TISSUE CULTURED OIL PALM
TS (Elaeis guineensis Jacq.)
INOCULATED WITH PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

AZLIN CHE OM

UNIVERSITI SAINS MALAYSIA

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SUE CULTURED OIL PALM PLANTLETS cq.) INOCULATED WITH PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

by

AZLIN CHE OM

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Dedicated to the one who irrationally enough, never gave up on meé

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LIST OF ABBREVIATIONS

1.	ARA	Acetylene Reduction Assay
2.	ANOVA	Analysis of Variance
3.	BTB	Bromothymol Blue
4.	BNF	Biological Nitrogen Fixation
5.	cfu	Colony forming unit
6.	C_2H_2	Acetylene
7.	C_2H_4	Ethylene
8.	CRD	Completely Randomized Design
9.	D	Day
10.	E	Endophytes
11.	FGC	Friable growing callus
12.	GC	Gas Chromatography
13.	g	gram
14.	h	hours
15.	H	Hydrogen
16.	ha	Hectare
17.	HMDS	Hexamethyldisilazane
18.	HPLC	High Performance Liquid Chromatography
19.	IAA	Indole-3-acetic Acid
20.	K	Potassium
21.	kg	Kilogram
22.	KOH	Potassium hydroxide
23.	l	liter
24.	LSD	Least Significant Difference
25.	L-tryp	L-tryptophan
26.	mg	milligram
27.	ml	mililiter
28.	MOP	Muriate of Potash
29.	MS	Murashige and Skoog

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n

32. N_2	Nitrogen gaseous
33. NAA	Naphthaleneacetic acid
34. Nfb	Nitrogen free semisolid medium
35. OD	Optical Density
36. pp	page
37. P	Phosphorus
38. PGPR	Plant Growth Promoting Rhizobacteria
39. PE	Polyembryoids
40. R	Rhizosphere
41. RC	Red Congo
42. R12	Locally isolated *Acetobacter diazotrophicus* (R12)
43. SEM	Scanning electron microscope
44. Sp7	*Azospirillum brasilense* (Sp7)
45. Sp7k	Killed *Azospirillum brasilense*
46. spp.	Species
47. TEM	Transmission electron microscope
48. TSP	Triple Superphosphate
49. Z78	*Herbaspirillum seropidacea* (Z78)

LIST OF SYMBOLS

1.	%	per cent
2.	<	smaller than
3.	℃	Degree Celsius
4.	ε	micro

...ANTLET TISU KULTUR KELAPA SAWIT

...) DENGAN INOKULASI RHIZOBAKTERIA

PENGGALAK PERTUMBUHAN (PGPR)

ABSTRAK

Sebanyak 29 isolat yang berkebolehan mengikat gas nitrogen telah berjaya dipencilkan daripada akar kelapa sawit (bahagian rhizoplan, rhizosfera dan tisu akar). Mikroorganisma diazotrof yang telah berjaya dicamkan berdasarkan ujian biokimia ialah *Paenibacillus durus* (terdahulu dikenali sebagai *P. azotofixans*), *Paenibacillus polymyxa*, *Azospirillum lipoferum*, *Herbaspirillum seropedicae* dan *Acetobacter diazotrophicus*. Isolat juga berkebolehan menghasilkan asid indol-3-asetik dalam medium kultur. Seterusnya, kajian dijalankan bertujuan untuk melihat kesan inokulasi PGPR dalam menggalakkan pertumbuhan tisu kultur kelapa sawit. Keputusan eksperimen yang dijalankan menunjukkan bahawa embroid yang diinokulasi oleh rhizobakteria pencilan tempatan *A. diazotrophicus* (R12) dan *A. brasilense* (Sp7) telah menghasilkan pertambahan berat (sehingga 1.51 dan 2.37 g) dan bilangan pucuk (5) berbanding rawatan kawalan (+Sp7k). Teknik ini turut dapat mengurang dan menghapuskan kesan negatif yang disebabkan oleh inokulasi ke atas pertumbuhan embriod. *A. brasilense* (Sp7) dan isolat R12 turut diuji keberkesanannya untuk menggalakkan perkembangan akar pucuk kelapa sawit. Berdasarkan penemuan yang diperolehi, inokulasi Sp7 dan R12 ke atas pucuk kelapa sawit mencatatkan pertambahan berat basah daun (3.14 g), berat basah akar (0.10 g), bilangan akar sekunder (5), kandungan protein daun (25.88 mg BSA/ ml protein) dan klorofil (0.24 mg klorofil /mg berat basah daun) yang tinggi bebanding rawatan kawalan (+Sp7k). Tambahan lagi, plantlet yang diinokulasi dengan Sp7 dan R12 juga menunjukkan aktiviti pengikatan

Sp7 dapat mengikat N_2 sehingga 0.965 µmol C_2H_4 g^{-1} 1.181 µmol C_2H_4 g^{-1} berat basah h^{-1}. Plantlet yang telah diinokulasi kemudian diaklimatisasikan ke rumah kaca dan menunjukkan kebolehan adaptasi yang tinggi terutamanya pokok yang diinokulasi dengan R12 (100% kadar hidup). Pokok ini diberikan rawatan inokulasi Sp7 dan R12 dan dibiarkan bertumbuh sehingga 280 hari (D_{280}) di rumah kaca. Pokok yang diinokulasi dengan R12 juga menunjukkan berat kering (10.5 g) dan isipadu akar (43.0 cm^3) yang tertinggi diikuti dengan rawatan lain (+N, +Sp7 dan +Sp7k). Di samping itu, kandungan protein dan klorofil daun pokok yang diinokulasi dengan R12 dan Sp7 adalah lebih tinggi berbanding rawatan kawalan (+Sp7k) sebanyak 8.44 dan 8.53 mg BSA/ ml protein dan 0.23 dan 0.24 mg klorofil /mg berat basah daun, masing - masing. Kesan positif yang ditunjukkan oleh inokula ke atas tumbuhan perumah adalah disebabkan kolonisasi yang efektif oleh Sp7 dan R12 di permukaan embroid dan akar kelapa sawit. Keputusan yang diperolehi, menunjukkan rhizobakteria pengikat nitrogen yang diuji (Sp7 dan R12) boleh digunakan sebagai biobaja dalam bentuk inokulum mikrob untuk memperbaiki dan meningkatkan pertumbuhan pokok tisu kultur kelapa sawit.

