IDENTIFICATION OF NITROGEN FIXING MICROSYMBIONTS FROM *Mucuna bracteata* NODULES

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by

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LIST OF ABBREVIATIONS

BTB	Bromothymol Blue
ANA	Apparent Nitrogenase Activity
Cfu	Colony forming unit
EAC	Electron Allocation Coefficient
H_2 Evolution	Hydrogen Evolution
N_2 Fixation	Nitrogen Fixation
PCR	Polymerase Chain Reaction
CR	Congo Red
TAE	Tris-Acetate-EDTA
TNA	Total Nitrogenase Activity
YEMA	Yeast Extract Mannitol Agar
YEMB	Yeast Extract Mannitol Broth

PENGECAMAN MIKROSIMBION PENGIKAT NITROGEN DARIPADA NODUL *Mucuna bracteata*

ABSTRAK

Mucuna bracteata adalah tumbuhan kekacang penutup bumi yang baru diperkenalkan yang menghasilkan nodul dan mewujudkan pengikatan nitrogen secara simbiosis. Walau bagaimanapun, maklumat tentang mikroorganisma pengikat N_2 tumbuhan ini adalah terhad. Rhizobia merupakan bakteria pengikat nitrogen yang menghasilkan nodul pada akar tumbuhan kekacang. Ia juga merupakan ahli Famili *Rhizobiaceae* dalam Kelas *α*-proteobakteria. Terdapat penambahan bilangan bakteria yang boleh menghasilkan nodul dan mengikat N_2 pada tumbuhan kekacang yang bukan merupakan genus *Rhizobium* dalam Famili *Rhizobiaceae* mahupun dalam tradisi filogenetik rhizobia. Justeru, pemencilan dan pencirian mikrosimbion daripada nodul *M. bracteata* telah dijalankan berdasarkan ujian-ujian biokimia. Seterusnya, pengenalpastian terperinci identiti pencilan telah dilaksanakan berdasarkan kaedah analisis jujukan 16S rDNA. Sebanyak 13 pencilan mikrosimbion berjaya menghasilkan nodul dan mengikat N_2 apabila diinokulasikan kepada pokok *M. bracteata.* Untuk penilaian evolusi bakteria pengikat N_2, analisis gen *nifH* yang mengkod enzim nitrogenase, telah digunakan dengan pelbagai primer PCR. Selain itu, amplifikasi gen *nifH* dan 16S rRNA telah berjaya diperoleh melalui kaedah PCR. Analisis jujukan menunjukkan bahawa pencilan mikrosimbion dari *M. bracteata* terdiri daripada *Burkholderia* sp., *Brevundimonas* sp., *Achromobacter* sp., *Stenotrophomonas* sp. dan *Bacillus* sp.. Justeru, berdasarkan keputusan yang diperoleh menunjukan bakteria yang mengikat N_2 secara simbiosis di dalam akar dikenali sebagai rhizobia, dan ia bukan hanya daripada kelas *α*-proteobacteria tetapi juga daripada *β*-proteobacteria (*Burkholderia* sp. dan

Achromobacter sp., 99-100% persamaan) dan γ-proteobacteria (*Stenotrophomonas* sp., 99% persamaan).

IDENTIFICATION OF NITROGEN FIXING MICROSYMBIONTS OF *Mucuna bracteata* NODULES

ABSTRACT

Mucuna bracteata is a new leguminous cover plant which forms nodules and establises a nitrogen-fixing symbiosis. However, there is little information available on the N_2-fixer of *M. bracteata.* Rhizobia are used to be recognized for all the bacteria that are able to produce nodule and fix N_2 in legume's root and collectively a member of the *Rhizobiaceae* in *α*-class of proteobacteria. There have been an increasing number of bacteria that can nodulate and fix N_2 in legume which are not belong to the original genus *Rhizobium* in *Rhizobiaceae* family and traditional rhizobial phylogenetic lineages. Thus, isolation and characterization of microsymbiont from nodules of *M. bracteata* was carried based on biochemical tests. Furthermore, clearer identification of the isolates was performed based on 16S rDNA sequence analysis. Thirteen microsymbionts could nodulate and promote N_2 fixation activity in association with *M. bracteata*. For evaluation of N_2-fixing bacteria, analysis of *nifH*, the gene encoding nitrogenase enzyme, has been used with various PCR primers. Amplified *nifH* and 16S rRNA gene were successfully obtained from PCR technique. The sequence analysis revealed that root nodules of *M. bracteata* consisted of *Burkholderia* sp., *Brevundimonas* sp., *Achromobacter* sp., *Stenotrophomonas* sp. and *Bacillus* sp. The results presented here suggested that bacteria which were able to form N_2-fixing symbioses with root known as rhizobia is not only from the *α*-class of proteobacteria but also from *β*-class of proteobacteria (*Burkholderia* sp. and *Achromobacter* sp., 99-100% of similarity) and gamma(γ)-class of proteobacteria (*Stenotrophomonas* sp., 99% of similarity).

