UNIVERSITI PUTRA MALAYSIA

ISOLATION AND IDENTIFICATION OF RHIZOBACTERIA FROM PADDY LAND AND THEIR BENEFIT AS A BIOFERTILIZER

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Biological nitrogen fixation (BNF) studies have been emphasized following the problem faced in the application of chemical nitrogen fertilizer in wetland rice cultivation. Among fertilizer inputs, nitrogen is the major limiting nutrient for crop production. Besides energy intensive, chemical nitrogen fertilizer also cause groundwater pollution. One of the approaches to alleviate this problem is by isolating the beneficial rhizobacterial from wetland rice which have the capability to fix nitrogen biologically and produce plant growth hormones, indole-3-acetic acid (IAA) *in vitro*.

The study consisted of three experiments. Experiment I was the isolation and identification of rhizobacterial strains from paddy land and their reactions toward several biochemical tests. Experiment II was the determination of IAA production by the rhizobacterial using modified colorimetric methods. Experiment III was to determine the effects of IAA production by rhizobacterial isolates on growth of rice variety MR 211, the colonization of

these isolates on rice roots and nitrogenase activity of rhizobacteria through Acetylene Reduction Assay.

From the isolation procedures, several strains of bacterial were isolated from the rice rhizosphere. The population of these rhizobacterial ranged from 10^5 to 10^9 cfu/mL. By using Biolog Identification System, these rhizobacterial have been identified as *Corynebacterium spp.*, *Proteus mirabilis* and *Spirillum spp.*, which showed their unique characteristics to Gram staining, pellicle formation, motility, starch hydrolysis, catalase, antibiotic test and carbohydrate fermentation with different carbon sources.

The experimental results indicate that *Corynebacterium spp.*, *Proteus mirabilis* and *Spirillum spp.* were able to produce IAA in culture medium supplemented with L-tryptophan as a precursor, which ranged between 1-10 μg/mL. The presence of precursor was essential for detection of IAA production by the bacteria. IAA production increased in the presence of 0.1 mg/L, 10 mg/L and 100 mg/L of precursor.

These rhizobacterial were able to stimulate the growth of rice root system. A significant increase in root volume (*Corynebacterium spp.*, 63%; *Proteus mirabilis*, 86.7%; *Spirillum spp.*, 83.3%), root area (*Corynebacterium spp.*, 23.1%; *Proteus mirabilis*, 9.1%; *Spirillum spp.*, 53.5%), root length (*Corynebacterium spp.* and *Proteus mirabilis*, 30%; *Spirillum spp.*, 59.3%) and root weight (*Corynebacterium spp.*, 94.1%; *Proteus mirabilis*, 96.9%;

Spirillum spp., 97%) was recorded compared to uninoculated control. However, there was no significant difference in the leaf length and area.

In the presence of plant growth promoting rhizobacteria (PGPR) on the root surface, at approximately 10^5 - 10^9 cfu/mL, ARA rate of rice is considered positive. Ethylene production for *Corynebacterium spp.*, *Proteus mirabilis* and *Spirillum spp.* were 13.8, 73.1 and 14.1 nmol/hr/mL, respectively for the first hour of incubation time. The ARA rates for all isolates were higher when the incubation period was prolonged and continued to increase until 10 hours of incubation.

Results from this study showed that the groups of Coryneform, Enterobacteriaceae and Spirillaceae are abundant in Kelantan rice areas. These rhizobacterial strains were able to fix atmospheric nitrogen for plant-N requirement and release auxin as a secondary metabolite, essential for root growth stimulation.

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

PENGASINGAN DAN PENGECAMAN RIZOBAKTERIA DARIPADA SAWAH PADI SEBAGAI BAJA BIOLOGI YANG BERPOTENSI

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Kajian mengenai pengikatan nitrogen secara biologi (BNF) mendapat perhatian berikutan daripada masalah penggunaan baja kimia dalam penanaman padi sawah. Di antara baja yang digunakan, nitrogen merupakan nutrien yang paling mengekang dalam pengeluaran padi. Selain penggunaan tenaga yang berlebihan, baja kimia khasnya nitrogen juga menyebabkan pencemaran air tanah. Salah satu pendekatan yang digunakan untuk menyelesaikan masalah ini ialah dengan mengasingkan rizobakteria yang berfaedah daripada sawah padi yang mempunyai kebolehan untuk mengikat nitrogen secara biologi dan dapat menghasilkan hormon tumbuhan, indole-3-acetic acid (IAA) secara *in vitro*.