UE CULTURED OIL PALM PLANTLETS

g.) INOCULATED WITH PLANT GROWTH

PROMOTING RHIZOBACTERIA (PGPR)

ABSTRACT

Twenty-nine isolates of N_2 fixing rhizobacteria were successfully isolated from oil palm roots (rhizoplane, rhizosphere and the root tissues). These diazotrophs were successfully identified based on biochemical tests as *Paenibacillus durus* (formerly known as *P. azotofixans*), *Paenibacillus polymyxa*, *Azospirillum lipoferum*, *Herbaspirillum seropedicae* and *Acetobacter diazotrophicus*. The isolates produced indole-3-acetic acid in the growth media. Experiments were carried out with the purpose of observing the effects of PGPR inoculation on growth enhancement of tissue-cultured oil palm. Results using oil palm embryoids showed that locally isolated *A. diazotrophicus* (R12) and *A. brasilense* (Sp7) produce higher biomass (up to 1.51 and 2.37 g) and shoot formation (5) increment compared with the Control treatment (+Sp7k). Moreover, the deleterious effects of bacterial inoculation on embryoids development could be delayed and eliminated through this technique. *A. brasilense* (Sp7) and isolate R12 were also tested for the effectiveness to improve and induce rooting of oil palm shoots. The results indicated that inoculation of Sp7 and R12 on oil palm shoots had enhanced shoot fresh weight (3.14 g), root fresh weight (0.10 g), number of secondary roots (5) and higher leaf protein (25.88 mg BSA/ ml protein) and chlorophyll (0.24 mg chlorophyll / mg leaf fresh weight) contents compared to Control treatment (+Sp7k). Furthermore, the plantlets inoculated with Sp7 and R12 also showed associative nitrogen fixation activities that Sp7 could fix up to 0.965 µmol C_2H_4 g^{-1} fresh weight h^{-1} of N_2 and R12 fixed N_2 at 1.181 µmol C_2H_4 g^{-1} fresh weight h^{-1}. The

then acclimatized in the glasshouse and successfully cially the plantlets inoculated with R12 (100% survival rate). The plants were inoculated with Sp7 and R12 grown for 280 days (D_{280}) in the glasshouse. Results revealed that the plants inoculated with R12 showed the highest root dry weight (10.5 g) and root volume (43.0 cm^3) compared to other treatments (+N, +Sp7 and +Sp7k). Besides that, higher leaf protein and chlorophyll contents were detected for plants inoculated with R12 and Sp7 compared to the Control (+Sp7k) accounted 8.44 dan 8.53 mg BSA/ ml protein and 0.23 and 0.24 mg chlorophyll /mg leaf fresh weight, respectively. Positive influence of the inocula on growth of the host plants were due to effective colonization of Sp7 and R12 on embryoid tissues and root surfaces of oil palm. The results successfully showed that the tested diazotrophic rhizobacteria (Sp7 and R12) could serve as biofertilizer in the form of microbial inoculants to improve growth of tissue cultured oil palm plants.

CHAPTER ONE

INTRODUCTION

Various studies have been conducted, using Plant Growth Promoting Rhizobacteria (PGPR) for improving growth of the host plants towards sustainable agricultural practices (Lucy *et al.*, 2004). As of today this technique has been used to encourage the studies of using PGPR to promote growth and development of oil palm. The PGPR will colonize the plant roots and promote growth of the host plants using their potential ability in fixing atmospheric nitrogen and excretion of plant growth regulators (e.g. indole-3-acetic acid (IAA)) (Park *et al.*, 2005; Baldani and Baldani, 2005; Hoque *et al*., 2001; Chong-Min *et al.*, 2005). Besides that, indirect mechanisms also occurred including antibiotic excretion for protection against pathogenic bacteria, reduction of iron availability to phytopathogens in the rhizosphere, synthesis of fungal cell wall-lysing enzymes and competition with detrimental microorganisms for colonization sites on plant roots (Lucy *et al*., 2004; Vestberg *et al*., 2004).

Nitrogen fixing rhizobacteria (diazotrophs) are one of the most favourable alternative biofertilizer used today to replace the use of chemical fertilizers for crops. It will offer economical as well as ecological benefits to the agricultural systems (Khairuddin, 2002). The diazotrophs are often referred to as biofertilizer, which could increase crops yields due to the ability to convert nutritionally important elements (N) from unavailable to available form through biological nitrogen fixation process (Cakmakci *et al*., 2006). Biofertilizer is a natural product which contains different types of living cells of microbial inoculants such as bacteria

ıbilizing rhizobacteria), algae and fungi (Vessey, 2003). en successfully applied especially to rice, sugarcane and oil palm seedlings (Elbeltagy *et al*., 2001; Biswas *et al*., 2000; Sevilla *et al*., 2001; Amir *et al*., 2001). Inoculation of the host plants with *Azospirillum* spp. and locally isolated PGPR can maximize crop yields and quality while minimizing application of chemical fertilizers which can be harmful to the environment and society (Bashan and Levanony, 1991; Baldani *et al*., 1997). The contribution of fixed nitrogen to the plants is mainly through N_2 fixing activities by free-living and associated diazotrophs with the host plants or from death and lysis (mineralization) of nitrogen-fixing bacteria. The fore mentioned facts are supported by Zimmer and Bothe (1989) indicated that *Azospirillum* could improve nitrogen supply for cereal by mean of N_2 fixation process and thereby to enhance crop productivity. *Azospirillum* could also supply IAA, causing increased rooting, which in turn enhanced mineral uptake by the host plants (Steenhoudt and Vanderlayden, 2000).

