EVIDENCE OF ENDOPHYTIC DIAZOTROPHS MIGRATION FROM ROOT TO LEAF TISSUES OF OIL PALM USING PADDY AS A PLANT MODEL

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by

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LIST OF ABBREVIATIONS

ARA	Acetylene Reduction Assay
BNF	Biological Nitrogen Fixation
bp	base pair
BTB	Bromothymol Blue
C_2H_2	Acetylene
C_2H_4	Ethylene
cfu	Colony forming unit
DNA	Deoxyribonucleic acid
g	gram
GC	Gas Chromatography
H	Hydrogen
h	hours
ha	Hectare
HPLC	High Performance Liquid Chromatography
IAA	Indole-3-acetic Acid
IPTG	Isopropylthio-β-D-galactosidase
K	Potassium
kb	kilobase pair
kg	Kilogram
KOH	Potassium hydroxide
kPa	kilo Pascal
l	litre
LB	Luria Bertani
L-tryp	L-tryptophan
mg	milligram

ml	millilitre
MS	Murashige and Skoog
N	Nitrogen
N_2	Nitrogen gaseous
NAA	Naphthalene acetic acid
Nfb	Nitrogen free semisolid medium
OD	Optical Density
P	Phosphorus
PCR	Polymerase Chain Reaction
Psi	pounds per square inch
RC	Red Congo
rDNA	ribosomal Deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
SEM	Scanning electron microscope
TAE	Tris-acetate-EDTA
TEM	Transmission electron microscope

BUKTI KEHADIRAN DIAZOTROF ENDOFITIK DALAM TISU DAUN KELAPA SAWIT

ABSTRAK

Diazotrof endofitik adalah bakteria yang hidup dalam nic mikro di dalam tisu tumbuhan. Dalam hal ini, diazotrof endofitik mampu mengikat nitrogen dan menyediakan sumber N kepada tumbuhan tanpa tekanan daripada persekitaran luaran. Kajian ini telah di lakukan untuk memencil dan mengecam diazotrof endofitik daripada tisu daun kelapa sawit dan pencilan tersebut kemudiannya ditanda dengan gen *gfp* dengan tujuan untuk menjejak pengkolonian mereka di dalam perumah tumbuhan yang lain iaitu pokok padi. Pencilan dicamkan sebagai pengikat nitrogen berdasarkan keputusan Asai Penurunan Asetilena (ARA) dan amplifikasi PCR bagi gen *nifH.* Potensi pencilan untuk menghasilkan asid indol-3-asetik juga dianggarkan dengan ujian kolorimetrik Salkowski. Identifikasi pencilan dipastikan dengan analisis penjujukan DNA ribosom 16S (16S rDNA), ciri-ciri fenotip berdasarkan keupayaan pencilan untuk menggunakan sumber karbon yang berbeza dan perbezaan morfologi pada agar Congo Red, Nfb malat pejal dan separa pejal dan juga ciri-ciri pencilan di bawah mikroskop cahaya. Eksperimen ini telah berjaya memencilkan 9 strain diazotrof endofitik daripada tisu daun kelapa sawit. Berdasarkan kepada ciri-ciri molekular dan fenotip, diazotrof endofitik ini mempunyai leluhur yang hampir kepada genera *Azospirillum* sp., *Acinetobacter* sp., *Acetobacter* sp., *Enterobacter* sp. dan *Phytobacter* sp. Bagi mengkaji bentuk kolonisasi, strain bakteria yang boleh disesuaikan dengan vektor pGFPCR iaitu *Enterobacter* sp.

ditandakan dengan gen *gfp* dari plasmid tersebut. *Enterobacter* sp. strain USML2 yang telah ditandakan diinokulasikan pada sistem akar pokok padi. Sebaik sahaja bersentuhan dengan sistem akar pokok padi, strain bakteria ini menunjukkan penyebaran yang luar biasa yang mana selepas 24 jam selepas penginokulasian, $3.44x10^{6}$ $CFUgram^{-}$ (berat basah) *Enterobacter* sp. USML2 yang telah di-tag telah dilihat pada bahagian akar. Manakala $3.21x10^{4}$ $CFUgram^{-}$ (berat basah) *Enterobacter* sp. USML2 yang telah di-tag dijumpai di bahagian batang pokok padi dan sejumlah $3.44x10^{4}$ $CFUgram^{-}$ (berat basah) *Enterobacter* sp. USML2 yang telah di-tag dijumpai di bahagian atas batang pokok padi. Dengan $5.31x10^{4}$ $CFUgram^{-}$ (berat basah) *Enterobacter* sp. USML2 yang telah di-tag dijumpai di bahagian tisu daun,dapat disimpulkan bahawa migrasi bakteria berlaku dari akar ke daun pokok padi. Kolonisasi yang padat diperhatikan di akar primer dan akar sekunder dan juga pada cabang pertunasan akar lateral. Hasil kajian menunjukkan bahawa kolonisasi bakteria *Enterobacter* sp. strain USML2 adalah sama dengan kolonisasi endofitik pencilan bukan-legum. *Enterobacter* sp. strain USML2 dapat memasuki tisu akar pada tapak pertunasan akar lateral, tanpa pembentukan serangan jangkitan seperti pada rhizobakteria simbiotik.

