



**UNIVERSITI PUTRA MALAYSIA**

***GROWTH AND ANTIOXIDANT CONTENT OF PEGAGA (*Centella asiatica* (L.) Urban), INOCULATED WITH PLANT GROWTH- PROMOTING RHIZOBACTERIA***

**ADILAH BINTI SURIMIN**

**FP 2017 23**



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RHIZOBACTERIA**

**By**

**ADILAH BINTI SURIMIN**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in  
Fulfillment of the Requirements for the Degree of Master of Science**

**November 2016**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

**GROWTH AND ANTIOXIDANT CONTENTS OF PEGAGA (*Centella asiatica* (L.) Urban) INOCULATED WITH PLANT GROWTH-PROMOTING RHIZOBACTERIA**

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**November 2016**

**Chairman : Professor Zulkifli Hj. Shamsuddin, PhD**  
**Faculty : Agriculture**

Pegaga (*Centella asiatica*) has been recognized as a medicinal plant which contains valuable antioxidant compounds; phenolic and flavonoid, the pharmacologically active ingredients which are beneficial for human body system. Unfortunately, these bioactive compounds are present at lower concentrations in plant extracts. Plant Growth-Promoting Rhizobacteria (PGPR) which work as elicitors, can be an effective method to stimulate growth and expression of antioxidants in Pegaga. The experiments were conducted under soilless conditions to clearly observe the effect of PGPR on plant growth and bioactive compound. Beneficial PGPR were isolated from Pegaga roots and was evaluated based on plant biochemical assays, formation of phytohormone Indole-3-acetic acid (IAA), N<sub>2</sub>-fixation, and P and K-solubilizations. From the 16 isolates, two bacterial strains C3E2 (UPMB30) and C1R1 (UPMB31) showed maximum plant growth promoting abilities and were further identified based on colony morphology, Gram-staining and characterized through 16S rRNA gene sequencing. UPMB30, an endobacterial isolate was 99% identified as *Bacillus subtilis* and UPMB31, a rhizobacterial isolate, as an *Stenotrophomonas maltophilia*. To achieve the second objective, a few factors and critical issues were verified to ensure these PGPR isolates could function under soilless condition. The selected PGPR strains were then exposed to various soilless culture conditions: pH (4.0, 5.0 and 6.0), fertilizer concentrations (50%, 100%, 200%) and bacterial population densities (10<sup>4</sup>cfu/mL, 10<sup>9</sup>cfu/mL and uninoculated) to determine the optimum combination of fertilizer and inoculum sized. The results showed high survivability and growth of bacterial strains in the following descending order; pH 4.0>5.0>6.0 and fertilizer concentration 50%>100%>200%. Application of 10<sup>4</sup>cfu/mL bacteria with 50% fertilizer rate increased 12% of yield and saves 50% of chemical fertilizer input. The high inoculum size, 10<sup>9</sup>cfu/mL however, reduced plant growth length and led to over colonization and possibly became harmful to the plant. Antioxidant and their interactions with PGPR were evaluated in the final experiment. Free radical scavenging assay (DPPH): 1,1- diphenyl-2- picrylhydrazyl; Total Phenolic Content

(TPC): Folin-Ciocalteu assay; and Total Flavonoid Content (TFC): Aluminium chloride method assay were analyzed. Results demonstrated high concentrations of DPPH, TPC and TFC in extracts taken from all the three parts of Pegaga (roots, leaves, stems), irrespective of PGPR strains. The increase in antioxidant synthesis presumably represents a defensive response to colonization by PGPR. Results showed all strains highly colonized in the roots ( $10^6$  to  $10^7$  CFU/mL). Highly reduced levels of proline content were observed in the inoculation treatment for all strains compared to the uninoculated control; UPMB10 (52.06  $\mu\text{g}/\text{fw}$ ), UPMB31 (44.33  $\mu\text{g}/\text{fw}$ ), UPMB30 (31.54  $\mu\text{g}/\text{fw}$ ) and uninoculated control (98.69  $\mu\text{g}/\text{fw}$ ). In conclusion, this study clearly indicated that two potential isolated strains (UPMB30 and UPMB31) could be applied as elicitors to significantly increase vegetative growth of Pegaga and antioxidant contents in the plants.