In search for natural fertilizer to enrich soil fertility, isolation of PGPR is very important. The relationship between PGPR and the host plants can be categorized into two level of complexity; rhizospheric and endophytic. It is interesting to compare the nitrogen fixing ability of endophytes and rhizospheric rhizobacteria. The diazotrophic rhizobacteria can fix atmospheric nitrogen (N_2) and supply it to the host plants. It is also important to isolate local endophytic microbes that have the capability to fix nitrogen within the root tissues of the host plants. The endophytic microorganisms can biologically fixed nitrogen and it is transferred directly to the host plants without requiring an excess pool of nitrogen. The ammonia produce would efficiently assimilated by the host plant (James, 2000).

uch as *Acetobacter diazotrophicus*, *Azoarcus* spp., ne strain of *Azospirillum brasilense* tend to colonize the root cortex and may penetrate the endodermis and colonize the stele. The endophytes may be subsequently translocated to the aerial parts of the host plants (James, 2000). It is highly beneficial to isolate local diazotrophic rhizobacteria since it is more preferable and effective for inoculation to the tested crops under tropical conditions. A locally isolated diazotroph can be more competitive and well adapted to the extreme environment.

The high nutrient demand and high cost of chemical fertilizers (e.g. urea) for oil palm industry have encouraged the growers to find cheaper alternatives that may contribute to greater nutrient use efficiency. Better profit could be achieved through extensive biofertilizer applications to the agricultural industry (e.g. oil palm which required large quantities of fertilizers). The recommended rate of N fertilizer suggests an annual application of 4.2 kg nitrogen (ammonium sulfate) $palm^{-1}$ $year^{-1}$ to meet the nutrient demands of oil palm (Tarmizi and Mohd Tayeb, 2006). However, the use of high level of chemical fertilizers and plant growth hormones has several drawbacks. High inorganic nitrogen fertilizer usage can contribute to health hazards and environmental pollution (Stoltzfus *et al.*, 1997). High concentrations of IAA input can inhibit the hypersensitive response and may suppress plant defense genes expression. Subsequently it will lead to malfunctioning, which includes epinasty, tumor formation and plant organ deformation (Maor *et al.*, 2004). Thus, biofertilizer approach is becoming increasingly highlighted and important as many agricultural chemicals undergo intense scrutiny with regards to human toxicity and environmental impact.

ovement strategy for oil palm is through application of at any stage of tissue cultured process for the oil palm production. Struz and Nowak (2000) have reported that, introduction of diazotrophic rhizobacteria to the plant tissues during *in vitro* propagation process would maintain the beneficial organism within tissues of the host plant. It could be introduced as early as the embryoids and the shoot production stages of oil palm through tissue culture techniques. Several studies have been performed for establishing the bacteria within tissues of the host plants (Preininger *et al*., 1997; de Mayolo *et al*., 2003; Kumria *et al*., 2001). The early attachment of microbial within the root cells can help to protect and achieve full establishment of diazotrophs with the host plants. Pandey *et al*. (2000) have tested microbial inoculants (e.g. *Bacillus subtilis*, *Bacillus* sp. and *Pseudomonas corrugata*) for hardening of tissue cultured tea plants. The inoculation resulted in enhanced survival of the host plants up to 100%, 96% and 88%, respectively, compared to the control plants.

This study was on oil palm plantlets, which are readily colonized with diazotrophic rhizobacteria. Exploitation of biological nitrogen fixation (BNF) process and plant growth stimulation involving N_2 fixing bacteria for oil palm could fulfill the aim of agricultural industry to be more sustainable and environmental friendly.

tudy were:

1. To isolate diazotrophic rhizobacteria from oil palm roots and determine the ability to fix N_2 and produce essential phytohormones (Chapter 4)

2. To observe the effects of bacterial inoculation on oil palm embryoids proliferation and multiplication (Chapter 5)

3. To enhance rooting initiation of oil palm shoots which inoculated with PGPR under *in vitro* conditions (Chapter 6)

4. To observe growth of bacterized oil palm plantlets under glasshouse conditions (Chapter 6)

CHAPTER TWO

LITERATURE REVIEW

2.1 Plant Growth Promoting Rhizobacteria (PGPR)

Bacteria categorized as Plant Growth Promoting Rhizobacteria (PGPR) can promote plant growth through mechanisms such as i) asymbiotic biological nitrogen fixation, ii) the ability to produce plant growth hormones, e.g. indole-3-acetic acid (IAA), gibberellic acid and cytokinin, iii) increased nutrient and water uptake, iv) antagonism against phytophatogenic microorganisms by producing siderophore and v) solubilization of mineral phosphates and mineralization of other nutrients (Mantelin and Touraine, 2004). The successful effects of promoting plant growth by the PGPR depend on the effectiveness of bacterial colonization within the host plants.

Plant growth promoting rhizobacteria have been used as biofertilizer for over a century in the agricultural systems. It had successfully shown that PGPR had affected growth of the host plant significantly. Results from different experiments showed that up to 50 ï 70 % yield increase were reported due to PGPR inoculation (Lucy *et al*., 2004). Other reports on influence of PGPR for enhancing crops yields were also recorded for sugar beet, sugarcane, wheat, oil palm, maize, pineapple, kallar grass and rice (Amir *et al.*, 2001; 2003; Malik *et al.*, 1997; Elbeltagy *et al.*, 2001; El Zemrany *et al.*, 2006; Suman *et al*., 2005; Cakmakci *et al.*, 2006; Baldani *et al*., 2000).

n proposed as microbial inoculants which could enhance e applications of nitrogen fertilizer. It will directly and indirectly improve plant growth while moving towards environmental friendly agriculture systems. The most promising PGPR for the agricultural sector are N_2 fixing bacteria also collectively known as biofertilizer. The biofertilizer could ensure continuous supply of nutrients (N) to maintain optimal yields of the host plant (Kennedy *et al.*, 2004).