EVIDENCE OF ENDOPHYTIC DIAZOTROPHS IN LEAF TISSUES OF OIL PALM

ABSTRACT

Endophytic diazotrophs are bacteria that occupy the microniches in plant tissues. In this case, they are able to fix nitrogen and provide N source to the plant with less environmental constraints. Research was conducted to isolate and identify endophytic diazotrophs from oil palm leaf tissues. The isolates were then tagged with *gfp* gene to track their colonization inside rice plant tissues. The isolates were identified as nitrogen fixers based on the results of the Acetylene Reduction Assay (ARA) and successful PCR amplification of the *nifH* gene. The potential of isolates to produce indole-3-acetic acid was also estimated by Salkowski's colorimetric test. Identification was performed using 16S ribosomal DNA (rDNA) sequence analysis as well as phenotypic characteristics of the isolates on Red Congo agar, Nfb solid and semi solid malate media and under light microscope were also observed. Nine bacterial strains of endophytic diazotrophs were successfully isolated from oil palm leaf tissues. Based on molecular and phenotypic characterizations, the endophytic diazotrophs were phylogenetically close to genera *Azospirillum* sp., *Acinetobacter* sp., *Acetobacter* sp., *Enterobacter* sp. and *Phytobacter* sp. To study the colonization pattern, the bacteria *Enterobacter* sp. strain USML2, was transformed with pGFPCR vector to tag it with *gfp* gene. The tagged *Enterobacter* sp. strain USML2 was introduced to the root system of rice plant. After coming into contact with the rice plant root system the bacteria showed an unusual spreading to other plant

part where in 24 hours after inoculation, $3.44x10^6$ $CFUgram^-$ (fresh weight) of *gfp*-tagged *Enterobacter* sp. USML2 were observed in root section. While $3.21x10^4$ $CFUgram^-$ (fresh weight) of *gfp*-tagged *Enterobacter* sp. USML2 found in stem section and $3.44x10^4$ $CFUgram^-$ (fresh weight) of *gfp*-tagged *Enterobacter* sp. USML2 found in upper stem section. With $5.31x10^4$ $CFUgram^-$ (fresh weight) of *gfp*-tagged *Enterobacter* sp. USML2 found in leaf tissues, it is concluded that bacteria migration happens from roots to leaf of rice plant. Dense colonization was observed on the primary and secondary roots and also on the junction of emergence of the lateral roots and subsequently to stem and leaf tissues. Results showed that the colonization pattern of *Enterobacter* sp. strain USML2 was similar to that of other endophytic bacteria isolated from non-legumes. *Enterobacter* sp. strain USML2 reached entry inside the root at the sites of emergence of lateral roots, without formation of infection threads as in the case of symbiotic rhizobacteria.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Nitrogen is the main source of minerals for plant growth in agriculture. Therefore, the use of nitrogen fertilizer is one of the factors that influence the growth of a crop. The use of N_2-fixing endophytic bacteria as biofertilizer has been proven could enhance the growth and yield of crops (Chanway *et al.,* 2000). It is one of the most effective and efficient method to replace the excessive use of chemical fertilizers that can harm the environment. Moreover, with the rise in fertilizer prices nowadays, the use of chemical fertilizers especially nitrogen, are very expensive for farmers and thus, the alternative is to use bacteria inoculants in crop plants as biofertilizer (Govindarajan *et al.*, 2008).

Plant-associated bacteria play an important role in growth progress of a plant, both in the natural and controlled ecosystems (Sturz *et al.,* 2000; Liu *et al.,* 2006; Govindarajan *et al.,* 2008). Interactions between beneficial bacteria and host plants can have profound effects on crops health and yield and also quality of rhizosphere occupied by the bacteria. Beneficial microbes support plant growth and health by increasing the soil fertility through several mechanisms including increasing nutrient availability and improving soil structure. They also benefit the plants by inducing the plants defence mechanisms trough their ability to produce antibiotic against

outcompeting pathogens and also providing the host plants with growth-stimulating substances such as phytohormones (Ciccillo *et al.,* 2002; Lodewyckx *et al.,* 2002; Van Loon *et al*., 1998).

The appearance of endophytic diazotrophs as frequent colonizer of some important crops and grasses have been widely studied (Stoltzfus and de Bruijn, 2000; Zinniel *et al.,* 2002). This variety of endophytes colonization gives higher yield to the host plants even without the supplementation of any chemical fertilizer (Lodewyckx *et al.,* 2002). It has been suggested that the low partial oxygen pressure (pO_2) condition exist in the interior part of the plants render it as suitable niche for nitrogen fixation (Govindarajan *et al.,* 2008). Endophytic bacteria have the advantages in terms of being protected from the competitive, high-stress environment of soil as compared to rhizospheric bacteria. They also are believed to provide more fixed nitrogen to the plants as the fixed nitrogen itself has direct accessibility to the plants (Sturz *et al.,* 2000; Whipps, 2001). Moreover, endophytic bacteria are of particular interest as they would induce greater plant growth promoter rather than bacteria restricted to the rhizosphere and root surfaces (Chanway *et al.,* 2000; Regupathy *et al.,* 2010).

Various genera of nitrogen fixing bacteria including *Azoarcus, Herbaspirillum, Azospirillum, Burkholderia, Rhizobium, Serratia, Enterobacter* and *Klebsiella* have been isolated from crop plants such as rice, maize, wheat and certain grasses (Sharma *et al.,* 2005; Azlin *et al.,* 2005; Singh *et al.,* 2006; Forchetti *et al.,* 2007; Jha and Kumar, 2009). It is reported in isolation and identification studies, that this endophytes have a broad range of host, for example, *Herbaspirillum seropedicae*

has been found in variety of crops, including maize, sorghum, sugarcane and other gramineae plants (Olivares *et al.,* 1996; Rosenblueth and Martinez-Romero, 2006; Senthilkumar *et al.,* 2011). *Enterobacter* genera have been shown to have an association with different host plants. This indicates that an endophyte isolated from one host family member can colonize other nonhost members which suggestive of non-specific host plants (Dong *et al.,* 2003; Senthilkumar *et al.,* 2011). For example colonization of *Burkholderia* sp. isolated from onion has been demonstrated in grapes (Compant *et al.,* 2005), potatoes and vegetables (Nowak *et al*., 1995).