CHAPTER 1

INTRODUCTION

In recent years, legume covers have received considerable attention due to their ability to enhance the productivity and sustainability of agricultural systems. Legumes tend to be good succession species which are widely cultivated as cover crops and green manure for oil palm and rubber plantations. These cover crops are leguminous creepers, which are easy to establish. Application of legumes in farming systems is not only as a food source but also for soil enrichment regimes especially in the humid tropics (Ojo, 2001). Common characteristics of all cover crops include their ability to control soil erosion as well as limit the competition from other weedy vegetation and improve soil organic matter and N status (Alley *et al.,* 1999; Nicholls *et al*., 2001). Also in this category is the wild legume *Mucuna bracteata,* which grows fast and covers the field quickly. In Malaysia, *M. bracteata* is a new legume cover crop that has been established in young and replanted oil palm and rubber plantations. This newly introduced legume species was to replace the conventional legume cover crops such as *Calapogonium mucunoides* and *Pueraria javanica* (Pushparajah, 1977; Wahab, 1997). Many authors have reported the benefit of this superior legume cover in conserving the soil and in improving or maintaining the soil structure and fertility on oil palm plantations (Mathews, 1998; Alley *et al*., 1999; Giller, 2001 and Mendham *et al.,* 2004).

The legume cover species can be used as a ground cover in plantations because it has the ability to fix atmospheric nitrogen which can enrich the soils with fixed N (Graham, 2008) which later will be used by the immature or young plants. Cover cropping with legumes during the early phase of plantation growth may be a useful mechanism to enhance nitrogen in the soil and optimize the synchrony between nitrogen

supply and nitrogen uptake by plants. The nitrogen supply and competition characteristics in these systems depend to a large extent on the species of legume that is used (Alley *et al*., 1999). Based on a study of three legume species in Kerala, India, *M. bracteata* released the highest nitrogen to the soil compared to other two legumes; *Pueraria phaseoloides* and *Stylosanthes hamata* (Mendham *et al.,* 2004). In addition, Versteeg *et al.* (1998) found that maize yield in fields with *Mucuna* sp. was 70% greater than in fields without *Mucuna* sp. and similar study was observed by Sanginga *et al.* (1996).

All plants require nitrogen in order to survive as well as to promote plant growth. Nitrogen gas (N_2) constitutes approximately 78.1% of the earth's atmosphere (Gerald, 1987), which represents an abundant supply to those organisms that can access to it. The process of N_2 fixation is the reduction of N_2 to biologically useful ammonia (NH_3), requiring nitrogenase enzyme activity. At the same time, hydrogen gas (H_2) is released and this process is known as Hydrogen Evolution (H_2 evolution). N_2 is very stable, that is why N_2 fixation requires high energy with help from N_2-fixing organisms. The exploitation of N_2 by legumes is due to the presence of N_2-fixing bacteria in the root nodules. This bacteria was given the generic name *Rhizobium* and was formally adopted in 1926 by Buchanan (Holt *et al.,* 1994; Brenner *et al.,* 2005).

Bacteria of the genus *Rhizobium* play a very important role in agriculture by inducing N_2 fixation in root nodules of legumes such as peas, beans, clover, alfalfa and *Mucuna*. The expression of nodulation genes in the bacteria is activated by signals from plant roots and the corresponding bacteria synthesis signals that induce nodule formation. This symbiosis can relieve the requirements for added nitrogenous fertilizer during growth of leguminous crops. For that reason, there is a need to determine the most effective diazotrophic microsymbiont which could induce formation of nodules

and further promote symbiotic N_2 fixation activities for the host plant (Ojo, 2001; Appunu and Dhar, 2008). Isolated microsymbionts from nodules of *Mucuna* sp. have been shown to be slow grower and some were able to nodulate cowpea and soybean effectively (Allen and Allen, 1981; Sanginga *et al*., 1996). Based on a study of *Mucuna* sp. (velvetbean), the microsymbiont for it seems to thrive more in alkaline rhizosphere. *Mucuna deeringiana* had influenced the diversity of bacteria in soil including *Bacillus* sp., *Arthrobacter* sp. and *Burkholderia* sp. in soil (Vargas-Ayala *et al.,* 2000). Hence, Ojo (2001) had reported that *Mucuna pruriens* microsymbionts consisted of *Rhizobium* sp. and *Bradyrhizobium* sp.