Tiga kajian telah dijalankan untuk melihat kesan bakteria ini. Kajian I ialah pengasingan dan pengecaman rizobakteria daripada sawah padi dan tindak balasnya terhadap beberapa ujian biokimia. Kajian II ialah penentuan IAA yang dihasilkan oleh rizobakteria dengan menggunakan kaedah 'colorimetric'

yang diubahsuai. Kajian III ialah menentukan penghasilan IAA terhadap tumbesaran pokok padi variati MR 211, corak pencolonian bakteria pada akar padi dan menentukan aktiviti enzim nitrogenase dengan menggunakan ujian 'Acetylene Reduction Assay' (ARA) atau Asai Penurunan Asetilin.

Daripada prosedur pemencilan, beberapa jenis bakteria telah diasingkan daripada rizosfera padi. Populasi bakteria adalah antara 10^5 kepada 10^9 cfu/mL Dengan menggunakan Sistem Pengecaman Biolog, rizobakteria daripada padi dikenalpasti terdiri daripada *Corynebacterium spp.*, *Proteus mirabilis* dan *Spirillum spp.* di mana ia menunjukkan sifat unik masing-masing terhadap pewarnaan Gram, pembentukan pelikel, pergerakan, hidrolisis kanji, katalase, uijan antibiotik dan fermentasi karbohidrat dengan menggunakan sumber karbon yang berlainan.

Hasil kajian menunjukkan *Corynebacterium spp.*, *Proteus mirabilis* dan *Spirillum spp.* mampu menghasilkan IAA di dalam media kultur yang dibekalkan dengan L-tryptophan sebagai pemangkin, iaitu penghasilan IAA di antara 1-10μg/mL. Kehadiran pemangkin adalah diperlukan untuk mengesan penghasilan IAA oleh bakteria. Penghasilan IAA meningkat dengan kehadiran pemangkin sebanyak 0.1 mg/L, 10 mg/L dan 100 mg/L.

Rizobakteria tersebut berkebolehan untuk menggalakkan tumbesaran pada sistem akar padi. Peningkatan yang ketara dapat diperhatikan pada isipadu akar (*Corynebacterium spp.*, 63%; *Proteus mirabilis*, 86.7%; *Spirillum spp.*, 83.3%), keluasan akar (*Corynebacterium spp.*, 23.1%; *Proteus mirabilis*,

9.1%; *Spirillum spp.*, 53.5%), kepanjangan akar (*Corynebacterium spp.* dan *Proteus mirabilis*, 30%; *Spirillum spp.*, 59.3%) dan berat akar (*Corynebacterium spp.*, 94.1%; *Proteus mirabilis*, 96.9%; *Spirillum spp.*, 97%) berbanding dengan kawalan. Walau bagaimanapun, tiada peningkatan yang ketara terhadap kepanjangan dan keluasan daun padi.

Kadar ARA pada padi dianggap positif dengan kehadiran rizobakteria yang menggalakkan tumbesaran pokok (PGPR) pada permukaan akar sebanyak 10^5 – 10^9 cfu/mL. *Corynebacterium spp.*, *Proteus mirabilis* dan *Spirillum spp.* menunjukkan penghasilan etilena sebanyak 13.8, 73.1 dan 14.1 nmol/hr/mL masing-masing dengan inkubasi pada jam pertama. Kadar ARA untuk semua bakteria bertambah apabila masa inkubasi meningkat secara berterusan hingga 10 jam.

Daripada kajian ini, dapat disimpulkan bahawa kumpulan Coryneform, Enterobacteriaceae dan Spirillaceae banyak terdapat di kawasan padi di Kelantan. Rizobakteria ini mampu mengikat gas nitrogen untuk keperluan nitrogen dalam tumbuhan dan menghasilkan auksin sebagai metabolit sekunder sebagai penggalak utama dalam perkembangan sistem akar.