In isolation part of this study, it was hypothesized that isolates harbored from leaf tissues of oil palm would be endophytic diazotrophs that are common in interior tissues of plant such as *Gluconacetobacter diazotrophicus* in sugarcane (Tapia-Hernández *et al.,* 2000), *Herbaspirillum seropedicae*, endophytes in maize, rice seedlings and sugarcane (Roncato-Maccari *et al.,* 2003; James *et al.,* 2002). However, after 16S rDNA identification was done, almost all of isolates were common diazotrophic bacterial in soil and rhizosphere. This finding triggers an interest to investigate another hypothesis that the soil/ rhizosphere diazotrophs has the ability to migrate from the soil to the leaf tissues.

Molecular technique is an approach of interest for studying the presence of endophytic diaztrophs in plants. The practice of conventional methods maceration and centrifugation of surface-sterilized plant tissues could be used for detection and isolation of endophytes inside the plants. However, these methods only provide an estimation of bacterial population with some extent of colonization, but could not reveal the site of infection during colonization process (Govindarajan *et al.,* 2008).

Visualization and localization of microbes in plant tissues are particularly interesting in studying plant-microbe interaction in detail. This attractive study can be facilitated by using tagging methods that allow us to track the bacteria of interest in environment or plant system. Variety of marker system can be used like chromogenic (*gusA* and *lacZ*), luminescent (*luxAB*) and fluorescent markers like green fluorescent protein (*gfp*) (Ramos *et al.,* 2002; Shiveta *et al.,* 2008). Compare to other molecular markers for tracking bacteria in any systems, the green fluorescent protein (*gfp*) marker conversely, has been proven to be a very attractive, simple and yet excellent marker system in studying the plant-microbe interactions (Chalfie *et al.,* 1994; Errampalli *et al.,* 1999; Sharma *et al.,* 2005). For example the *gusA*, *lacZ*, and *luxAB* markers have the disadvantages like time-consuming procedures, the needs of a suitable substrates and the occurring of problem when the colour derivative diffuse to places other than the site of bacterial colonization (Sessitsch *et al.,* 2002). On the other hands, the green fluorescent protein (*gfp*) (Prasher *et al.,* 1992), a 27 kDa polypeptide fluoresces directly when excited by UV or shortwave blue light. This direct non-substrate detection can be easily detectable, eliminating the use of exogenous cofactors which later on might give some drawbacks to the experiments (Shiveta *et al.,* 2008; Valdivia *et al.,* 1998). Furthermore, it has been proven that the use of green fluorescent protein (GFP) in combination with confocal laser scanning microscopy (CLSM) is a powerful approach and has been used for visualizing plant microbe interactions in rice (Chi *et al.,* 2005; Verma *et al.,* 2004; Liu *et al.,* 2006) wheat (Ramos *et al.,* 2002; Sharma *et al.,* 2005), maize (Newman *et al.,* 2003; Liu *et al.,* 2006; Shiveta *et al.,* 2008), etc.

1.2 Objectives of the research

As the study of endophytic colonization pattern of locally isolated endophytic diazotrophs in rice plant is much needed, thus the objectives of this study are:

- To identify endophytic diazotroph bacteria from leaf tissues of oil palm by PCR amplification of *nifH* gene and partial sequence of 16S rDNA.
- To tag one of the isolates with green fluorescence protein (*gfp*) to facilitate its localization inside plant tissue.
- To observe the process of colonization of one of these isolates using rice plant as a model.

CHAPTER 2

LITERATURE REVIEW

2.1 Diazotrophic microorganisms

Diazotrophs are the microorganisms that capable of fixing N_2 and converted it to ammonia (NH_3) by nitrogenase enzyme. 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 bacteria provide beneficial effects to the host plants (Senthilkumar *et al*., 2008). Rhizosphere (soil region sheltered by plant roots) and rhizoplane (root surfaces) are another part of a plant being occupied by complex microbial communities depending on the nutrients released by the roots (Bais *et al.*, 2004; Senthilkumar *et al*., 2006).

Previous reports have shown various nitrogen-fixing bacteria which associated with cereals and grasses (Rai *et al.*, 2007; Rothballer *et al.*, 2008). Endopyhtic diazotrophs such as *Acetobacter diazotrophicus*, *Azoarcus* spp., *Herbaspirillum* spp. and some strains of *Azospirillum brasilense* are reported to colonize the host plant by colonizing the root cortex and later penetrate the endodermis to colonize the stele and other aerial parts of plant (James, 2000; Rai *et al.*, 2007; Rothballer *et al.*, 2008).

2.2 Endophytic colonization

Endophytic bacteria are defined as bacteria that reside within the plant tissues and pathogenically harmless to the host plants (Hardoim *et al*., 2008). Endophytic bacteria have been isolated from the surface-sterilized flowers, fruits, leaves, stems, roots, and seeds of various plant species (Madhaiyan *et al.*, 2004; Mocali *et al.*, 2003). The isolates have demonstrated an incredible amount of diversity in plant hosts and in bacterial taxa as well (Bacon & Hinton, 2006). Many factors, such as plant genotype (Pamela *et al.*, 2002; Surrette *et al.*, 2003), plant development (Mocali *et al.*, 2003), and other biotic and abiotic environments (Assigbetse *et al*., 2005; Graner *et al*., 2003) are considered to influence the population structures of endophytic bacteria.