The significant changes in various plant growth parameters of sugarcane such as plant height, tiller number, dry matter yield and N uptake were influenced by inoculation of various nitrogen fixing and plant growth promoting rhizobacteria (Suman *et al.*, 2005). The inoculation of sugarcane with *Gluconacetobacter diazotrophicus* had increased shoot and root biomass and total N content of the host plants after 60 days of growth (Muthukumarasamy *et al*., 2006). Amir *et al.* (2003) also showed that inoculation of oil palm plant with rhizobacteria (*Azospirillum* and locally isolated *Bacillus* spp.) stimulated root growth and developments (root volume and root dry weight) of the host plants.

2.2 Diazotrophic rhizobacteria

Microorganisms that are capable of fixing N_2 are called ódiazotrophsô as they convert N_2 to ammonia (NH_3) by electron reduction and protonation of N_2 by their nitrogenase enzyme complex. Diazotrophic systems can be grouped into exophytic (diazotrophs that remains outside the host plant) and endophytic systems (when diazotrophs that are found within the host plant) (Shenoy *et al*., 2001). The

he zone of microbial stimulation at or near the plant root e related more specifically to root surfaces (Elliot *et al*., 1984). The colonization of roots by bacteria is an important step and involves a preliminary contact between beneficial bacteria and the host plant. They then penetrate deeply into the plant tissues (Barbara and Thomas, 1998). However, it is a complex phenomenon influenced by many biotic and abiotic parameters, some of which are now apparent. That is the reason why sometimes the use of these bacteria to protect crops failed because new rhizobacteria are unable to recolonize the rhizosphere of inoculated plants (Benezri *et al*., 2001).

The diazotrophs could be active as free living (diazotrophs are not in direct contact with the host plant) or associated (diazotrophs reside mostly on the external root surfaces or in the intercellular spaces) with the host plants. In this association, the inocula provide beneficial effects to the host plants (Shenoy *et al*., 2001). Previous report has shown various endophytic nitrogen-fixing bacteria associated with cereals and grasses (Barbara and Thomas, 1998). Endopyhtic diazotrophs such as *A. diazotrophicus*, *Azoarcus* spp., *Herbaspirillum* spp. and some strains of *Azospirillum brasilense* tended to colonize the root cortex and might even penetrate the endodermis to colonize the stele and may be subsequently translocated to the aerial parts of the host plants (James, 2000).

The discovery of endophytic bacteria inside the plant tissues is an exciting phenomenon and it is a challenge to understand their possible functions. In addition, endophytic colonization can prevent competition with indigenous soil bacteria. Endophytic colonization of diazotrophs is important to maximize N_2 fixation

petition from other microorganisms. Bacilio-Jimenez *et rasilense* could be excluded from the rhizoplane of the host plants due to competition with endophytic bacteria.

Initially the bacteria infected the root surface of the host plant before penetrating it and colonizing the root tissues. The primary colonization occur at the root tips and root elongation and differentiation zones where the bacteria can invade and penetrate into the central root tissues that later differentiate into stele (James *et al.*, 2001). Another route of entry appeared to be the points of emergence of lateral root junctions where the bacteria cells have been detected between the cell layers of the lateral root and the cortex of the main roots. Similar patterns of invasion have been observed for *A. diazotrophicus*, *Herbaspirillum* spp. and *Azospirillum* spp. Besides that, endophytes were also observed in the intercellular spaces when the mucilaginous layer was disrupted (Bacilio-Jimenez *et al*., 2001)

Although the initial steps of interior colonization consisted of bacterial attachment to the root surfaces subsequent steps involve several infection mechanisms. For intracellular colonization, the primary or secondary cell wall barrier has to be overcome, suggesting the involvement of cellulolytic enzymes. The enzymatic digestion might facilitate vertical spreading of endophytes. *Acetobacter* spp. and *Herbaspirillum* spp. have been found in large population in certain varieties of sugarcane which have grown for many years without nitrogenous fertilizer (Gough *et al*., 1997). Scanning Electron Micrographs (SEM) frequently identified endophytic bacteria at the base segment of secondary roots between the epidermis and mucilaginous layer.

ation within the plant tissues could ensure complete ed nitrogen to the host plant. It could provide protection from being impaired by environmental factors such as rain and flood that could reduce the number of bacteria population adhered on the root surfaces (Elliot *et al*., 1984). Earlier findings by Olivares *et al*. (1996) showed that the population of *Herbaspirillum* spp. on the root surfaces of graminae decreased below the detection limit (<100 cells g^{-1}) after 30 days of growth. The prospect is bright to apply this concept for economic crops like oil palm and rice in Malaysia. Application of endophytes as a biofertilizer and bioenhancer is a new approach in farming practices and serve as an alternative strategy to avoid excess applications of mineral fertilizers to plants.

2.2.1 *Azospirillum* spp.

Azospirillum spp. are Gram-negative bacteria which tested positive for oxidase test and are aerobic heterotrophs that fix N_2 under microaerobic conditions (in a range of 120-170 $\pm$ 40 nmol C_2H_4 per test tubes) (Ropper and Ladha, 1995). Strains of *Azospirillum* dissimilate nitrite to nitrous oxide and dinitrogen based on nitrate reductase test and detection of N_2O reductase gene involved in the denitrification. The *Azospirillum* spp. is mucoid, colourless and colonies are convex with regular margins on the nutrient agar after incubation for two days at 30°C (Galdagi *et al*., 2002). It appears as small pink colonies, which later turn scarlet, on Congo red solid media (Rodriguez-Caceres, 1982). Phase contrast light microscopy and transmission electron microscopy revealed curved to S-shaped motile rods, with

racellular granules (poly-ɓ-hydroxybutyrate, PHB) could were grown in N-free medium (Tarrand *et al*., 1978).

