COLONIZATION OF *ORYZA SATIVA* ROOTS BY PLANT GROWTH-PROMOTING RHIZOBACTERIUM, *BACILLUS SPHAERICUS* UPMB10 AND SUBSEQUENT INOCULANT FORMULATION

By

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

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I dedicate the fruits of my labour to my late father, Pakirisamy Suppiah.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy.

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Chairman : Professor Zulkifli Hj. Shamsuddin, PhD

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The *Bacillus sphaericus* UPMB10 plant growth-promoting strain was selected for colonization and inoculant formulation studies. The UPMB10 strain is a Gram positive, endospore forming bacteria. The biochemical assay results showed that this strain was able to hydrolyze complex carbon source (potato starch) but did not utilize simple sugars. The UPMB10 strain was able to grow in the presence of 10 µg mL^{-1} of the antibiotic streptomycin and 10U of bacitracin which may be an important inherent characteristic in the competitiveness of this plant growth-promoting rhizobacterium. The results of the *in vitro* colonization study showed that *B. sphaericus* strain UPMB10 could successfully proliferate on and colonize the roots of *Oryza sativa* L. MR220 for up to 28 days after inoculation. Enumeration of bacteria colonizing the rice root indicated that the UPMB10 strain colonized both the rhizoplane and the root interiors at up to 10^8 and 10^4 cfu g^{-1} root tissue, respectively. The production of root cell wall degrading enzymes, cellulase

and pectinase, by this strain may have facilitated cellular entry. Scanning electron microscopy of colonization showed dense colonization at areas rich in root exudates specifically the elongation zone, junction of lateral roots and junctions of epidermal cells for up to 21 days. Clusters of microcolonies formed a mucigel within the mucilaginous sheath of the root. Colonies were also seen colonizing beneath what could possibly be the epidermal cuticle of the roots, especially in the relatively older primary roots. The colonization of the rice roots by this strain displayed adhesion structures which attached the cells with the root surface of rice and between each other. The process of root colonization also elicited a varying pattern of bacterial morphology during temporal colonization of rice roots. Normal rods of this strain were sized 0.5 – 0.6 µm x 1.5 – 1.9 µm. Some colonies of cells appeared rounder and fatter (0.9 – 1.1 µm x 1.3 – 1.8 µm) with widths twice that of the normal rods. Results also showed colonies elongating (length 5.3 – 6.2 µm) in readiness for cell replication possibly as a result of receiving sufficient nutrients from the colonized sites on the roots. The UPMB10 strain was successfully tagged with the green fluorescent protein (GFP) marker in the plasmid specifically constructed for this study (pSV101*gfp3*) by electroporation. The presence of GFP was confirmed by PCR, SDS-PAGE and Western blotting. The fluorescence signal of the transformants however, was too weak to be used to follow colonization on roots in non-sterile environments.

The effects of different bacterial liquid media, carrier materials (coirdust, ground oil palm fronds [GOPF] and GOPF amended with Kusokom®

compost), temperatures (20, 30 and 40° C), moisture potentials (pF 2.19 and 2.54) and storage period (6 months) on viable cell growth were evaluated. Tryptic soy broth (TSB) was found to be suitable for the cultivation of a high number of viable bacterial cells (10^8 cfu mL^{-1}) for long term inoculant production. Ground oil palm fronds (GOPF) + 25% Kusokom® compost was found to be a suitable carrier for maintaining and increasing the number of *B. sphaericus* UPMB10 viable cells (Log_{10} 9.28 cfu g^{-1}) at near room temperature (30 °C) for up 6 months. The results indicated that there were interactions between the factors studied for the inoculant formulation. Consequently, the growth and survival of UPMB10 in the carriers depended on the storage temperature and time (p=0.05). Highest viable growth in GOPF, coir dust, GOPF + 50% Kusokom® compost and GOPF + 25% Kusokom® compost was recorded at temperatures 20° C, 30° C, 40° C and 30° C respectively, and at storage time D21, D7, D28 and D14, respectively. Results of interactions between carrier and temperature indicated that growth and viability was more favourable at 30 ° C in GOPF and GOPF + 25% Kusokom® compost, while GOPF + 50% Kusokom® compost treatment was able to tolerate storage temperature of 40 ° C. In all treatments the viable population was significantly higher at D161 (Log_{10} 8.61 cfu g^{-1}) and D21 compared to D1 (Log_{10} 8.00 cfu g^{-1}). The outcome of this study showed the potential of producing a commercial inoculant from GOPF+ 25% Kusokom® compost for Malaysia and other tropical countries with high numbers of *B. sphaericus* UPMB10 that showed high colonizing ability.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah.