It is an exciting and challenging phenomenon to discover the endophytic bacteria inside the plant tissues and understand their possible functions (Reinhold-Hurek and Hurek, 1998). Interior colonization of endophytic diazotrophs is important to maximize N_2 fixation potential in the course of less competition from other dominant microorganisms. The interior colonization of diazotrophs within plant tissues could ensure complete transferring of biological fixed nitrogen to the host plant. The interior environment could also provide protection to the endophytes from being impaired by outside environmental factors such as heavy rain and flood that can reduce the number of bacteria population adhered on the root surface (Elliot *et al*., 1984). Similar finding was also observed by Olivares *et al*. (1996), where by the population of *Herbaspirillum* spp. after 30 days of inoculation decreased below detection limit (<100 cells g^{-1}). In addition, other reports also showed that bacteria

colonization can be inhibited by other bacteria. Findings by Bacilio-Jimenez *et al*. (2001), *Azospirillum brasilense* seems to be expelled from the rhizoplane by the endophytic bacteria suggesting that endophytes compete with *Azospirillum* in colonizing the root surfaces.

Initially the bacteria infected the root surfaces of host plant before penetrating the roots and colonizing the root tissues (Prieto and Mercado-Blanco, 2008). The bacteria invade roots by some pathways of infection, the primary colonization site is root tips and root elongation and differentiation zones where the bacteria can invade and penetrate into the central root tissues that later differentiate into steles (Rosenblueth and Martínez-Romero, 2006). The points of emergence of lateral root junctions appears to be another route of entry, the bacteria cells have been detected between the cell layers of the lateral roots and the cortexes of the main roots (Prieto *et al.,* 2009). The initial step of bacterial interior colonization started with bacterial attachment to the root surfaces, followed by specific infection mechanisms (Mercado-Blanco and Bakker, 2007). Similar models of invasion have been observed for *A. diazotrophicus*, *Herbaspirillum* spp. and *Azospirillum* spp. Besides that, the endophytes were also observed in the intercellular spaces when the mucilaginous layer was disrupted (Bacilio-Jimenez *et al*., 2001). The involvement of cellulolytic enzymes has been suggested for intracellular colonization, where the primary or secondary cell wall barrier of the host plant has to be overcome. The enzymatics digestion might facilitate vertical spreading of endophytes.

Bacterial colonization on root surfaces is an important step or the preliminary intact ion between beneficial bacteria and the host plant before penetrating deeply into the plant tissues. Many biotic and abiotic parameters have influenced this phenomenon (Prieto and Mercado-Blanco, 2008) which can cause the application of these bacteria to the plants sometimes failed as rhizobacteria are unable to recolonize the rhizosphere of inoculated plants in controlled condition (Benezri *et al*., 2001).

2.3 Nitrogen fixation

Nitrogen fixation is a process to reduce and change the nitrogen gas into ammonia (NH_3). This reaction is mediated by oxygen-sensitive nitrogenase enzyme, requires energy (16 mol of ATP/ mol N_2 reduced) (Giller, 2001) and produces hydrogen gas. Then, the H_2 will be used to reduce molecular oxygen and generate electrons and ATPs. The equation for this reaction in most nitrogen fixing microorganisms is as follows:

Equation 1: Nitrogen fixation

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

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

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

N_2 reduction stoichiometry indicated the use of ATP.

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

Majority of organisms can only use N in the form of ammonium (NH_4^+) or nitrate (NO_3^-). Most plants can absorb NH_4^+ whereas NO_3^- is taken up into the plant cell by the action of permease. Nitrate, which is the preferred sources of exogenous inorganic nitrogen for most plants mainly from ammonium, liberated decaying organic matter. The conversion is called nitrification, which involving two bacteria, *Nitrosomonas* sp. and *Nitrobacter* sp. *Nitrosomonas* converts NH_4^+ into NO_2^-. *Nitrobacter* oxidizes NO_2^- to NO_3^- (Antoniou *et al*., 1990). Living organisms cannot utilize N_2 without the help from diazotrophic (nitrogen fixing) bacteria via Biological Nitrogen Fixation (BNF) process. This BNF process could also enhance resource conservation such as organic or chemical nitrogen and improve the environmental sustainable. A smart utilization of BNF in agriculture also benefits farmers from the economic burden of purchasing nitrogen fertilizer for high crops yield.

2.4 Nitrogenase

Nitrogenase is an enzyme that catalyzes the conversion of nitrogen to ammonia in nitrogen-fixing organisms. This conversion requires hydrogen as well as energy from ATP. The occurrence of this enzyme in legumes is only within the bacteroids. According to Postgate (1982), nitrogenase complex is sensitive to oxygen and becoming inactivated when exposed to it. The enzyme consists of two components; 1) MoFe-protein or dinitrogenase contains the active sites where N_2 is reduced and 2) dinitrogenase reductase (Fe-protein) provides electrons to MoFe-protein for N_2 reduction (Giller and Wilson, 1991).

MoFe-protein contains active site for substrate reduction, and is organized as an α2β2 tetramer of 240 kDa molecular weight. Affiliated with this protein are two superb metalloclusters the FeMo-cofactor and P-cluster. The P-cluster is suspected as initial acceptor of electrons from the Fe-protein while the FeMo-cofactor represents the site of substrate reduction. It is believed that the coupling process of ATP hydrolysis to electron transfer is mediated by Fe-protein to support substrate reduction by MoFe-protein (Rees *et al*., 2005).