Abstrak tesis ini dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

**PERTUMBUHAN SERTA PENGHASILAN ANTIOKSIDA PEGAGA (*Centella asiatica* (L.) Urban), MELALUI BAKTERIA PENGGALAK PERTUMBUHAN POKOK**

Oleh

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Pegaga (*Centella asiatica*) telah dikenali sebagai tumbuhan ubatan yang mengandungi bahan antioksidan yang tinggi seperti fenolik dan flavonoid, yang juga merupakan bahan farmakologi aktif yang bermanfaat untuk sistem badan. Walaubagaimanapun, bahan bioaktif ini hadir di dalam ekstrak tumbuhan pada kuantiti yang amat sedikit. Dengan memanipulasikan tekanan pada pokok Pegaga dengan menggunakan PGPR boleh dijadikan sebagai kaedah berkesan dalam merangsang pertumbuhan pokok dan penghasilan antioksidan dalam Pegaga. Eksperimen telah dijalankan dibawah keadaan tanpa tanah untuk melihat lebih jelas keberkesanan PGPR pada kadar pertumbuhan dan penghasilan bahan bioaktif Pegaga. Bakteria berfaedah PGPR telah diasingkan dari akar pokok Pegaga dan dinilai menerusi aktiviti biokimia; pengikatan nitrogen, penglarutan P dan K dan penghasilan hormone asid indol-3-asetik (IAA). Daripada 16 bakteria, dua daripadanya C3E2 (UPMB30) dan C1R1 (UPMB31) telah menunjukkan penggalakan pertumbuhan pokok yang maksima dan seterusnya dikenalpasti melalui morfologi koloni, pewarnaan Gram dan melalui penjujukan gen 16SrRNA. Endobakteria UPMB30 telah dikenalpasti 99% kesamaan dengan *Bacillus subtilis*, manakala rhizobakteria UPMB31 sebagai *Stenotrophomonas maltophilia*. Bagi mencapai objektif kedua, ada beberapa faktor dan isu kritikal yang perlu dikenalpasti bagi memastikan PGPR mampu berfungsi di bawah keadaan penanaman tanpa tanah. PGPR yang terpilih ini telah didedahkan kepada pelbagai pH (4.0, 5.0, 6.0), kepekatan baja (50%, 100%, 200%) dan saiz inokulasi bakteria ( $10^4$  cfu/mL,  $10^9$  cfu/mL dan tanpa inokulasi). Kombinasi optima antara baja kimia dan saiz inokulasi ditentukan. Keputusan menunjukkan pertumbuhan dan ketahanan bakteria pada turutan menurun seperti berikut; pH 4.0 > 5.0 > 6.0 dan kepekatan baja, 50% > 100% > 200%. Kombinasi  $10^4$  cfu/mL inokulasi bakteria bersama 50% kepekatan baja telah meningkatkan hasil tanaman sebanyak 12% dan ini menjimatkan sebanyak 50% penggunaan baja kimia. Walaubagaimanapun, kepekatan saiz inokulasi yang tinggi,  $10^9$  cfu/mL, telah merencatkan pertumbuhan pokok, mungkin disebabkan oleh kepekatan inokulasi yang menjurus kepada kolonisasi yang tinggi dan terlalu padat dan mengakibatkan pokok

lemah dan mati. Interaksi antara PGPR dan antioksidasi Pegaga dinilai dalam eksperimen terakhir. Penangkapan radikal bebas: 1,1-difenil-2-pikrilhidrazil (DPPH), jumlah kandungan fenolik (TPC): asai Folin-Ciocalteu, dan jumlah kandungan flavonoid (TFC): asai Aluminium chloride, telah dianalisa. Endobakteria. Kandungan antioksidasi DPPH, TPC dan TFC yang tinggi telah diekstrak daripada tiga bahagian pokok Pegaga (akar, daun, batang), hasil dari inokulasi. Peningkatan kandungan antioksidasi mungkin daripada tindakbalas pertahanan pokok terhadap kolonisasi PGPR yang tinggi dibahagian akar ( $10^6$  to  $10^7$  CFU/mL). Kandungan prolin yang rendah didapati pada semua rawatan inokulasi; UPMB10 (52.06 µg/berat basah), UPMB31 (44.33 µg/berat basah), UPMB30 (31.54 µg/berat basah) dan tanpa inokulasi (98.69 µg/berat basah). Kesimpulannya, dua bakteria berpotensi ini (UPMB30 and UPMB31) mampu digunakan sebagai elisitor bagi meningkatkan hasil vegetatif Pegaga dan kandungan antioksidasi dalam pokok.



## ACKNOWLEDGEMENTS

Alhamdulillah to the uncreated the creator Almighty Allah, who granted me the opportunity and for His willingness that made the completion of this study possible. My unreserved gratitude goes to my supervisory committee, Prof. Dr. Zulkifli Hj. Shamsuddin, Assoc. Prof Dr. Wong Mui Yun and Assoc. Prof Dr. Radziah Othman for their numerous contributions, guidance, advice, suggestions and assistance. I would like to thank the Ministry of Higher Education for the KPT grant, which provided me the opportunity to pursue my Master degree. Acknowledgment also goes to my beloved parents and relatives for their love, support and encouragement. Special thanks for the help, cooperation and joyful time to my friends, colleagues and students: Mrs. Nur Farhana Che Hassan, Mrs. Siti Suheda Sofi, Ms. Zahidah Abd Razak, Ms. Nur Fatimah Ibrahim, Mr. Kuan Khing Boon and Mr. Mohd Yamin Shaari.



