time. In addition, they concluded that it is possible for a diabetic person who experiences sexual dysfunction to use *Eurycoma longifolia* concurrently with an anti-diabetic agent such as rosiglitazone so interactions occur may affect in enhancing the therapeutic effect.

### 2.5.4 Antimicrobial activities

Farouk and Benafri (2007) evaluated various extracts namely methanol, ethanol, acetone and aqueous extracts from *Eurycoma longifolia*. Different parts of this plant were used in their study like leaves, stem, and roots to test against antibacterial activity by using gram-positive and gram-negative bacteria. Their result proposed that, the extracts of leaves and stem were active on both gram-positive and gram-negative bacteria, except for two gram-negative bacteria namely *Escherichia coli* and *Salmonella typhi*. Their studies also revealed that aqueous leaves extract of *Eurycoma longifolia* possesses antibacterial activity against *Staphylococcus aureus* and *Serratia marscesens*. Surprisingly the root extracts did not show any antibacterial activity (Rajeev and Karim, 2010).

## 2.6 Current propagation method of *Eurycoma longifolia*

*Eurycoma longifolia* being the indigenous medicinal plant is popularly sought after for herbal remedies. Currently, the most common method of propagating *Eurycoma longifolia* plant is through seeds (Sobri et al., 2005a). However, being a recalcitrant plant, the seeds have a low precentage of germination and it takes a long

time to germinate due to extremely immature state of zygotic embryo at the time of dispersal. The gradual dissapearance of this plant due to the indiscriminate collection of taproot as the raw material for the drug preparations. Hence, it needs to be rapidly mass-multiplied on a commercial scale to comply with the need of the herbal and pharmaceutical industry (Sobri et al., 2005a,b). Therefore, *in vitro* techniques can be an important alternative approach to produce useful secondary metabolites from *Eurycoma longifolia.*

Currently, there are efforts to propagate this plant by the means of tissue culture either by regeneration of somatic embryogenesis or manipulations of hormones for callus induction from various explants of *Eurycoma longifolia* (Luthfi and Chan, 2002; Sobri et al., 2005a; Luthfi et al., 2009; Rosli et al., 2009; Maziah et al., 2010). Luthfi and Chan (2002) reported on the *in vitro* shoot organogenesis of *Eurycoma longifolia* Jack using suspension and callus cultures. In addition, the first successful micropropagation protocol for this important recalcitrant plant was developed by Sobri et al. (2005a). Lutfi et al. (2009) studied on the effects of various macronutrients, micronutrients and sucrose on growth and canthinone alkaloid (9-hydroxycanthin-6-one and 9-methoxycanthin-6-one) production in cell suspension cultures of *Eurycoma longifolia* Jack. Subsequently, they also reported on the optimization of macronutrients and micronutrients treatment for the cell suspension cultures and also for the canthinone alkaloid production.

In addition, Rosli et al. (2009) reported on the improvement of 9-methoxycanthin-6-one productivity, a compund believed to posses an anticancer

property, from callus cultures of *Eurycoma longifolia* and concluded that there were some factors affecting 9-methoxycanthin-6-one production in callus cultures such as different media compositions and physical factors. Subsequently, Maziah and Rosli (2009), reported on the methods of callus induction and extraction of 9-methoxycanthin-6-one from *Eurycoma longifolia* Jack explants with the emphasis on the tap and fibrous roots. Furthermore, Maziah et al. (2010) details on the success on generating callus cultures using leaf, petiole, rachis, stem, tap root, fibrous root, cotyledon and embryo segments of *Eurycoma longifolia* using various concentrations and types of auxin.