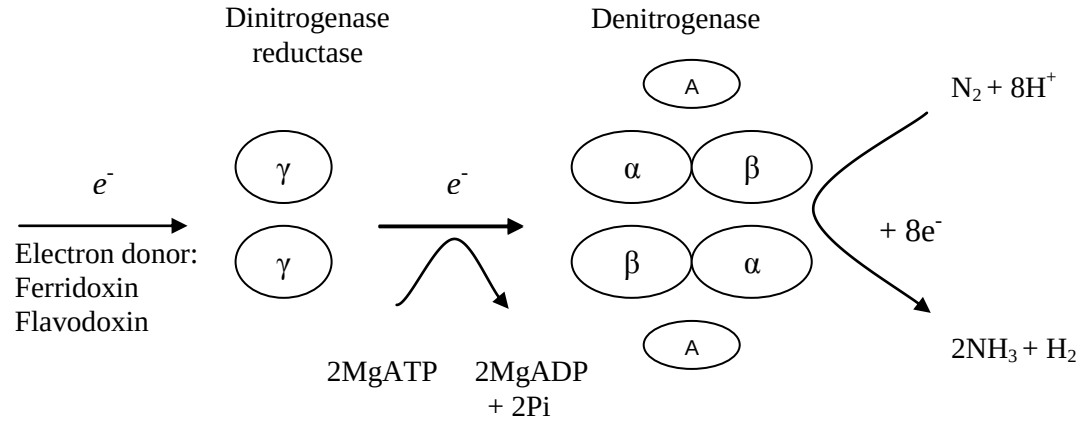

Figure 2.1: Subunit structure of the Mo nitrogenase and the reaction of N_2 fixation (Giller and Wilson, 1991).

As the nitrogenase is sensitive towards the oxygen, the anaerobic diazotrophs have their own mechanisms to protect this nitrogenase complex, including the high rates of metabolisms and some physical barrier (Postgate, 1982). Free-living diazotrophs like *Azotobacter*, maintaining a low level of oxygen in its cells by having a very high respiration rates. While the nodulate diazotrophs like *Rhizobium* controls the oxygen level in nodule with leghaemoglobin (red, iron-containing protein) that

has a similar function to that of heamoglobin; binding to oxygen. The legheamoglobin prevents the accumulation of free oxygen that would destroy the nitrogenase activity, but in the same time provides sufficient oxygen for metabolic function of the bacteroids. Leghaemoglobin is formed in the interaction of the plant and rhizobia as in leguminosae plant (Giller and Wilson, 1991).

2.4.1 Nitrogenase activity detection

One of the methods to measure nitrogenase activity is by radioactive isotope labeling. There is no well-handy radioactive isotope of N_2, but several methods have been developed. An example is the reduction of nitrogen-15 isotope. However, the reduction of nitrogen-15, a heavy but none-radioactive isotope to NH_3 is difficult to measure and requires a mass spectrometer. In this process, the gas phase of a culture is filled with ^{15}N and let the cells digested. Ammonia (^{15}N-labeled NH_3) that produced will be distilled off and assayed for its ^{15}N content as proof of nitrogen fixation (IAEA, 2001).

Another method to estimate N_2 fixation is based on a nutrient anomaly parameter, N*, which is defined as the nitrogen concentration in excess or in deficit of phosphorus, relative to the Redfield ratio of 16 (N:P). Areas with a positive N* indicate N_2 fixation. This method is particularly useful for global estimation of N_2, but it is unsuitable for studying in cultures or natural samples under controlling factors (Gruber and Sarmiento, 1997; Michaels *et al.*, 1996).

2.4.2 Acetylene reduction assay (ARA) as a method for nitrogenase activity detection

Enzyme that plays the main role in the reduction of nitrogen gas is nitrogenase. Apart from the reduction of nitrogen gas to ammonia, nitrogenase also reduces other substrates, which contain triple bonds; e.g. acetylene gas (C_2H_2), N_2O, cyanide, methyl isocyanide, azide, cylopropene and diazirine (Giller, 2001).

In practice, the most applicable method for nitrogenase activity detection is the Acetylene Reduction Assay (ARA), where nitrogenase activity is measured by its ability to reduce acetylene C_2H_2 to ethylene, C_2H_4, and later detected by gas chromatography. Less energy is needed for this process since the reduction of acetylene by nitrogenase is only a two-electron process, and ethylene is produced (Burgess and Lowe, 1996). Apart from that, acetylene gas reduction by nitrogenase is more dominant over the N_2 reduction; it is due to high water solubility and high enzyme affinity towards acetylene.

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

Nitrogen — Ammonia

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

Acetylene — Ethylene

The acetylene reduction is a more rapid, sensitive and yet the easiest way to measure the nitrogenase activity. Nitrogenase activity of bacteria can be measured

both in *in vivo* as well as *in vitro* by the acetylene reduction assay (ARA) with the fact that acetylene and ethylene permeable to the bacterial envelope (Burgess and Lowe, 1996).

The Acetylene Reduction Assay (ARA) of nitrogenase activities is carried out by incubating the test materials in an atmosphere containing 10% acetylene in an air proof container of known volume. The amounts of accumulated ethylene (C_2H_4) after the incubation periods are then measured by gas chromatography. The nitrogenase enzyme activity is expressed directly as μM C_2H_4 produced $plant^{-1}$ h^{-1}.