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CHAPTER 1

INTRODUCTION

Rice, *Oryza sativa* L., is one of the most important cereal crops in the world (Ladha *et al*., 1997). It feeds well in excess of more than two billions people in Asia and many millions in Latin America (Flinn and De Datta, 1984; Ladha *et al*., 1997). It had been cultivated in India and China for about 8,000 years (Poehlman and Sleper, 1995). Now, the cultivation area is extended to Northeastern India, Banglade.
Southern China.

The world's annual rice production must be increased from the present 520 million tonnes to 760 million tonnes in order to feed the fast increasing global population by the year 2020 (Flinn and De Datta, 1984; Cassman and Pingali, 1994). Tropical lowland rice agriculture is now responsible for 86% of the total world rice crop, and the yields are typically in the range of 2-3.5 t/ha (Ladha *et al*., 1997). The productivity (yield/ha) must be enhanced in order to solve the reduction in land area. Yield stagnation or even decline has been observed in some rice growing areas of Asia since the early 1980s (Flinn and De Datta, 1984; Cassman and Pingali, 1994).

Observations have been made and they showed that the rice yield decline is commonly associated with decrease in crop nitrogen uptake. Among fertilizer inputs, nitrogen is the major limiting nutrient for crop

production (Regan, 1988). It can be supplied through chemical or biological means, but the production of chemical nitrogen fertilizer is energy intensive as two tonnes of fuel oil are consumed to produce one tonne of fertilizer nitrogen (Regan, 1988).

In normal farming practice, the nitrogen fertilizers are chemically synthesized by Haber-Bosch process which is very expensive due to the requirement of high temperature (400-600°C) and pressure (100-200 atm). Besides, in this process the carbon dioxide (CO_2) gas is also released to the atmosphere causing the Greenhouse Effect. Another greenhouse gas is nitrous oxide (N_2O), which is generated by denitrification of spilled fertilizers on land. In groundwater, there are reports of pollution by nitrate (NO_3^-) dissolved from fertilizers (Regan, 1988).

An alternative to the increased use of mineral nitrogen fertilizers is to use more N derived from biological nitrogen fixation (BNF). Indeed, at present, the low input systems typical of much tropical rice agriculture add little or no mineral N, subsisting mainly on nitrogen provided by BNF from free-living and plant-associated diazotrophs. A variety of organisms are responsible for this BNF, ranging from cyanobacteria and photosynthetic bacteria in the floodwater and soil surface to heterotrophic bacteria within the soil and root zone (Roger and Ladha, 1992; Ladha and Reddy, 1995; Boddey *et al.*, 1995).

Microorganisms are considered the primary sources of biologically active substances in soil. They are able to synthesize a diverse group of substances that stimulate plant growth by direct or indirect modes of action (Frankenberger and Arshad, 1995). The metabolites produced by soil microorganisms may influence the growth and development of plants through plant growth regulators, vitamins and inhibitors (Strzelczyk and Burdziej, 1984; O'Gara *et al.*, 1994 and Astrom *et al.*, 1993). Besides, these plant growth regulators also enhance root development, improve mineral and water uptake by the plant system (Umali-Garcia *et al.*, 1980; Okon and Kapulnik, 1986; Frankenberger and Arshad., 1995). One of the phytohormones that can be synthesized by microorganisms is indole-3-acetic acid (IAA).

Currently, inoculation of these bacteria increased the yield, biomass, nitrogen content and improved root development of cereal crops extensively. Okon, (1981) obtained 77 kg more N/ha of inoculated corn with nitrogen fixing bacteria compared to uninoculated plants. By using estimation data obtained from N-balance studies, suggested that indigenous cyanobacteria may contribute as much as 80 kgN/ha/crop, averaging 30 kgN/ha/crop and when inoculated into the rhizosphere, they can increase rice yields by an average of 337 kgN/ha/crop (App *et al.*, 1980; Watanabe, 1986; Roger and Ladha, 1992).