KOLONISASI AKAR *ORYZA SATIVA* OLEH RIZOBAKTERIUM PENGGALAK-PERTUMBUHAN POKOK, *BACILLUS SPHAERICUS* UPMB10 DAN FORMULASI INOKULAN

Oleh

PREMALATHA A/P PAKIRISAMY

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Pengerusi : Profesor Zulkifli Hj. Shamsuddin, PhD

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Kajian telah dijalankan untuk mengesan kolonisasi dan menghasilkan formulasi biobaja dengan bakteria penggalak-pertubuhan pokok iaitu *Bacillus sphaericus* UPMB10. Beberapa ujian yang mudah dijalankan seperti pewarnaan Gram, morfologi endospora dan koloni di atas plat agar serta ujian-ujian biokimia ke atas *B. sphaericus* UPMB10 untuk mengenalpasti strain tersebut dan mempastikan tidak berlaku mutasi atau kontaminasi yang boleh mempengaruhi keputusan kajian-kajian selanjutnya. Ujian biokimia menunjukkan bahawa strain ini dapat menghidrolisis sumber karbon kompleks seperti kanji kentang, tetapi tidak dapat menurunkan gula asas. Pertumbuhan strain UPMB10 tidak dicegah oleh 10 μg mL^{-1} antibiotik streptomisin, yang merupakan ciri penting dalam keupayaan penyaringan bakteria penggalak-pertubuhan pokok ini. Keputusan kajian kolonisasi akar padi (*Oryza sativa* L. MR220) oleh *B. sphaericus* UPMB10 secara *in vitro* menunjukkan strain ini berjaya mengkolonisasi dan berkembang pada

permukaan dan di dalam akar padi sehingga 28 hari selepas penginokulatan. Keputusan menunjukkan strain UPMB10 mengkolonisasi akar padi pada kadar sehingga 10^8 and 10^4 cfu g^{-1} tisu akar, masing-masing pada permukaan dan di dalam tisu akar. Bakteria UPMB10 ini juga dapat menghasilkan enzim penghakis dinding sel akar iaitu selulase dan pektinase yang boleh memudahkan kemasukkan sel-sel backteria ke dalan tisu akar pokok. Kajian menggunakan mikroskop pengimbas elektron (SEM) menunjukkan kepadatan koloni sel-sel bakteria UPMB10 yang tinggi sehingga 21 hari selepas penginokulatan pada zon pemanjangan akar, persimpangan pengeluaran akar sisi dan antara sel epidermis akar, iaitu di tempat-tempat "pembocoran nutrien" akar pokok berlaku. Pengumpulan mikrokoloni pada permukaan akar menghasilkan musijel dalam litupan musilaj akar. Koloni bakteria UPMB10 juga dikesan di bawah kutikel epidermis akar terutamanya pada kawasan akar yang lebih tua. Kajian ini juga menunjukkan kolonisasi bakteria UPMB10 menghasilkan struktur perlekatan sel bakteria dengan permukaan akar dan antara satu sama lain. Kajian-kajian sebelum ini dengan *Bacillus sphaericus* UPMB10 belum pernah menunjukkan struktur perlekatan sebegini. Kajian proses kolonisasi akar juga menampilkan bentuk sel bakteria UPMB10 yang luarbiasa. Sesetengah sel bakteria ini berbentuk bujur dan bersaiz lebih besar (0.9 – 1.1 µm x 1.3 – 1.8 µm) berbanding dengan bentuk rod biasa (0.5 – 0.6 µm x 1.5 – 1.9 µm). Terdapat juga sel berbentuk memanjang (panjang 5.3 – 6.2 µm) untuk proses replikasi. Perubahan bentuk sel sebegini mungkin menunjukkan kehadiran nutrien yang secukupnya untuk pertumbuhan sihat dan replikasi sel bakteria pada kawasan akar yang dikolonisasikan.