I certify that a Thesis Examination Committee has met on 28 November 2016 to conduct the final examination of Adilah binti Surimin on her thesis entitled "Growth and Antioxidant Content of Pegaga (*Centella asiatica* (L.) Urban), Inoculated with Plant Growth-Promoting Rhizobacteria" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15. March 1998. The Committee recommends that the student be awarded the Master of Science.

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## LIST OF ABBREVIATIONS

IUN	International Union for Conservation of Nature
NADH	Nicotinamide adenine dinucleotide
AMF	Arbuscular Mycorrhizal Fungi
PGPB	Plant growth-promoting bacteria
iPGPR	Intracellular plant growth promoting rhizobacteria
ePGPR	Extracellular plant growth-promoting rhizobacteria
TE	Trace elements
Nfb	Nitrogen fixing bacteria
KSB	Potassium solubilizing bacteria
PSB	Phosphate solubilizing bacteria
PCR	Polymerase chain reaction
BLAST	Basic Local Alignment Search Tool
PBS	Phosphate buffer saline
ROS	Reactive oxygen species
DPPH	Free radical scavenging assay
TPC	Total phenolic compound
TFC	Total Flavonoid compound
GAE	Gallic acid equivalents
CAE	Cathecin equivalent
TAE	Tannic acid equivalent
UNiCC	Microbial Culture Collection Unit

## CHAPTER 1

### INTRODUCTION

Pegaga is a perennial creeping plant of the genus *Centella* from the Apiaceae family (Hashim, 2011). It has been recognized as a medicinal plant and listed as the five prominent herbs in Malaysia. Pegaga contains valuable bioactive compounds such as antioxidants and triterpenoids, the pharmacologically active ingredients which are beneficial for body and brain system and potentially useful in the prevention of cancer, diabetes, and arteriosclerosis (Subathra et al., 2005). Pegaga has gained worldwide demand for pharmaceutical and cosmetic products in the last few years (James and Dubery, 2008; Devkota et al., 2010, Singh et al., 2010). International Union for Conservation of Nature (IUN) has reported Pegaga as an endangered plant (Sharma and Kumar, 1998). Thus, a well-directed cultivation of Pegaga is to ensure plant materials with high quality and quantities of biological compounds are continuously produced. A collection of Pegaga from natural populations contains various composition and concentrations of secondary metabolites due to different growth conditions and genetic distinction (Randriamampionona et al., 2007; Thomas et al., 2010; Devkota et al., 2010).

It is a very challenging process to get a balanced direct correlation between high secondary metabolite content and plant growth yield. From the primary pathways, various compounds are derived such as polysaccharides, sugars, proteins and fats which make up the bulk of the plant. However, the secondary products are present at a much lower concentration. A few techniques have been developed as an alternative to produce these metabolites such as plant cell culture. However, this technique is faced with another problem where the secondary metabolites produced could be minimally extracted. Plant cell culture on a large scale produced only a few compounds that satisfy the commercial and biological criteria. Based on similar principles but in combination with a new technique, some strategies for triggering the production of secondary metabolites have been developed by using elicitor, signal compound and abiotic stress (Yukimune et al., 1996; Zhao et al., 2000, 2001a, b and Zhang et al., 2004). Application of beneficial microorganisms as biotization has been tried. Biotization is a metabolic response of plant materials to microbial inoculation under *in vitro*-culture, thus, leading to biotic and abiotic stress resistance through development and physiological changes enhancement. PGPR co-cultured plantlets produce high yield vegetative and secondary metabolite production (Soundarapandian and Dhandayuthapani, 2010).

The inoculation of Plant Growth-Promoting Rhizobacteria (PGPR) has been reported to induce yield enhancement and secondary metabolite production in various crops. Many research with PGPR have shown beneficial effects with increase in vegetative growth through the production of plant growth regulators (Zahir et al., 2004), fixation of atmospheric nitrogen, increased root growth and nutrient uptake (nitrogen, phosphate, potassium), and production of siderophores (Lucy et al., 2004). PGPR also synthesize phytohormone which can trigger secondary metabolite production. Lorena et al (2013)

reported *Azobacter brasilense* and *Pseudomonas fluorescens* gave beneficial impacts by increasing essential oil (EO) composition and phenolic content in Marigold (*Tagetes minuta*).

To get a consistent result without interference from the physical and chemical interactions in soil, the studies were conducted using soilless culture as a growth medium. In justification, the study offers the possibility of enhancing plant growth rate and biological compound production in Pegaga by PGPR under soilless condition. Thus, the general objective of this study is to increase plant growth and bioactive compounds of Pegaga (*Centella asiatica*) under hydroponic condition. The specific objectives of the study are:

1. To isolate and characterize beneficial biochemical abilities of indigenous beneficial PGPR from Pegaga (*Centella asiatica*).
2. To determine the effects of varying PGPR dosages and fertilizer concentrations on vegetative growth and yield of Pegaga (Glasshouse)
3. To evaluate the effect of PGPR application at appropriate dosages and fertilizer concentration on antioxidant compounds of Pegaga (Glasshouse)

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