Azospirillum spp. has been found either on the surface of roots, particularly the root hair, elongation zones or within disrupted epidermises and outer cortices. *Azospirillum* spp. are not wholly endophytes but root-associated, soil-dwelling bacteria that are often found within plants, probably entering host plants *via* seeds or *via* wounds or cracks at lateral root junctions (James and Olivares, 1997). It is notable that *Azospirillum* spp. can be isolated from rhizosphere of sugarcane, grasses, wheat and rice (Van *et al*., 1997; Baldani and Baldani, 2005). The ability to produce phytohormones such as indole-3-acetic acid has been investigated particularly in *A. brasilense*. On the other hands, *A. irakense* is a low producer of indole-3-acetic acid and it is unique among *Azospirillum* species for its ability to grow on pectin and tolerates high concentration of salts (3%) (Baldani *et al*., 1997).

2.2.2 *Acetobacter diazotrophicus*

Acetobacter diazotrophicus is a Gram negative, acid tolerant obligate aerobe shaped as a straight rod with rounded end (0.7 ï 0.9 ε m) length. The cells can be seen under microscope as a single, pair of chain-like structures without endospore (Muthukumarasamy *et al*., 2002). The most common physiological characteristics of *A. diazotrophicus* are high sucrose tolerance (10%), growth and nitrogen fixation at low pH (pH 5.5), absence of nitrate reductase and production of acetic acid (Baldani *et al*., 1997; Sevilla *et al*., 1996). The formation of an initial thick white pellicle near the surface of N-free semisolid malate medium (Nfb medium) is one of the

rophicus (Muthukumarasamy *et al*., 2002). It has been coffee, pineapple, sorghum, finger millet and several species of tropical grasses (Emtiazi *et al*., 2003).

A. diazotrophicus has been recognized as an aerotolerant diazotroph in which oxygen is instrumental for the generation of ATP required for N_2 fixation. It has the ability to fix N under aerobic and microaerophilic conditions (Muthukumarasamy *et al*., 2002). Emtiazi *et al*. (2003) reported that *A. diazotrophicus* fixes N_2 at a wide range of atmospherics pO_2 and can adapt to maintain activity in response to both long-term and short term changes in atmospheric pO_2. Previously, González *et al*. (2006) demonstrated that for optimal nitrogen fixation activity, *A. diazotrophicus* demands high aerobic conditions. Therefore, *A. diazotrophicus* provide itself with an efficient mechanism for protection of its nitrogenase enzymes from the deleterious action of oxygen.

Two major protection systems exist for aerobic diazotrophy *A. diazotrophicus*; 1) respiratory protection mechanism (high respiratory activity in N_2 dependent grown cells) and 2) conformational protection mechanism (interaction of nitrogenase with FeSII protein leading to inactivate but protected nitrogenase) (González *et al*., 2006; Dong *et al*., 2002; Ureta and Nordlund, 2002). Apparently it was able to transfer 50% of fixed nitrogen in an *in vitro* system which modeling a plant-bacteria interaction (Cohjo *et al*., 1993). It was estimated that *A. diazotrophicus* fixed up to 150 kg N ha^{-1} $year^{-1}$ in sugarcane (Boddey *et al*., 1991). Besides that, Ureta and Nordlund (2002) reported that *A. diazotrophicus* exhibited antagonistic potential against *Colletrotrichum falcatum* (redroot causal

trophicus could also produce indole-3-acetic acid (IAA) lants (Bastian *et al*., 1998; Lee *et al*., 2004).

2.2.3 *Herbaspirillum* spp.

Herbaspirillum spp. are Gram-negative bacteria, positive for oxidase, catalase and urease test and grow well at pH ranging from 5.3 to 8.0. Organic acids and sucrose are favoured carbon substrates for *Herbaspirillum* spp. (Muthukumarasamy *et al*., 1999). *Herbaspirillum* spp. comprise of three species including *H. seropedicae*, *H. rubrisubalbicans* and *H. frisingense*. *Herbaspirillum* spp. form white, veil-likes pellicles in N free semisolid malate medium due to aerotactic attraction to appropriate oxygen concentration. They use organic acids like malic, fumarics, aspartic, citric, succinic, 2-ketogluconic acid and sugars like glucose, fructose, manitol, sorbitol, glycerol, *meso*-erythritol, galactose and sodium gluconate for sustaining their growth. Valverde *et al.* (2003) reported that *Herbaspirillum* cells grown on nutrient agar for 48 hours were motile with one or two flagella and had a short and curved rod morphology 0.6 ï 0.7 µm in diameter. It fixed atmospheric N_2 under microaerophilic condition. Kirchhof *et al.* (2001) reported that *Herbaspirillum frisingense* was able to reduce acetylene to ethylene with a mean ratio of 130 nmol C_2H_4 h^{-1} per 10^8 cells at an incubation temperature of 30°C. These data were in good agreement with the acetylene reduction ability of *Herbaspirillum seropedicae* at 37 °C (Baldani *et al*., 1986). You *et al.* (2005) also reported that the best nitrogen fixing activity of *Herbaspirillum* at 2.0% (v/v) O_2, was 56 nmol C_2H_4 h^{-1} per 10^9 cells. Phytohormone production by *Herbaspirillum* sp. is one of the other mechanisms that could contribute to plant growth promotion of host plant (Bastian *et al*., 1998).

acultative endophytic bacterium was well studied with m but very little is known on the association of *H. seropedicae* with maize and wheat. *Herbaspirillum* spp. are endophytic diazotrophs found in roots, stems and leaves of several plants such as rice, sugarcane and *sorghum bicolor* (Elbeltagy *et al*., 2001; James *et al*., 1997; James *et al*., 2002). An observation under Scanning Electron Microscopy (SEM) showed heavy colonization of *H. seropedicae* on root surfaces and within root and aerial tissues of maize and wheat (Roncato-Maccari *et al.*, 2003). *H. seropedicae* also attached and colonized on the root surface progressively from 3-15 days after inoculation. Besides that, Roncato-Maccari *et al*. (2003) also observed that *H. seropedicae* often colonized the site of secondary root suggesting that this site offer an opportunity for the bacteria to occupy the vascular cylinder after cortical invasion.