The assay is feasible for determination of nitrogen fixation activities by associative and free-living bacteria (Anderson *et al*., 2004). Principally, nitrogenase activity occupied the fixing ability of bacteria during certain incubation times. Aerobic respiration and ATP synthesis during O_2 dependent growth need only minimal O_2 concentration to meet their energy demand for nitrogenase activity. Conversely, high O_2 concentration can lead to irreparable damage of nitrogenase (Marchal and Vanderleyden, 2000). Some bacteria have the ability to protect the nitrogenase enzyme from the inhibitors, high oxygen concentration. Nevertheless some bacteria tolerate with 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.

2.5 Gene *nifH* as diazotrophs diversity biomarker

The *nifH* gene is a biomarker commonly used to explore the diversity of diazotrophs in the environment such as wetland plants and rice roots (Lovell *et al.,* 2000; Ueda *et al.,* 1995). A range of plant-associated diazotrophs, including the alpha, beta and gamma proteobacteria and also the Gram-positive anaerobes have successfully detected by the *nifH* gene fishing method from the plant roots (Engelhard *et al.,* 2000; Knauth *et al.,* 2005; Ueda *et al.,* 1995). Even though the studies of *nifH* gene pools associated with plant have been reported (Engelhard *et al.,* 2000, Hurek *et al.,* 2002), the dynamics of *nifH* genes in plant under field conditions have rarely determined (Wu *et al*., 2009). It has been studied that the community structure of diazotrophs are greatly influenced by the physiological properties of plants due to close relations between diazotroph and plant roots (Engelhard *et al.,* 2000, Knauth *et al.,* 2005, Tan and Reinhold-Hurek, 2003).

2.6 Diversity and characterization of *nifH* gene

Culturing methods have long been used in microbiology to study the nitrogen fixer diversity; however these methods have a drawback which identifies only 5–10 dominating genera of diaztrophs at a time (Sarita *et al.,* 2008). This strategy conversely has limited the diversity description of free-living soil diazotrophs or other environment samples due to unculturability of many prokaryotes (Amann *et al*., 1995). These drawbacks in culture-based approaches have been improved with molecular techniques that provide a more complete picture of diazotrophic

community in environments (Hugenboltz *et al.,* 1998; Zehr *et al.,* 2003). Cloning of PCR-amplified products, PCR-based restriction fragment length polymorphism (RFLP), fluorescently labeled terminal restriction fragment length polymorphism (t-RFLP) and denaturing gradient gel electrophoresis (DGGE) (Sarita *et al.,* 2008) becoming a more dominant techniques in characterizing the diversity of *nifH* gene pools from various environments (Poly *et al.,* 2001a; Rosch *et al.,* 2002; Piceno *et al.,* 1999). Various diazotrophic communities diverse in soil systems have been described by these molecular techniques, such as forest soils (Widmer *et al.,* 1999), pasture, agricultural soils (Shaffer *et al.,* 2000), wetland soils (Poly *et al.,* 2001b) and rhizosphere soils (Chelius, 1999).

At present, attention is focused on so-called functional genes, whose analysis allows the assessment of the diversity of microorganisms that perform certain ecological functions in an ecosystem. The *nifH* gene is one of the most rigorously conserved functional genes and the phylogeny of prokaryotes based on the analysis of this gene sequences agrees well with the data obtained when analyzing 16S rRNA (Ueda *et al.,* 1995; Zehr *et al.,* 2003). This allowed the molecular-biological approaches based on the analysis of the *nifH* gene sequences to be applied to study the biodiversity of both nitrogen-fixing isolates and the whole natural microbial community (Ben-Porath and Zehr, 1995; Okhuma *et al.,* 1996).

In order to determine *nifH* gene nucleotide sequences, the oligonucleotide primers which make it possible to amplify fragments of this gene sufficient for identifying microorganisms must be designed. Various types of *nifH* primers have been designed to study the diversity of diazotrophs (Poly *et al.,* 2001a; Rosch *et al.,*

2002; Widmer *et al.*, 1999). However, there are certain difficulties connected with the successful use of these primers. First, the wide phylogenetic diversity of nitrogen-fixing microorganisms result in considerable variability of even such conserved gene as *nifH* which sometimes can be found at 360bp in certain microorganisms while in other microorganisms 470bp. That is why the set of conserved sites of this gene suitable for constructing oligonucleotide primers is rather limited. Second, the high degree of degeneracy of the *nifH* gene nucleotide sequences virtually rules out the possibility of the existence of sufficiently extended invariant sequence sites. Therefore, most of the currently existing oligonucleotide primers were designed to amplify *nifH* gene of individual taxonomic groups of prokaryotes or even for individual microorganisms, particularly cyanobacteria (Kirshten *et al.*, 1991). Apart from this, the pool of the *nifH* nucleotide sequences available from GenBank is relatively small (in comparison with the number of available 16S rRNA sequences). This also hinders the development of primers that are sufficiently universal.

2.7 Markers for tracking introduced bacteria

To track the bacteria that have been introduced in complex environments such as soils and plants needs complex discriminate mechanisms in order to distinguish between the inoculums and the indigenous microflora. Several criteria are needed in a marker used for tracking purposes and it should obviously be specific. The specificity and the stability of marker must be verified within the environment where the bacteria are introduced. Thus, loss and/or its transfer to other

microorganism can be avoided. Besides, the marker should not change the behavior of introduced bacteria. Surprisingly, very little comparison study on the activity of the marked and wildtype strains have been done (Blot *et al.*, 1994; Gotz *et al.*, 2006; Ramos *et al*., 2002; Thornton, 2008). In the same way, perturbation of the system since the alteration should be kept as low as possible to avoid substrate amendment during the marker expression. Furthermore, the tracking process of markers chosen should be easy and hassle-free whereby its appearance in soils or plants or other environments can be distinguished. The expression conditions favorable (pO_2, pH, etc.) by the markers also should be considered (Gotz *et al.*, 2006).