The purpose of this study was to examine the ability of the isolated microsymbionts to fix N_2 in association with *M. bracteata* nodules. The isolated microsymbionts were identified based on microscopic and biochemical tests. In addition, this study will observe the N_2 fixation process in *M. bracteata* inoculated with the isolated microsymbionts based on H_2 evolution determination. The *M. bracteata* roots were inoculated with the isolated microsymbiont species which can develope nodules to fix N_2 and eventually release H_2. The rate of H_2 released from the nodules provides a measurement of nitrogenase activity that occurres in the N_2 fixation process. The great advantage of measuring nitrogenase activity by H_2 evolution is that measurements can be made with minimal disturbance on the plant tissues. Additionally, 16S rDNA sequencing was carried out to identify the potential microsymbionts that could enhance growth of *M. bracteata.* Consequently, for evaluation of N_2-fixing populations in the environment, analysis of the *nifH* gene, the gene encoding nitrogenase reductase, has been used with various PCR primers that amplify this gene from both microorganisms and environmental samples (Zehr and McReynolds, 1989; Zehr *et al.,* 1995; Ueda *et al.,* 1995; Ohkuma *et al.,* 1996; Choo *et al.,* 2003).

1.1 Objectives

1) To isolate, identify and characterize the symbiotic N_2-fixing microsymbionts from root nodules of *M. bracteata*

2) To estimate the N_2 fixation rate of these microsymbionts based on production of H_2 gas (H_2 Evolution).

3) To identify and characterize the potential microsymbionts based on molecular approach.

CHAPTER 2

LITERATURE REVIEW

2.1 The Superiority of *Mucuna bracteata* As a Cover Crop

Mucuna sp. has commonly known as velvetbean. However, the taxonomy of this pan-tropical genus *Mucuna* sp. is confusing and there are many different species. The confusion arose in part from the enormous variability in colour and size of the seed and wide range in growth durations within this cultivar. The taxonomic of *M. bracteata* is as follows:

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Fabales
Family	:	Fabaceae
Subfamily	:	Faboideae
Tribe	:	Phaseoleae
Genus	:	*Mucuna*
Species	:	*bracteata*

In terms of best conditions for physical growth, the most suitable weather for *Mucuna* sp. is during the hot season, and that is why *Mucuna* sp. is among the more successful green manure species found in various tropical and subtropical settings (Broughton, 1983; Daniel *et al.,* 1998; Mathews, 1998). Their large seed gives relatively rapid early growth and establishment of soil cover (Carsky *et al.,* 1998). *Mucuna* sp. are grown as a cover crop and green manure. The term 'green manure' refers to the plants use as an organic fertilizer, whereas the principal use of a cover crop is to protect the soil from erosion or to suppress weeds by maintaining a dense canopy close to the

ground (Giller, 2001). The desirable characteristics of green manures and cover crops include their ability to stabilize the soil and improve agronomic productivity in uplands and tropical lowlands.

The desirable characteristics of *M. bracteata* include its vigorous and fast growth. It is easy to establish and needs low labor and chemical requirements for high biomass production. This leguminous plant is easy to plant and maintain in plantations as it is non-palatable to cattle. In addition, it can cover a field quickly, drought resistant and shade tolerant. It can be used as a cover crop because of its competitive ability against competing weed growth *via* the presence of chemicals and physical suppression. Thus, it is suitable to apply in agro farming systems especially in rubber and oil palm plantations (Mathews, 1998; Mendham *et al.,* 2004).

Mucuna sp. can be grown without extensive fertilization if nodulated by an effective rhizobial strain (Ojo, 2001). Thus, the advantage of legume covers is their ability to fix atmospheric N through their symbiotic relationship with *Rhizobium* sp. which usually associated with the root system and developed nodules (Giller, 2001).

2.2 Nodulation of Leguminous Plant

Leguminosae (Fabacaea) is a well known plant group that able to produce nodules and perform N_2 fixation activity in roots (Soltis *et al.,* 2000). According to Lavin *et al.,* (2005), legumes emerged about 60 million years ago. The International Legume Database and Information Service (ILDIS) is one of the organizations that is responsible for determining and verifying the taxonomy of legumes (Sprent, 2008). Legumes produce a special interaction known as 'symbiosis' with specific bacteria. Sprent and James (2007) suggested that legumes are very versatile in this symbiosis relationship. With this relationship, nodules will be formed and induced by the bacteria

on the plant root to prepare a place for N_2 fixation activity. Nodules are root-like structures of central vascular tissue which have been infected by compatible bacteria (Giller, 2001, Sprent, 2008). The most well known bacteria that can create symbiosis relationship and induce nodule formation in legumes are called rhizobia (Spencer *et al.*, 1994).