2.2.4 *Paenibacillus* spp.

Paenibacillus spp. were initially labeled as *Bacillus* spp. and are nonpathogenic endophytic bacteria and plant growth enhancer by producing plant growth regulators such as indole-3-acetic acid (Bacon and Hinton, 2002). *Paenibacillus* spp. were identified based on two characteristics: aerobic growth and by virtue of their capacity to form endospores in aerobic conditions (Mavingui *et al*., 1992). *P. polymyxa* is the most easily distinguished species amongst the bacilli owing to its very characteristics spores, which have heavily ribbed surfaces and are star-shaped in cross sections. *Paenibacillus* spp. are Gram-positive to Gram variable bacteria and showed positive results in catalase, Voges-Proskauer, starch, casein hydrolysis and nitrate reductase tests (Seldin *et al*., 1983). Most of *Paenibacillus*

. Different species of *Paenibacillus* such as *P. polymyxa*, formerly *P. azotofixans*) were able to fix nitrogen under anaerobic conditions. Seldin *et al*. (1998) found that *P. durus* were facultatively anaerobic, positive for catalase, Voges-Proskauer and starch hydrolisis, failed to reduce nitrate, effectively reduced acetylene to ethylene and were able to solubilize calcium phytate (organics phosphate). It had the ability to ferment three carbohydrates (starch, sorbitol and dulcitol).

Panibacillus spp. only fix N_2 anaerobically and respire oxygen rapidly to keep the amount of free oxygen low which is preferable for nitrogenase activities. Mollica *et al*. (1985), established ARA methods for screening of N_2 fixation activity in semisolid medium for *P. polymyxa, P. macerans* and *P. durus.* Several species of *Paenibacillus* (e.g., *P. macerans* and *P. polymyxa*) could protect the host plants against pathogenic fungus and nematode by producing growth inhibitory toxins (McSpadden Gardener, 2004; Bacon and Hinton, 2002; Landa *et al*., 2001).

2.3 Auxin production by Plant Growth Promoting Rhizobacteria (PGPR)

The potential used of Plant Growth Promoting Rhizobacteria (PGPR) for controlling and improving growth of host plant always related to the ability to produce beneficial plant growth regulators or phytohormones. Auxins are a class of phytohormone that play a central role in growth and development of plants (Lugwig-Müller *et al*., 1995; Patten and Glick, 2002). The plantôs response to IAA through roots extends from elongation of primary root, formation of lateral root and adventitious roots (Leveau and Lindow, 2005). Indole-3-acetic acid (IAA) and

are examples of auxins produced by the rhizobacteria in *et al.*, 2000; Martinez-Morales *et al*., 2003). Up to 80 % of rhizobacteria can synthesize IAA and have been identified as *Azotobacter* spp., *Acetobacter diazotrophicus*, *Paenibacillus polymyxa*, *Bacillus* spp., *Azospirillum* spp., *Rhizobium* spp., *Bradyrhizobium* spp., *Agrobacterium tumefaciens*, *Pseudomonas* spp. and *Xanthomonas* spp. (Dobbelaere *et al*., 1999; Lebuhn *et al*., 1997; Patten and Glick, 2002; Barazani and Friedman, 1999; Somers *et al*., 2005; Bastian *et al*., 1998; Kawaguchi and Syöno, 1996). Another two major groups of phytohormones produced by rhizobacteria are gibberellins and cytokinins (Bastian *et al*., 1998).

Bacterial - IAA production has been studied not merely due to its physiological effects on plants, but also due to its possible roles in plant-microbe interactions through IAA synthesis pathway and their regulation. It has been found that bacteria synthesize IAA by several pathways and the operation of more than one pathway in certain species has been proposed (Lambrect *et al*., 2000; Vandeputte *et al*., 2005). Lambrect *et al*. (2000) proposed three IAA synthesis pathways existed in *Azospirillum brasilense*. The pathways involved two tryptophan dependent pathway (indole-3-acetamide, IAM and indole-3-pyruvic acid, IpyA) and one tryptophan independent pathway. The IAM pathway initiated from L-tryptophan to IAM followed by IAA. While, the IpyA pathway is as follows; L-tryptophan → indole-3-pyruvic acid → indole-3- acetaldehyde → IAM → IAA. L-tryptophan (L-trp) is generally considered as an IAA precursor. Its addition to bacterial culture will release greater quantities of IAA and other related compounds (Asghar *et al*., 2002; Glickmann and Dessaux, 1995; Lee *et al*., 2004).

p. provide excellent model systems to investigate the biosynthetic pathways in azospirilla (Zakharova *et al*., 1999). Prior work has provided evidence for the existence of IAM pathway in *Azospirillum brasilense* and has shown biochemical and genetic evidence that azospirilla can utilize the IPyA pathway. It was also suggested that 90% of *Azospirillum* IAA synthesis followed a Trp-independent route (Lambrect *et al*., 2000).

Colorimetric technique derived by Salkowski is a well-known method to determine the level of indole-3-acetic acid in a growth medium (Meudt and Gaines, 1967; Somers *et al*., 2005; Asghar *et al*., 2002; Patten and Glick, 2002). It is a simple, rapid, cheap and not time-consuming method which allows faster daily analysis of IAA in bacterial cultures (Glickmann and Dessaux, 1995). The method permitted detection of IAA at a very low amount (lower detection limit, 0.3 µg/ml; 1.7 x 10^{-6} M). Patten and Glick (2002) found that in the presence of tryptophan, the wild type strains of *Pseudomonas putida* produced higher level of IAA. Asghar *et al*. (2002) revealed that IAA detected in a liquid medium of *Brassica* cultures were increased when supplemented with L-trp (24.6 µg/ml IAA equivalent) compared to the medium without L-tryp (11.40 µg/ml IAA). Addition of high concentrations of L-trytophan in the liquid medium could produce higher levels of IAA up to 68.3 µg/ml. Zakharova *et al*. (1999) reported that during 8-72 hours of incubation periods, IAA synthesis by *Azospirillum brasilense* ranged from 0.3 to 15 mg IAA equivalent per ml culture medium. Likewise, 18 strains of isolated *Acetobacter diazotrophicus* from rhizosphere of sugarcane had the ability to produce IAA under *in vitro* conditions ranging from 0.14 to 2.42 µg/ml (Fuentes-Ramirez *et al*., 1993).