In addition, Monica et al. (2011a, b) describes in detail the possibility of inducing hairy roots from *Eurycoma longifolia* using wild strains of *Agrobacterium rhizogenes* strains MAFF 106590, 106591, 210265, 301726 and 720002. In the first report, Monica et al. (2011a) describes on the seed morphology and histology analysis of *Eurycoma longifolia* by light microscopes, which revealed seeds structures of this important medicinal plant at different growing stages. According to the published paper, the seed structures of *Eurycoma longifolia* consist of main regions such as epidermis, hypodermis, storage parenchyma and procambium. The cotyledon of *Eurycoma longifolia* develops into a complex and reticulate vascular system. The seed development phases and the development of the vascular system on the progression of germination provide the insight of the actual and accurate information on the *Eurycoma longifolia* cotyledon development period. This information is essential for using the seed as the source of inoculums for the production of the hairy root cultures. Seeds being the storage organ may facilitate the generation of the hairy roots,

as it evidently has the essential features like tracheas, which are the main site of infection for *Agrobacterium rhizogenes*.

Subsequently, Monica et al. (2011b) reported on chemotaxis movement assay of *Eurycoma longifolia* using wild and disarmed strains of *Agrobacterium rhizogenes.* They concluded that, strong positive chemotactic response was observed in most of the tested bacteria strains and all the tested strains of *Agrobacterium rhizogenes* showed positive chemotactic response towards the tested root and somatic embryos of the valuable medicinal plant, *Eurycoma longifolia*. Therefore, in the report it was concluded that induction of hairy roots is possible in *Eurycoma longifolia*.

## 2.7 *Agrobacterium rhizogenes*

*Agrobacterium rhizogenes* is a gram-negative, rod-shaped soil bacterium that belongs to the genus *Agrobacterium* (Hu and Du, 2006). *Agrobacterium rhizogenes* causes hairy-root disease which also known as root-mat disease. This bacterium induces the neoplastic growth of any parts of the plant cells to differentiate to form hairy roots (Veena et al., 2007). *Agrobacterium rhizogenes*-induced hairy roots are very similar in structure to wild-type roots with a few observable difference like root hairs are longer, more numerous and root systems are more branched and exhibit an agravitropic phenotype (Veena et al., 2007). Hairy roots are highly differentiated and can proliferate on phytohormone-free media, which distinguishes it from undifferentiated plant cell cultures. The hairy root disease mechanism has been exploited to develop a valuable biotechnological application known as hairy root cultures.

Wild-type *Agrobacterium rhizogenes* strains have previously been employed to incorporate foreign genes into plants (Kouchi et al. 1999; Narayanan et al.1999). Because of their virulence and host range, *Agrobacterium rhizogenes* have the potential to serve as a new vehicle for standard plant transformation purposes for wide variety of crop species for which efficient transformation systems are available (Porter, 1991). In nature, the *Agrobacteria* can be parasitic on a wide range of dicotyledenous plants, while *Rhizobia* can be narrowly symbiotic on legumes (Kazuki et al., 2001).

*Agrobacterium rhizogenes* can be divided basically into 3 major groups of agropine, mannopine, and cucumopine type strains. The grouping is based utilization of opines which is a low-molecular-weight molecules that can be metabolized by *Agrobacterium rhizogenes* that are produced by transformed root cells (Ono and Tian, 2011).

### 2.7.1 *Agrobacterium rhizogenes* mediated transformation

*Agrobacterium rhizogenes* strains contain a single copy of a large root inducing (Ri) plasmid. The Ri plasmid possesses transfer DNA (T-DNA) which consist of left T-DNA ($T_L$ -DNA) and right T- DNA ($T_R$ - DNA). $T_R$ -DNA contains genes homologous to the Ti plasmid tumor – inducing genes and the *tms* loci genes, two morphogenic loci of $T_R$ -DNA corresponds to the *tms* loci of Ti plasmid (White et al., 1985), which can directly synthesize auxin (Capone et al., 1989b). Genes of $T_L$- DNA, *rolA, rolB, rolC* and *rolD* direct the synthesis of a substance that reprograms the cells to differentiate

into roots under the influence of endogenous auxin (Zhu et al., 2000). T- DNA is transferred to the wounded plant cells and becomes stably integrated into the host genome (Chilton et al., 1982). This is because the repair mechanisms of the wounded plant cells such as plant recombination processes and DNA repair enzyme activities stimulates the intergration of T-DNA (Villemont et al., 1997; Daniel et al., 2011).

