UNIVERSITI PUTRA MALAYSIA

RADIATION MUTAGENESIS OF MICROBIAL BIOFERTILIZER FOR FUNCTIONALITIES IMPROVEMENT IN CUCUMBER AND GREEN MUSTARD

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FP 2019 69

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By

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

July 2019

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

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Gamma irradiation has been utilised to induce mutagenesis in microorganisms to enhance enzyme production in the fermentation industry. However, radiation mutagenesis of microorganisms to induce multifunctional activities, especially in the biofertilizer industry, has yet to be reported in Malaysia. Eight biofertilizer bacteria, which were isolated from compost, soil, and plants, were subjected to gamma irradiation from a gamma cell at Malaysian Nuclear Agency (Nuclear Malaysia) to improve their plant growth promoting activities, such as N_2 fixation, phosphate solubilisation, potassium solubilisation and growth hormone production. Isolates were identified as *Acinetobacter calcoaceticus* (M100), *A. baumannii* (AP1), *Klebsiella pneumonia* (AP2), *Enterobacter* bacterium (AP3), *Pseudomonas putida* (C2, V15 and M99), and *Pantoea stewartii* (V3) by using 16S rRNA method. These isolates were gamma irradiated at doses of 50–400 grey (Gy). Survival curves revealed that bacterial levels (log number of cfu/mL) decreased when gamma irradiation dose was increased. LD_{50} of the isolates ranged from 400 Gy to 500 Gy. Among the variants, *A. calcoaceticus* (M100/200) and *A. baumannii* (AP1/200) irradiated after being given a dose of 200 Gy of gamma irradiation exhibited an improvement in N_2 fixation, phosphate solubilisation, and potassium solubilisation. There was no N_2 fixation *nif* gene amplified in *A. baumannii* (AP1) and *A. calcoaceticus* (M100). Only phosphate solubilisation *pqq* genes were amplified by primer pqq7. BLAST results showed only minor changes of the wild type and mutant sequences. Gamma irradiation did not cause major changes of these *pqq* genes. There could be other *pqq* genes or molecular changes may occur after gamma irradiation. *A. calcoaceticus* (M100) and its mutant, M100/200, were tested in greenhouse by using a complete randomised design (CRD) experimental design and field experiment by using complete randomised block design (CRBD) experimental design on cucumber. The mutant enhanced 47.28 % of fresh and 52.63 % of dry weight of five week old cucumber seedlings in the greenhouse experiment and 6.38 % of yields in the field experiment as compared with wild type. Nitrogen and phosphorus are important

plant nutrients. The effects of gamma irradiated *A. calcoaceticus* on the uptake of N and P of green mustard (*Brassica chinensis*) were investigated in a greenhouse experiment by using complete randomised design (CRD) experimental design. N, P and K contents, as well as pH, were determined using the soil nutrient analyser. Two-week-old seedlings were treated with biofertilizer (mutant or wild type) with either N (urea) or P (phosphate rock) source alone or in combination. Crops were harvested after two months. Fresh and dry weight, height, chlorophyll content, leaf area and total N and P of the crops were determined. Treatment T8 (Mutant M100/200 with N and P source) showed the highest results in plant height (28.75 cm), fresh and dry weights of top part of green mustard (66.33 g and 3.83 g), root length (17.38 cm), fresh and dry weighs of root (4.90 g and 0.97 g), leaf area (938.62 cm^2), SPAD reading (40.48) and total chlorophyll (1.97 mg cm^{-2}) in comparison to those of the samples given other treatments in the greenhouse experiment. The highest total N (4.69%) showed in treatment T5 (mutant M100/200 with N source). The highest total P (11.29 mg/L) showed in treatment T7 (M100 with N and P source). Nitrogen input did not enhance growth, whereas phosphorus input and phosphate solubiliser administration increased plant growth indirectly through a better rooting system. In conclusion, *Acinetobacter* sp. could be improved as multifunctional biofertilizer inoculants through gamma irradiation. Whole genome sequencing for the wild type and mutant may be carried out in determined future investigation of mutagenesis by gamma irradiation.