Strain UPMB10 telah berjaya ditandakan dengan “green fluorescent protein” (GFP) melalui plasmid yang dibina khas untuk kajian ini (pSV101gfp) yang dimasukkan dengan kaedah “elektroporation”. Kehadiran GFP dalam sel UPMB10 telah ditentukan melalui kaedah PCR, SDS-PAGE dan Western Blotting. Namun demikian, isyarat cahaya dari sel yang mengandungi GFP sangat lemah dan oleh itu, tidak dapat digunakan untuk kajian kolonisasi akar dalam keadaan semulajadi (tidak dinyahjangkit).

Kesan-kesan media cecair, bahan pembawa (sabut kelapa, pelepah hancur kelapa sawit [GOPF] dan kompos Kusokom®), suhu (20, 30 dan 40° C), kelembapan (pF 2.19 dan 2.54) dan tempoh penyimpanan (6 bulan) ke atas pertumbuhan *B. sphaericus* UPMB10 dikaji. Keputusan menunjukkan bahawa media Media “tryptic soy broth” (TSB) sesuai untuk pertumbuhan sel hidup strain UPMB10 sehingga tahap 10^8 cfu mL^{-1} untuk penghasilan inokulan jangka masa yang panjang. Bahan pembawa pelepah hancur kelapa sawit + 25 % (w/w) kompos didapati sesuai untuk meningkatkan dan menyokong pertumbuhan sel hidup strain UPMB10 pada suhu bilik (30° C) sehingga 6 bulan. Keputusan juga menunjukkan terdapat interaksi signifikan antara bahan pembawa dan suhu (p=0.01), bahan pembawa dan tempoh penyimpanan (p=0.05), serta suhu dan tempoh penyimpanan (p=0.05). Pertumbuhan sel hidup tertinggi dalam GOPF, sabut kelapa, GOPF+ 50% kompos Kusokom® dan GOPF+ 25% kompos Kusokom® masing-masing pada suhu 20° C, 30° C, 40° C dan 30° C; dan pada jangka masa D21, D7, D28 dan D14. Keputusan interaksi antara bahan pembawa dan suhu menunjukkan pertumbuhan lebih tinggi pada suhu 30° C di dalam GOPF

dan GOPF+ 25% kompos Kusokom®. Manakala, pertumbuhan tiggi pada suhu 40° C diperhatikan dalam GOPF+ 50% kompos Kusokom®. Dalam semua ujikaji, populasi sel hidup adalah lebih tinggi secara signifikan pada tempoh D161(Log_{10} 8.61 cfu g^{-1}) dan D21 (Log_{10} 8.00 cfu g^{-1}) berbanding pada permulaan eksperimen (D1). Kesimpulannya, hasil kajian ini menunjukkan potensi menghasilkan biobaja komersil untuk diguna di Malaysia dan negara tropika yang lain, daripada pelepah hancur kelapa sawit + kompos dengan *Bacillus sphaericus* UPMB10 yang mampu mengkolonisasikan akar pokok.

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This thesis 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 are as follows:

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DECLARATION

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

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Date: 13 NOVEMBER 2007

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