Hairy roots can be induced by the incorporation of a bacterial-derived segment of DNA transferred (T-DNA) into the chromosome of the plant cell (Taylor et al., 2006). The expression of genes encoded within the T-DNA promotes the development and production of roots at the site of infection. These are observed mostly on dicotyledonous plants. An important feature of *Agrobacterium rhizogenes*-induced roots is their unique ability to grow *in vitro* in the absence of exogenous plant growth regulators (Rao and Ravishankar, 2002). Transformants are selected by detecting the genes located in the T- DNA such as *rolA, rolB, rolC* and *rolD.*

### 2.7.2 Nucleotide sequence of plasmid PRi1724

The *Agrobacterium rhizogenes* strains used in this study were MAFF wild strains of *Agrobacterium rhizogenes* that were obtained from National Institute of Agrobiological Sciences, Japan. Thus, as in the case of other MAFF strains it possesses the pRi1724 plasmid. The complete nucleotide sequence of plasmid pRi1724 have been elucidated and reported by Kazuki et al. (2001). In their report, the pRi1724 plasmid were compared mainly with plasmids like symbiotic (Sym) plasmids Sym plasmid

(pNGR234a), nopaline-type Ti plasmid (pTi-SAKURA) and agropine-type Ri plasmid (pRiA4b). A mikimopine-type Ri plasmid, pRi1724 (Figure 2.1) of *Agrobacterium rhizogenes* MAFF301724 was selected (Shiomi et al., 1987) as a choice for nucleotide elucidation because of the following advantages such as pRi1724 is one of the most studied Ri plasmids and its physical map has been designed with the cosmid linking library (Tanaka and Oka, 1994) and lambda linking library (Moriguchi et al., 2000). Although, being smaller than a typical agropine-type Ri plasmid pRiA4b, the pRi1724 plasmid has high capability of T-DNA transfer and root induction (Huffman et al., 1984).