N_2 fixation in legumes starts with the formation of nodules. Thus, to form nodules, the compatible rhizobia will invade and enter the root through the epidermis or cracks (wounds) in the root. Then, infection threads will develop to transport the rhizobia and infect the inside cells. This infected cell is known as a bacteroid. The young infected cells will divide repeatedly and form new N_2-fixing tissues and finally develop a nodule structure. Within this nodule structure, rhizobia will fix N_2 and convert it to ammonia which later can be absorbed by the legume (Brewin, 2004; Sprent and James, 2007). When the nodule is young, the inside colour is usually white or grey as no N_2 fixation has yet occurred. As the nodule grows, the inside colour will turn pink or reddish and indicate that the nodule is actively fixing N_2. The pink and red colour is due to presence of leghemoglobin which is similar to hemoglobin in blood. Leghemoglobin controls O_2 flow inside the nodule by carrying O_2 from outer cells into inside cells for rhizobial respiration (Giller, 2001; Kalita *et al.,* 2006; Sprent, 2008). In addition, the legume supplies energy and necessary nutrients for rhizobia to survive, thus showing the importance of symbiotic relationships in the environment.

2.3 The Importance of N_2 Fixation Process

Nitrogen was discovered by Daniel Rutherford in 1772 (Cheng, 2008). N_2 constitutes approximately 78.1% of earth's atmosphere and is an abundant supply for life processes (Gerald, 1987). One of the most important biological process on earth is

N_2 fixation and it has been estimated about 175 million metric tons of global N_2 fixation activity occurs every year (Burns and Hardy, 1975). While, about 25-90 million metric tons of N_2 fixation is being contributed from grain, pasture and natural ecosystem legumes annually (Graham, 2008). A majority of organisms need a nitrogen source, especially from combined nitrogen, N_2 that is later converted to biologically useful form, ammonia (NH_3). Thus, biological N_2 fixation is an essential substance for every living thing on earth. On the other hand, N_2 fixation also occurs in nature as well as in blue-green algae, lichens and free living soil bacteria. This process contributes significant quantities of ammonia to natural ecosystems (Giller, 2001). Almost all of the fixed N_2 will go directly into the plant and some may leak into the soil for nearby non-legume plants. However, once the legume has decomposed, nitrogen eventually returns to the soil and can be useful for the neighboring plants (Graham, 2008).

N_2 is very stable and thus requires a lot of energy to be broken down in N_2 fixation process. The equation for N_2 fixation process is as follows (Giller, 2001; Cheng, 2008):

$$N_2 + 8H^+ + 8e^- + 16ATP \xrightarrow{\text{nitrogenase}} 2NH_3 + H_2 + 16ADP + 16Pi$$

This reaction is carried out by nitrogenase enzyme from specific bacteria coupled with Adenosine Triphosphate (ATP). From the above equation, six of the electrons are used to produce two molecules of ammonia, NH_3 and another two electrons for H_2 production. The N_2 fixation process requires the reduction of at least two protons to produce H_2, in a process known as H_2 evolution (Giller, 2001).

Biological N_2 fixation in plants requires an association with the N_2-fixing bacteria that are also known as diazotrophs. In fact, this ability in fixing N_2 seems to be exclusively the domain of limited prokaryotic species. Moreover, some species are free-

living N_2 fixers while others are symbiotic N_2 fixers. For example, leguminous plants as noted earlier need a symbiotic relationship between the plant root and the bacterial symbiont in order to fix N_2 from the atmosphere (Soltis *et al.,* 2000; Sprent and James, 2008). N_2-fixing bacteria in legumes are known as rhizobia and live in nodule where the N_2 fixation occurs. The rhizobial strains, *Rhizobium* sp. and *Bradyrhizobium* sp. are the most common N_2-fixing bacteria found in nodules of legumes. The interaction of the rhizobia with the host plant is very specific as this involves an extremely complex chemo-recognition process (Brewin, 2004).

2.4 Nitrogenase Enzyme

Nitrogenase is a multi-complex enzyme that can be found in prokaryotes and is usually oxygen liable (Cheng, 2008). Nitrogenase is needed by all N_2-fixing bacteria and varies between species. This enzyme consists of two components: Component 1 is MoFe protein and Component 2 is Fe protein. Component 1 (MoFe protein) structure is functioning as dinitrogenase, where N_2 is reduced while Component 2 (Fe protein) provides the electrons for Component 1 (MoFe protein) which is also known as dinitrogenase reductase (Giller, 2001; Einsle *et al.,* 2002; Howard and Rees, 2006).