2.7.1 Molecular marker

Various molecular markers such as chromogenic (*xyl*E, *gus*A, *lac*Z) (Kozaczuk *et al.*, 2000; Weller, 1988), antibiotic resistance (Glandorf *et al.*, 1992; Lemanceau *et al.*, 1992; Van Elsas *et al.*, 1998), fluorescent markers (*gfp* and unstable *gfp*) (Bloemberg *et al.*, 2000; Lowder and Oliver, 2001; Normander *et al.*, 1999) and luminescent (*lux*AB, *luc*) (Kragelund *et al.*, 1997; Ma *et al.*, 2001; Ramos *et al.*, 2000; Rasanen *et al.*, 2001; Turnbull *et al.*, 2001) have been developed and widely applied for tracking bacteria or microorganisms of interest in certain circumstances.

2.7.1.1 Fluorescent markers: stable and unstable green fluorescent protein

The effectiveness of green fluorescent protein (GFP) as a marker system to monitor bacterial cells in the environment is very well known. GFP will appear green under UV lights when this 27 KDa polypeptide converts the blue chemiluminescence of the CaC_2-sensitive photoprotein (aequorin from the jellyfish *Aequorea victoria*) into green light (Chalfie *et al.,* 1994). The vastness of using GFP as visual selection is due to its stable genetic transformation procedures compared to the classical choices that can take months and requires highly selected agents to prevent escapes. The use of g*fp* reporter gene-based protocol in plant-microbes study is now developed for a great number of plant species ranging from monocotyledons such as *Oryza sativa* (Saika & Toki 2009; Vain *et al.,* 2000) to perennial dicotyledons such as *Theobroma cacao* L. (Maximova *et al*., 2003), *Vitis vinifera* (Santos-Rosa *et al.,* 2008) and *Pyrus communis* L. (Yancheva *et al.,* 2006).

Recently, EGFP (enhanced green), ECFP (enhanced cyan) and EYFP (enhanced yellow) have been developed. These series of shifted GFP mutants have various excitations and emissions wavelengths and 20–35 times stronger than the wild type (Tsien, 1998). Errampalli *et al.* (1999) have suggested some of the advantages of using GFP are they are easily detectable, resistant to proteases that makes it extremely stable and does not require exogenous substrate for its expression. The simplicity of this tracking agent to be traced in environment allows the monitoring process of single cells even in real time. In addition, GFP is unrestricted in its expression and there is no background in indigenous bacterial populations that would distract its distinguishing appearance. However, with all

aforesaid advantages, variability of GFP expression in different species, interference of soil particles, lack of ability to be expressed in anaerobic conditions (expression of *gfp* demand oxygen) and the instability of plasmid should be put into consideration. All these limitations were overcome with preferentially bacteria strains used are chromosomally marked and thus the risk of plasmid transfer to other microorganisms also reduced. For that purpose, several Tn*5* transposon suicide delivery vectors have been developed (Suarez *et al.,* 1997; Tombolini *et al.,* 1997).

As the GFP are being used as a real-time reporter, new variant of GFP characterized by its short half-life has been developed by Andersen *et al.* (1998). The C-terminal end of intact GFP is introduced with short peptide sequences that will produce an unstable GFP structure that allows its degradation by bacterial endogenous proteases. With this degradation, the production of GFP does not accumulate during bacterial growth; it is possible to perform real-time analysis of the bacterial metabolic activity (Lowder & Oliver, 2001; Sternberg *et al.,* 1999). However, the expression level of protease may be differ depending on the microorganisms, thus care in environmental factors is needed and the growth phase of bacteria during expression must be applied in the interpretation of the results (Jansson *et al.,* 2000).

2.8 Green fluorescent protein

Gene encoding a protein called the green fluorescent protein (GFP) can be inserted into the genome of virtually any bacterium. The cell with this gene would appear green when observed under ultraviolet microscope or light. The cells with

GFP tagged can be introduced into environment, such as plant roots. The microscopes are used to follow the strain that has been tagged. By this method, the interaction of GFP-tagged strains with environment can be studied *in situ*. Green fluorescent protein also can act as a reporter gene, the gene that incorporated into vectors and encode proteins that are simple to detect.

Tagging with green fluorescent protein (GFP) (Elbeltagy *et al.*, 2001; Compant *et al.*, 2005; Prieto and Mercado-Blanco, 2008) gene marker has been particularly useful in facilitate the *in vivo* studies of bacterial infection pathways for determination of colonization behavior of diverse beneficial bacteria in tissue and organ of various plant hosts.

2.9 GFP as a marker of choice in bacterial localization in plant

The gene expression study both in the laboratory and in natural environments has been greatly enhanced by the fusing technology of promoters of interest to a reporter gene. Various reporter gene systems, including *luc* and *lux* (Prosser *et al.*, 1996), *inaZ* (Miller *et al.*, 2000), *lacZ* (Labes *et al.*, 1990), *gusA* (Prell *et al.*, 2002; Reeve *et al.*, 1999), as well as autofluorescent proteins (AFPs) (Gage, 2002; Stuurman *et al.*, 2000; Xi *et al.*, 2001) have all been used for molecular genetics analyses.