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

MUTAGENESIS RADIASI UNTUK PENGINGKATAN FUNGSI KEPELBAGAIAN MIKROORGANISMA BIOBAJA KE ATAS TANAMAN TIMUN DAN SAWI

Oleh

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Penyinaran gama telah digunakan dalam mutagenesis mikroorganisma untuk meningkatkan pengeluaran enzim dalam industri fermentasi. Walau bagaimanapun, mutagenesis sinaran mikroorganisma untuk peningkatan aktiviti pelbagai fungsi, terutama dalam industri biobaja masih belum pernah ada dijalankan di Malaysia. Lapan bakteria biobaja, yang telah dipencilkan daripada kompos, tanah, dan tumbuh-tumbuhan, telah disinarkan dengan menggunakan kemudahan penyinaran gama di Nuklear Malaysia untuk sasaran peningkatan aktiviti pertumbuhan, seperti pengikatan N_2, penguraian fosfat, kalium dan penggalak pengeluaran hormon pertumbuhan. Isolat dikenal pasti sebagai *Acinetobacter calcoaceticus* (M100), *A. baumannii* (AP1), *Klebsiella pneumonia* (AP2), *Enterobacter* bacterium (AP3), *Pseudomonas putida* (C2, V15 and M99) dan *Pantoea stewartii* (V3) dengan menggunakan kaedah 16S rRNA. Isolat-isolat ini telah disinarkan dengan sinaran gama pada dos 50-400 Gy. Graf kadar hidup menunjukkan tahap hidup bakteria (log nombor cfu/mL) menurun dengan peningkatan dos sinaran gama. LD_{50} isolat adalah di antara 400 Gy hingga 500 Gy. Isolat, *A. calcoaceticus* (M100/200) dan *A. baumannii* (AP1/200) yang disinarkan pada 200 Gy telah menunjukkan peningkatan dalam pengikatan N_2, penguraian fosfat dan kalium. Tiada gen *nif* pengikat N_2 baharu dijana oleh *A. baumannii* (AP1) dan *A. calcoaceticus* (M100). Hanya gen *pqq* pengurai fosfat yang ditonjolkan oleh primer pqq7 dijana. Keputusan perbandingan jujukan tidak menunjukkan perubahan ketara terhadap gen *pqq*. Sinaran gama tidak memberi kesan ketara terhadap gen *pqq* ini. Perubahan molekul akibat sinaran berlaku perubahan molekul selepas penyinaran dijalankan ataupun mungkin berlaku pada gen *pqq* lain. *A. calcoaceticus* (M100) dan mutan (M100/200) diuji pada timun di rumah hijau dan ladang. Mutan menunjuk peningkatan 47.28 % berat basah dan 52.36 % berat kering terhadap anak pokok timun berumur lima minggu di rumah hijau dan 6.38 % atas hasil timun dalam percubaan ladang. Nitrogen dan fosfat adalah nutrien penting untuk tanaman. Kesan mutan *A. calcoaceticus* terhdap

pengambilan nutrien N dan P oleh sawi hijau (*Brassica chinensis*) diuji dalam rumah hijau dengan mengguna reka bentuk rawak lengkap (CRD). Kandungan N, P dan K serta pH diukur menggunakan alat pengukur nutrien tanah. Anak pokok berumur dua minggu dirawat dengan biobaja (mutan atau asal) dengan unsur N (urea) atau P (batuan fosfat) secara tunggal atau secara gabungan. Pokok dituai selepas dua bulan. Berat basah, kering, ketinggian, kandungan klorofil, keluasan daun, N dan P diukur. Rawatan T8 (Mutant M100 / 200 dengan sumber N dan P) menunjukkan keputusan tertinggi dalam ketinggian tumbuhan (28.75 cm), berat segar dan kering bahagian atas sawi (66.33 g dan 3.83 g), panjang akar (17.38 cm) berat badan segar dan kering akar (4.90 g dan 0.97 g), keluasan daun (938.62 cm2), bacaan SPAD (40.48) dan jumlah klorofil (1.97 mg cm-2) berbanding dengan sampel yang diberi rawatan lain dalam eksperimen rumah hijau. Nilai tertinggi N (4.69%) ditunjukkan dalam rawatan T5 (mutan M100 / 200 dengan sumber N). Nilai tertinggi P (11.29 mg / L) ditunjukkan dalam rawatan T7 (M100 dengan sumber N dan P). Mutan (M100/200) dengan unsur N dan P atau P tunggal menunjukkan pengingkatan pertumbuhan serta pengambilan nutrien yang mengandungi N and P berbanding rawatan lain. Input N tidak meningkatkan pertumbuhan tetapi input P meningkatkan pertumbuhan secara tidak langsung dengan penggalakan kadar pertumbuhan akar yang baik. Kesimpulannya, *Acinetobacter* sp. boleh dipertingkatkan fungsi kepelbagaiannya melalui sinaran gama. Jujukan genom mutan dan asal yang lengkap diperlukan untuk keterangan lanjut tentang kesan sinaran gama terhadap mutagenesis.