This study hypothesized that rhizobacteria from paddy land could influence the growth and root development by producing the phytohormone

indole-3-acetic acid and through biological nitrogen fixation. The experiments were conducted with the following objectives:

(i) To isolate rhizobacterial strains from paddy land and identify the isolates based on biochemical tests.

(ii) To determine the production of indole-3-acetic acid as plant growth hormones by IAA producing rhizobacterial strains and their effects on rice growth.

(iii) To determine the colonization of rhizobacterial on rice roots.

(iv) To determine the nitrogenase activity of rhizobacterial through Acetylene Reduction Assay (ARA).

CHAPTER 2

LITERATURE REVIEW

2.1 The Role of Nitrogen in Biosphere

The growth of all organisms depends on the availability of mineral nutrients and none is more important than nitrogen, which is required in large amounts as an essential component of proteins, nucleic acids and other cellular constituents (http://helio.bto.ed.ac.uk/bto/microbes/nitrogen). There is an abundant supply of nitrogen in the earth's atmosphere, nearly 78% in the form of N_2 gas. However, N_2 is unavailable for use by most organisms because there is a triple bond between the two nitrogen atoms, making the molecule almost inert. In order for nitrogen to be used for growth, it must be 'fixed' (combined) in the form of ammonium (NH_4) or nitrate (NO_3) ions, therefore nitrogen is often the limiting factor for growth and biomass production (http://helio.bto.ed.ac.uk/bto/microbes/nitrogen).

2.1.1 Mechanism of Biological Nitrogen Fixation

A relatively small amount of ammonia is produced by lighting. Some ammonia is also produced industrially by the Haber-Bosch process, using an iron-based catalyst, with very high temperature (400-600°C) and fairly high pressure (100-200 atm). This process can also be achieved by certain

microorganisms through a process called biological nitrogen fixation (Regan, 1988; Janet and Peter, 1990).

Biological nitrogen fixation can be represented by the following equation (Figure 1), in which two moles of ammonia are produced from one mole of nitrogen gas, at the expense of 16 moles of ATP (adenosine triphosphate) and a supply of electrons and proton (hydrogen ions):

$$N_2 + 8H + 8e^- + 16ATP = 2NH_3 + H_2 + 16ADP + 16Pi$$

Figure 1: Mechanism of biological nitrogen fixation
Source: (http://helio.bto.ed.ac.uk/bto/microbes/nitrogen)

This reaction is performed exclusively by prokaryotes, using an enzyme complex known as nitrogenase. This enzyme consists of two proteins: an iron protein and a molybdenum-iron protein. The reaction occurs while N_2 is bound to the nitrogenase enzyme complex. The Fe protein is first reduced by electrons donated by ferredoxin. Then the reduced Fe protein binds ATP and reduced the Molybdenum-iron protein, which donates electrons to N_2, producing HN=NH. In two further cycles of this process (each requiring electrons donated by ferredoxin) HN=NH is reduced to H_2N-N H_2, and this in turn is reduced to $2NH_3$.

2.2 Associative/Free Living Nitrogen Fixing Bacteria (Diazotrophs)

Nitrogen fixation is the reduction of gaseous nitrogen (dinitrogen, N_2) to ammonia. Biological nitrogen fixation is apparently carried out only by certain prokaryotes (called diazotrophs), including various cyanobacteria, members of the Azotobacteriaceae, Methylococcaceae, Rhizobiaceae, Rhodospirillaceae, Enterobacteriaceae, Corynebacteriaceae, Actinomycetaceae, some Bacillus and Clostridium species (Janet and Peter, 1990).

Diazotrophs are nitrogen fixing bacteria which can colonize and contribute biological nitrogen to the crops (Kundu and Ladha, 1995). They might transmit as a continuum of root associated microorganisms from rhizosphere and rhizoplasm. They may be free living/associative in soil or water but many occur in mutualistic association with other organisms, which benefit from the supply of fixed nitrogen (Janet and Peter, 1990). Table 1 and Figure 2 show the nitrogen-fixing bacteria from soils and flooded system of rice.