2.4.1 What is nitrogen fixation process?

The nitrogen fixation is a process of reducing and converting atmospheric nitrogen gas into ammonia (NH_3). The reaction generates hydrogen gas which can be utilized to reduce molecular oxygen and generate electrons and ATP. The reaction is mediated by an oxygen-sensitive enzyme nitrogenase and requires energy, (16 moles ATP per mole of N_2 reduced) (Giller, 2001). The equations for this reaction in most nitrogen-fixing microorganisms are as follow:

Equation 1: Nitrogen fixation

$$N_2 + 8H^+ + 8H^- \rightarrow 2NH_3 + H_2$$

Equation 2: Net nitrogen fixation reaction (The net equation after the hydrogen is recycled).

$$N_2 + 6H^+ + 6e^- \rightarrow 2NH_3$$

N_2 reduction stoichiometry indicated the use of ATP.

$$N_2 + 8H^+ + 8\,e^- + 16ATP^+ \rightarrow 2NH_3^+ + H_2 + 16ADP + 16P_i$$

The majority of organisms can only use N in the form of ammonium (NH_4^+) or nitrate (NO_3^-). Most plants can absorb ammonium whereas nitrate is taken up into

on of a permease. Nitrate is mainly a product from

ication is the biological oxidation of ammonia with oxygen into nitrite followed by the oxidation of these nitrites into nitrates (Antoniou *et al*., 1990). Living organism cannot utilize nitrogen without the help from diazotrophic (nitrogen fixing) bacteria through Biological Nitrogen Fixation (BNF) process. The process improves nitrogen supply to the plant systems since the fixed nitrogen would be available to the plant directly with little or no loss. Such a system could also enhance resource conservation and environmental security besides freeing farmers from the economic burden of purchasing nitrogen fertilizer for crop production.

2.4.2 Nitrogenase enzyme

An enzyme called nitrogenase catalyses the conversion of nitrogen gas to ammonia and it is present in nitrogen-fixing organisms. In legumes, it only occurs within the bacteroids. The reaction requires hydrogen as well as energy from ATP. The nitrogenase complex is sensitive to oxygen and becoming inactivated when exposed to it (Postgate, 1982). The enzyme consists of two components; 1) MoFe-protein or dinitrogenase which contains the active sites where N_2 is reduced and 2) dinitrogenase reductase (Fe-protein) which provides electrons to MoFe-protein for N_2 reduction (Giller and Wilson, 1991).

MoFe-protein contains the active site for substrate reduction and is organized as a Ŭ2ƀ2 tetramer of molecular weight 240 kDa. Associated with this protein are two extraordinary metalloclusters designated as the FeMo-cofactor and the P-cluster. The FeMo-cofactor represents the site of substrate reduction, while the P-cluster is

or of electrons from the Fe-protein. The Fe-protein

TP hydrolysis to electron transfer and is the only known electron donor that can support substrate reduction by the MoFe-protein. The Fe-protein is a dimer of identical subunits (total molecular weight ca 60 kDa) (Rees *et al.*, 2005).

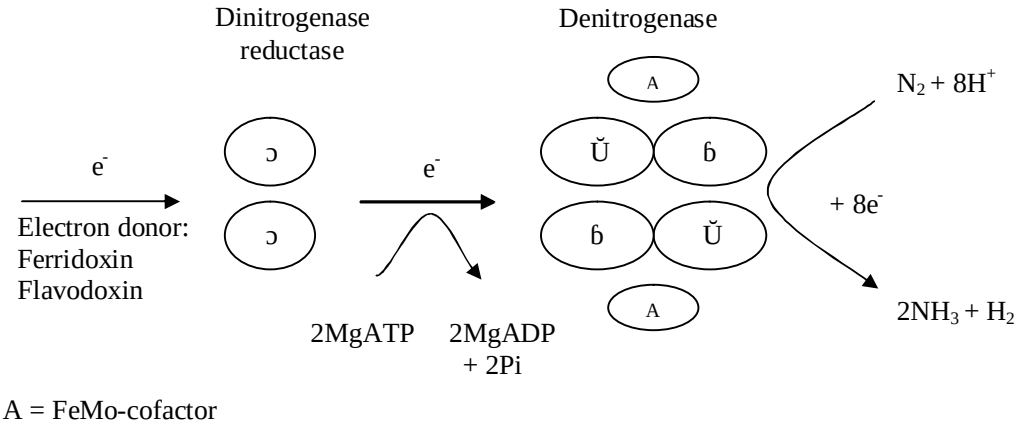

Figure 2.1: The subunit structure of Mo nitrogenase and the reaction of N_2 fixation (Giller and Wilson, 1991).

The sensitivity of nitrogenase to oxygen is not a problem with free-living anaerobic nitrogen-fixing bacteria such as *Clostridium* and *Azotobacter*. Free-living aerobic bacteria have a variety of different mechanisms for protecting the nitrogenase complex. Those include high rates of metabolism and physical barriers (Postgate, 1982). *Azotobacter* overcomes this problem by having the highest rate of respiration of any organism, thus maintaining a low level of oxygen in its cells. Meanwhile, *Rhizobium* controls oxygen levels in the nodule with leghaemoglobin. The leghaemoglobin (red, iron-containing protein) has a similar function to that of haemoglobin by binding to oxygen. It provides sufficient oxygen for metabolic

prevents the accumulation of free oxygen that would itrogenase. Leghaemoglobin is formed through the interaction of both plants and the rhizobia as neither can produce it alone (Giller and Wilson, 1991).