ACKNOWLEDGEMENTS

First and foremost, I would like to dedicate my sincere appreciation to my supervisor, Associate Professor Dr. Halimi Mohd Saud for his continuous guidance, suggestions throughout the project and for his encouragement, advice and remarkable patience in this project. My sincere thanks are also due to my supervisory committee members, Dr. Khairuddin Abdul Rahim; Professor Dr. Che Fauziah Binti Ishak and Dr. Puteri Edaroyati Megat Wahab for their valuable advice and support in this study.

I wish to express my gratitude to Universiti Putra Malaysia, Malaysian Nuclear Agency (Nuclear Malaysia), Ministry of Energy, Science, Technology, Environment & Climate Change (MESTECC) and the Public Service Department (JPA) for the technical and financial support. The able technical assistance of Dr. Ahmad Zainuri B. Mohd Dzomir, Latiffah Norddin, Abdul Razak Ruslan, Hazlina Abdullah, Shuhaimi B. Shamsudin, Sharpudin Md Nor and Nur Fariz Bt Hj Sulaiman was greatly appreciated.

My special thanks are dedicated to my friends, especially Chong Saw Peng, Hing Jan Nie, Dr. Yusuf Chong Yu Lok, Yeong Mei Chan and Ling Lai Kuan for their assistance, kindness and precious friendship in making this project a success.

Finally, my utmost gratitude is to my dearest family members, Alvin, Jessy and Jordan Wong for their love and encouragement. To Wong Kwai Fang and Phua Hong Moi thanks for their support and love throughout the years of my study.

I certify that a Thesis Examination Committee has met on 12 July 2019 to conduct the final examination of Phua Choo Kwai Hoe on her thesis entitled "Radiation Mutagenesis of Microbial Biofertilizer for Functionalities Improvement in Cucumber and Green Mustard" 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 Doctor of Philosophy

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
cfu	Colony forming unit
FNCA	Forum for Nuclear Cooperation in Asia
IAA	Indole-acetic acid
K	Potassium
N	Nitrogen
NA	Nutrient agar
NB	Nutrient broth
O.D.	Optical density
P	Phosphorus
PGP	Plant Growth Promoter

CHAPTER 1

INTRODUCTION

A biofertilizer is a substance composed of living microorganisms that colonise the rhizosphere or the interior parts of plants and promote growth by increasing the supply or availability of primary nutrients to host plants (Vessey, 2003; FNCA, 2018). Biofertilizer with arbuscular mycorrhiza fungi and nitrogen fixing bacteria (NFB) was developed in Malaysia in the late 1980s and early 1990s. Since then, phosphate solubilising microorganisms (PSM) and plant growth promoting microorganisms (PGPR) have been used as biofertilizer inoculants. In 2012, bioinoculants mainly nitrogen fixing bacteria (NFB) such as rhizobia, which constitute 79% of the global demand (Transparency Market Research, 2014). This shows that biofertilizer products target limited activities. Thus, multifunctional biofertilizers should be developed. Multifunctional activities include N_2 fixation, phosphate solubilisation, potassium solubilisation and plant growth promotion in single application (Youssef and Eissa, 2014). Current practices to produce multifunctional biofertilizers involve mixture of single microorganisms. As a consequence, this approach may cause other problems, such as contamination, antagonistic effects among microorganisms, and decreased biofertilizer shelf life (Kaur and Kaur, 2018). Mutagenesis or genetic recombination of single biofertilizer microorganisms may improve their multifunctional activities to satisfy market demands.