One of the most common reporter gene systems is green fluorescent protein (GFP) that has hexapeptide structure with internal natural chromophore which requires O_2 for cyclization (Chalfie *et al.*, 1994; Inouye & Tsuji, 1994). The cylinder

(or beta-can) three dimensional structure of GFP is built up by 11 antiparallel beta strands forming a beta-can structure that surrounds the chromophore that locates in inner alpha-helix (Yang *et al.,* 1996). This is the functional structure that confers the stability of native GFP protein. The autofluorescence of GFP does not require any cofactors or substrate for expression, therefore, GFP has the advantage of being detected via non-destructive sampling at the single-cell level. The excellence of GFP with its autofluorescent ability has allows it to be viewed under a wide range of conditions, such as in fluorescent plate readers, agar plates, as well as by fluorescence-activated cell sorting (FACS).

The levels of GFP autofluorescence emission also have been increased whereby many derivatives of GFP are being developed. These derivatives of GFP have features such as shifted excitation or emission spectra (Cormack *et al.,* 1996; Crameri *et al.,* 1996; Ellenberg *et al.,* 1998). The shuffle mutation of GFP at M153T, F99S and V163A, produced a GFPuv mutant, which have a 16-fold higher emission than wild-type GFP, but retains the wildtype excitation spectrum (Crameri *et al.,* 1996). The higher fluorescence emission of GFPuv appears because of its solubility is higher than wild-type GFP.

A site-directed mutant of wild-type *gfp* that has changes on F64L and S65T, GFPmut derivatives series have been developed with red shifted excitation spectrum (excitation maximum 488 nm) and a 35-fold increase in fluorescence. These characteristics close to those of FITC making them better suited to FAC sorters (Cormack *et al.,* 1996). Additionally, a suite of GFPmut proteins with different

stabilities have been created with addition of a protease-targeting signal to GFPmut (Andersen *et al.,* 1998).

An integration of GFPmut3.1 chromophore into the protein backbone of GFPuv produces another advance derivative, GFP+. This combination gives up to a 130-fold increase in fluorescence emission due to the red-shifted chromophore of GFPmut3.1 have the greater solubility of GFPuv (Scholz *et al.,* 2000). A cyan, blue and yellowish-green emission spectra of GFP mutants are now available, conversely with emission spectra wavelengths of not longer than 529 nm these mutants are not suitable for dual-labelling experiments with GFP (Baird *et al.,* 2000).

On the other hand, *DsRed* fluorescent protein, shares certain chromophore motifs and structural with GFP (Baird *et al.,* 2000). This fluorescent protein, isolated from corals of the genus *Discoma,* has an emission maximum of 583 nm that can be used in conjunction with GFP. However, because of its tetrameric structure, wild-type *DsRed* is slow to mature compared to GFP. However, mutant derivatives, DsRedT.4 and DsRedT.3 have been developed with ability to mature much faster than the wild-type (Bevis & Glick, 2002). In addition, a monomeric variant of DsRed that able to mature more rapidly called monomeric red fluorescent protein (mRFP1) has been developed (Campbell *et al.,* 2002).

A large number of vectors incorporating the AFPs have been made in conjunctions with their advantages as reporter proteins (Allaway *et al.,* 2001; Miller *et al.,* 2000; Stuurman *et al.,* 2000). Applications that are less trivial than the other tracking markers make the AFPs is preferred compared to other markers available.

2.10 16S rRNA

The evolutionary relationship of prokaryotes (bacteria and archea) can be obtained from the sequence of 16S rRNA which is a large polynucleotide that functions as a part of the small subunit (30S) of the ribosome in prokaryotes. The gene coding for it are referred to as 16S rDNA and are used in reconstructing phylogenies (Bavykin *et al*., 2004).

Knowledge about the comparison of DNA sequences as a very powerful method to study the evolutionary relationships between species has long been known by the systematist. However, with the limited access to genetic information at the time, the desire to exploit DNA sequences is not more than a figment of imagination. These days, with the existing of polymerase chain reaction (PCR) technology, accumulation of large sample of DNA sequences of organisms can be obtained with ease. Determination of DNA sequences becoming relatively a simple task with the help of sophisticate automated sequencing machinery. Thus, DNA sequencing, the determining process of sequence of nucleotides in segment of DNA, become widely available and comparatively cheap, easy and has been chosen as a primary tool for uncovering phylogeny (Rashidan *et al.*, 2004).

The use of 16S rRNA gene to estimate relationship among bacteria (phylogeny) has recently evolves and become important for identifying the unknown bacterium to genus or species level (Claudio *et al.*, 2002). With this knowledge, the method for phylogenetic classification and identification of prokaryotic species, genera and families have been standardized using the 16S rRNA sequences

determination. The 16S rRNA sequences data also being used to infer the evolutionary history of the organisms.

2.11 Auxin production by plant growth promoter

Auxin is a class of plant hormones (or plant growth substances) with some morphogen-like characteristics. Morphogen is a substance governing the pattern of tissue development. Coordination of many growth and behavioral processes in plant are mainly regulated by auxin and it is essential for plant development. Hirsch *et al.,* (1997) suggested that microorganisms play a very important role in changes of plant development mediated by manipulation of phytohormones balance. The microorganisms have the capacity in enhancing or inhibiting plant growth by producing plant growth substance on the roots and stems that they colonize and thus, alter the plant architecture (Ulrike, 2010).

According to Kepinski and Leyser (2003) and Vanneste *et al.* (2005), auxin regulates the cell cycle and plant organogenesis. Auxin is important in plant growth and organ formation by which the manipulation of auxin gradients leads to changes in plant development (Benkova *et al.,* 2003; Friml, 2003), therefore, the correct concentration, transportation and localization of auxin is very important in plant coordination. Hence, the development of plant organs such as leaves and flowers, plant response towards its environment and plant growth synchronization are mainly regulated by (dynamic and to environment responsive) distribution pattern of auxin within the plant. More recently, auxin has also been thought as plant immunity