2.4.3 Nitrogenase and Acetylene Reduction Assay (ARA)

The nitrogenase is the enzyme that plays the main role in the reduction of nitrogen gas. Other than reduction of nitrogen gas to ammonia, the nitrogenase will also reduce other substrates, which contain triple bonds; e.g. acetylene gas (C_2H_2), N_2O, cyanide, methyl isocyanide, azide, cylopropene and diazirine (Giller, 2001). The acetylene gas is the most useful alternative substrates for analyzing the nitrogenase activity. It could be reduce to ethylene gas (C_2H_4). The reduction of acetylene is dominant over the reduction of N_2 by nitrogenase, due to high water solubility and high enzyme affinity of acetylene.

$$N \equiv N + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$$

$$HC \equiv CH + 2H^+ + 2e^- \rightarrow H_2C = CH_2$$

Acetylene Ethylene

The acetylene reduction assay (ARA) of nitrogenase activities is carried out by incubating the test materials in an atmosphere containing 10% acetylene in a close container of known volume. The amounts of accumulated ethylene (C_2H_4) after the incubation periods are then measured by gas chromatography. In principle, the method measures the electron flux through nitrogenase in the sample materials

prevails during the assay period (Azam and Farooq, ...me activity is expressed directly as εM C_2H_4 produced plant^{-1} h^{-1}.

The assay is potentially useful for determination of nitrogen fixation activities by associative and free-living bacteria (Anderson *et al*., 2004). During O_2 dependent growth, a minimal O_2 concentration is necessary to support aerobic respiration and ATP synthesis to meet the high-energy demand of nitrogenase. However, high O_2 concentration can lead to irreversible damage of nitrogenase (Marchal and Vanderleyden, 2000). Some bacteria have the ability to protect the nitrogenase enzyme from high oxygen concentration. Nevertheless some bacteria tolerate the oxygen level by fixing N_2 under microaerophilic conditions. Higher nitrogenase activities (ARA) were recorded under microaerophilic conditions, especially for *Azospirillum* strains (Bashan and de Bashan, 2005). Likewise, *Herbaspirillum* spp., requires microaerobic environment to be capable of expressing the nitrogenase activities (You *et al.*, 2005). Han and New (1998) have shown that *A. lipoferum* had a higher average nitrogenase activity than *A. brasilense*, both in Nfb medium and in association with wheat roots at 79.9 nmol C_2H_4 mg^{-1} protein h^{-1}. N_2 fixing ability of free-living *Herbaspirillum* grown in semisolid medium at 2% (v/v) O_2 was recorded at 65.5 nmol C_2H_4 h^{-1} per 10^8 cells.

Acetylene reduction assay technique was also used to determine the nitrogenase activities of root-associated diazotrophs. Exploitation of ARA for grasses had indicated the contribution of BNF up to 50 kg/N/year and was confirmed by the ^{15}N isotope dilution technique (Baldani and Baldani, 2005). In association

baspirillum could fix up to 14.8 nmol C_2H_4 g^{-1} fresh 5). *Azosprillum* spp. are responsible for the nitrogenase enzyme activities in association with maize, wheat and rice (Rai and Gaur, 1982; Bashan and Holguin, 1997). Nitrogen fixation activities of associative *A. brasilense* with finger millet were recorded in a range of 34.92 to 57.97 εmol C_2H_4 g^{-1} fresh roots h^{-1}. Elbeltagy *et al*. (2001) had detected the nitrogenase activities of rice seedlings inoculated with *Herbaspirillum* spp. up to 71.3 nmol C_2H_4 g^{-1} wet weight h^{-1}.

2.5 Oil palm industry in Malaysia

The oil palm (*Elaeis guineensis* Jacq.) is the most important industrial crops in Malaysia. Oil palm is a monocotyledon tree, belong to the family *Palmae* and the genus *Elaeis*. Oil palm was commercially exploited as an oil crop when the first oil palm planting estate in Malaysia was established at Tennamaran Estate, Selangor in 1917 (Basiron and Chan, 2004). The palm oil is obtained from the pulp (mesocarp) of the fruit while the palm kernel oil is extracted from the endosperm of the kernel (Ruslan, 2005).

Approximately 90% of palm oil which is commercially fractionated into olein and stearin is used in food products. Its physical properties as a semi-solid vegetable oil make it particularly suitable for margarines, bakery shortenings and some confectionery fats. The remaining 10% are used as oleochemicals. Recent investigations suggest the potential use of palm oil fatty acid esters as a biodiesel for engines combustion, similar to diesel fuel (Ruslan, 2005). Currently, palm oil is the

etable oil, amounting almost up to 20% of the worldôs

According to the latest statistics, the planted area of oil palm in Malaysia as at the end of 2005 stood at 4.05 million hectares (Basiron, 2007). This represents about 60% of the total 6.075 million hectares of land designated for agriculture under the National Agriculture Plan (NAP3) (1998-2010) (Faridah, 2001). Malaysia is now the largest producer and exporter of palm oil in the world, accounting for 31.8 % of global production and 30.0 million tones of total Malaysia palm oil export to major countries from January ï May 2006 (MPOC, 2006).

Higher fertilizer cost in oil palm budget has to be faced by the planters in order to sustain higher yield of oil palm. Besides that, higher input of chemical fertilizers could also affect the environment, in term of air, soil and water pollutions. The use of inorganics nitrogen fertilizer can contribute to health hazards and environmental pollution (Stoltzfus *et al*., 1997). Nitrogen is commonly assimilated to nitrate, which often contaminates ground water and surface streams, subsequently causing environmental and human health problems (Gresshoff, 2003).

2.6 Oil palm tissue culture

The tissue culture technique enables productions of plantlets that are uniform and genetically identical to the parent plant (Wooi, 1984; Teixera *et al*., 1995). Therefore high yielding plants could be produced continuously. However, this technique needs great knowledge and skill and a longer time is required to produce oil palm plantlets. The main criteria for selecting the high yielding ortets are its