Microorganisms can acquire new genetic characteristics through mutation or genetic recombination. In genetic recombination, the efficacy of selected microorganism is improved through the genetic manipulation of wild type strains to produce genetically modified organisms (GMOs) or living modified organism (LMO) (Department of Biosafety, 2012). This approach requires a precise knowledge of the mechanisms affecting microbial traits and the structure and regulation of relevant genes. However, a complete understanding of these elements is difficult to obtain and can be very costly. Mutants can be generated by using chemical or physical mutagens (Satoh and Oono, 2019). Random mutagenesis is less costly than generating GMOs. This approach also does not require superior genetic knowledge to determine the desired features.

A gene is modified through spontaneous or induced mutation (Adrio and Demain, 2006; Sa). Chemical mutagenesis primarily produces single-base substitutions but not drastic mutations, such as large genomic deletions. Physical mutagens include ultraviolet (UV) light, ion beams, and gamma irradiation (Huma et al., 2012). Ion beams can generate localised irradiation in target organisms. Low energy loss allows a high-resolution control of penetration depth for relatively low-energy ions, which may induce local structural damage caused by atomic displacement (Tanaka et al., 2012). Ultraviolet rays (UV) elicit a moderate effect, which induces pyrimidine dimerisation through frame shift transition from GC to AT base pairs. Gamma rays are the most energetic and highly ionized form of radiation, which may cause mutations, such as single- or double-strand

breakage of DNA through deletion or structural changes, DNA–protein cross-links, oxidised bases, and basic sites (Huma et al., 2012). Microorganisms are mutated by ion beams (Chen et al., 2008; Li et al*.,* 2011), gamma irradiation (Afsharmanesh et al., 2013), UV light (Huma et al., 2012), or chemical mutagens to produce thermo-tolerant mutants in the fermentation industry and disease-control mutants in the agriculture industry (Mehdikhani et al., 2011; Huma et al., 2012; Afsharmanesh et al., 2013).

Among mutagens, ion beam and gamma irradiation are the most commonly used to produce mutants. However, an ion beam facility is not available in Malaysia but Nuclear Malaysia is equipped with two gamma irradiation facilities to support mutagenesis research. A gamma cell is an acute radiation facility, and a gamma greenhouse is a choric radiation facility. Plant mutants of orchids, hibiscus, and rice, have been generated out of these facilities. However, radiation mutant microorganisms in the biofertilizer industry have yet to be produced through gamma irradiation in Malaysia. Biofertilizer mutants with better multifunctionalities than wild type could be obtained through gamma irradiation. The present study aims to improve the multifunctional activities of biofertilizer inoculants through irradiation from gamma cell.

There has been no previous study on gamma irradiated *Acinetobacter calcoaceticus* for their effects on cucumber growth and on nitrogen and phosphorus uptake of green mustard (*Brassica chinensis*). Previous reports stated that phosphate solubilising bacteria played a major role in N_2 fixation and plant growth. The present study focused on N_2 fixation and phosphate solubilisation which, involved *nif* and *pqq* gene in wild type and mutant *A. calcoaceticus*. There is no nif and pqq gene information for mutant *A. calcoaceticus* in NCBI gene bank. Amplification of *pqq* genes of wild type and mutant *A. calcoaceticus* were carried out in this study.

The objectives of this study as follows:

- To obtain improved multifunctionalities biofertilizer microorganism mutant through gamma irradiation
- To amplify *nif* and *pqq* gene on wild type and mutant
- To determine the effectiveness of biofertilizer microorganisms mutant on plant growth on cucumber in greenhouse experiment
- To test the effect of biofertilizer microorganisms mutant on cucumber yield in field experiment
- To study correlation of N and P uptake on biofertilizer microorganisms mutant in greenhouse experiment by using green mustard

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LIST OF PUBLICATIONS

Phua, C. K. H, Rahim,K. A. and Saud, H.M. 2016. A review on microbial mutagenesis through gamma irradiation for agricultural applications. *Nuclear Science Journal of Malaysia Volume 28 (2): 20 – 29*

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