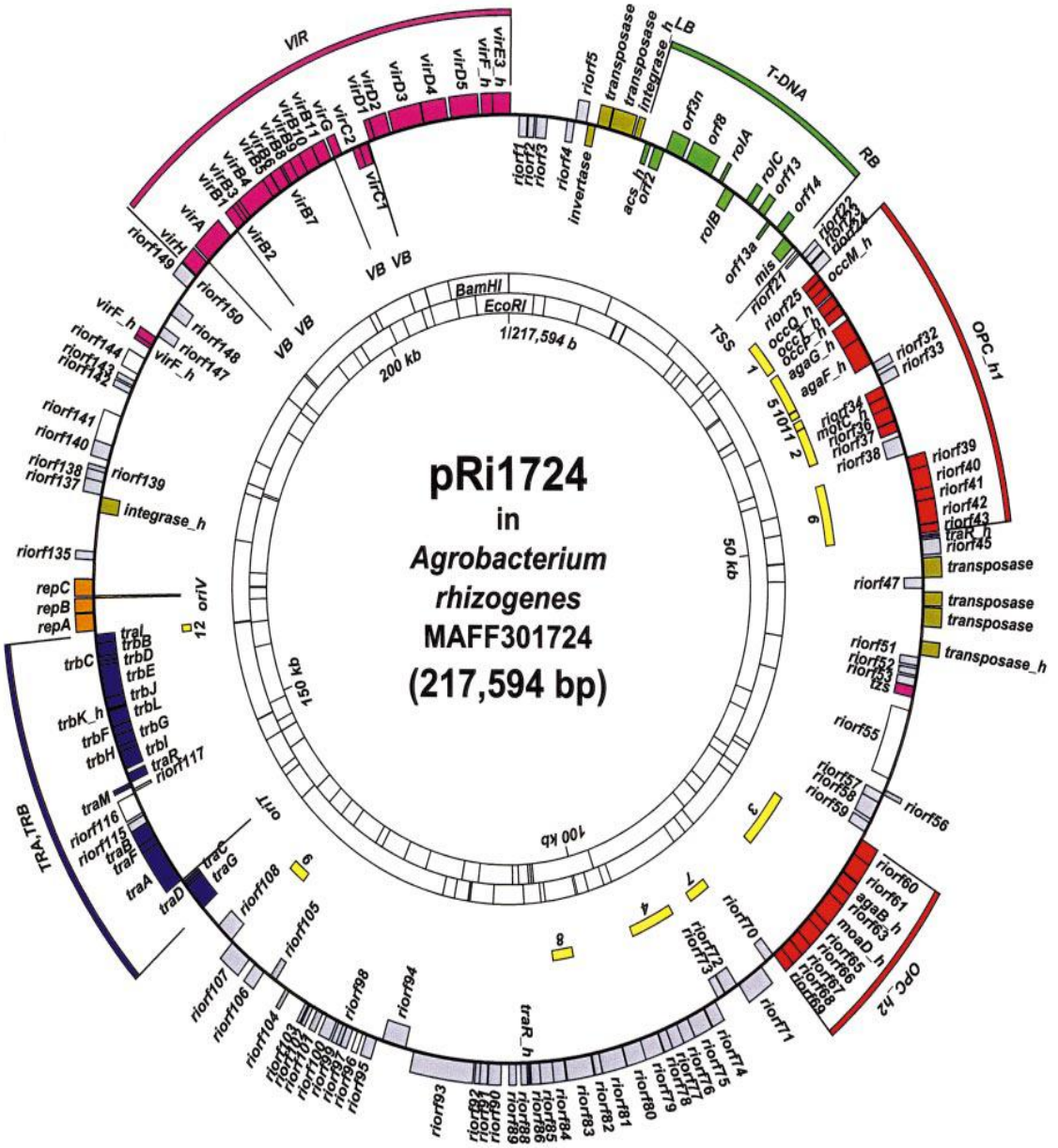


**Figure 2.1: Gene map of pRi1724 plasmid in *Agrobacterium rhizogenes* MAFF 301724.** The genes are illustrated with different colour to indicate different function, pink: virulence (*vir*) genes for plants, bright green: T-DNA-encoded genes including root-inducing genes, blue: conjugation (*tra* and *trb*) genes, red: putative opine catabolism (*opc*) genes, orange: vegetative DNA replication (*rep*) genes and dark green: *integrase*/*transposase* genes (Adapted from: Tsudzuki, 2000).

### 2.7.2.1 Features and Sequence of pRi1724 plasmid

As reported by Kazuki et al. (2001), a total of 173 open reading frames (ORFs) were estimated for pRi1724. These ORFs are asymmetrically distributed on the plasmid. About 115 ORFs are located at clockwise position and 58 ORFs are located at counter-clockwise position. In addition, the average G+C (guanidine and cytosine) content of pRi1724 is about 57.3 % is between the average G+C of tumor inducing (Ti) plasmid of pTi- SAKURA (56.0 %) and Sym (pNGR234a, 58.5 %) plasmids. The circular gene map of pRi1724 shows the distribution of G+C content of the entire pRi1724 DNA. In addition, the T-DNA region consist of 46.1 %, DNA replication (*rep*) genes region is 53.0 %, virulence genes regions are about 54.1 % and conjugation genes region (*tra* and *trb*) is about 60.4 % when compared to average G+C content of pRi1724 (Kazuki et al., 2001).

### 2.7.2.2 Gene organization and distribution

The ORFs in pRi1724 were grouped into 16 groups according to their predicted function and characteristics. Most of the ORFs show highest level of homology with chromosomal genes in other Gram-negative bacteria (Kazuki et al., 2001). About 58 % ORFs (100 ORFs) are involved in maintenance of the pRi1724 plasmid. The ORFs that are involved in the maintenance of this plasmid are virulence genes (*vir*), T-DNA region, conjugation genes region, replication (*rep*) genes and opine (*opc*) catabolism genes. The other ORFs that were not involved in maintaining the plasmid like encoding