Nitrogenase is encoded by a set of operons (*nifLA*), structural genes (*nifHDK*) and other supplementary genes (*nifE,J,M,N,Q,S,U,V*). The structural genes (*nifHDK*) are three important genes that form transcriptional units to construct nitrogenase enzyme. The Component 1 contains two α-subunits that are encoded by *nifD* gene while two β-subunits encoded by *nifK* gene. This protein component is a tetramer form and its molecular weight is 230 kDa. It is larger than protein Component 2 (Fe protein) which is 60 kDa and encoded by *nifH* gene (Giller, 2001; Dos Santos *et al.,* 2004).

In the N_2 fixation process, nitrogenase must have specific electron donors which are identified as feredoxins and flavodoxins and each must contain an electron carrier (Cheng, 2008). These electron donors will transfer the electrons to Component 2 (Fe protein) and at the same time two molecules of Mg-ATP will bind to Component 2 (Fe protein) and produce the reduced protein. In this binding process, the electrons will be transfered and donated to Component 1 (MoFe protein) while ATP molecule is converted to ADP (Schindelin *et al.,* 1997). In fact, for each N_2 fixation process, the electron transfer must occur six times. Besides that, two electrons are involved in H_2 evolution during the N_2 fixation. Therefore, 16 ATP and eight electrons are needed to fix one N_2 molecule. Thus, the process in evolving the hydrogen gas (H_2) is 'wasteful' rather than being allocated for N_2 reduction (Giller, 2001).

2.4.1 *nifH* gene

The N_2-fixing populations have *nifH* gene, the gene encoding nitrogenase reductase. The *nifH* gene codes Fe protein (Component 2) to form one transcriptional unit with a promoter located before the *nifH* gene site (Dos Santos *et al.,* 2004; Cheng, 2008). As a nitrogen fixer, the *nifH* gene was chosen in determination of rhizobial strains. This is because the larger of rhizobial sequences is available for comparison (Haukka *et al.,* 1998). It has been used with various PCR primers that amplify this gene from both microorganisms and environmental samples (Choo *et al*., 2003).

2.5 Evolution of Hydrogen During N_2 Fixation

During N_2-fixing process, a large amount of H_2 is released from legume root nodules and known as H_2 evolution. Based on N_2 fixation equation, one molecule of H_2 is evolved for each molecule of N_2 fixed. However, when measured the H_2 evolution through *in vivo* condition, at least two or more H_2 per one molecule N_2-fixed are produced (Layzell and Hunt, 1990). This H_2 evolution is also described as one of the factors in symbiotic N_2 fixation efficiency (Schubert and Evans, 1976; Curtis *et al.,* 2004). In addition, in this N_2 reduction process, two of the eight electrons allocated to nitrogenase always end up for H_2 production, whereas the other six electrons are used for ammonia production. Hence, about 75% of the electrons are involved in producing ammonia and about 25% for H_2 evolution. As a result, the nitrogenase enzyme plays an important role not only in producing ammonia but also subsequently in evolving the H_2 gas.

Measurement of H_2 evolution from the nodulated roots of legumes is one of the accurate methods of assaying nitrogenase activity (Hunt and Layzell, 1993). H_2 is a necessary by-product of N_2 fixation and that is why measurement of H_2 evolution is a valuable and non-invasive technique for use in root nodules of leguminous plants (Hunt, 1997; Witty and Minchin, 1998; Qubit Systems, 2004). On the other hand, this technique is only useful when implemented on plants inoculated with bacteria that lack of an enzyme called hydrogenase (Hup) (Curtis *et al.,* 2004). This Hup enzyme can recapture and reutilize H_2 produced by nitrogenase during the N_2 fixation process (Giller, 2001), thus preventing accurate detection of H_2 in gas flow analysis.

Thus, this technique can replace in some cases the conventional method for determining N_2 fixation through nitrogenase activity, known as Acetylene Reduction Assay (ARA). Similar information on nitrogenase enzyme activity can be obtained by

using this technique. The ARA technique is beneficial in measuring the nitrogenase activity for legumes inoculated with rhizobia contain hydrogenase enzyme in which H_2 evolution does not occur (Witty and Minchin, 1998). However, this assay can be potentially dangerous to perform as it uses acetylene an explosive gas as a non-physiological substrate for nitrogenase reaction. Another problem is the acetylene itself will induce a formation of diffusion barrier which controls O_2 flux into the nodule (Minchin *et al.,* 1986).

2.6 Uptake Hydrogenase Enzyme

As stated above, H_2 evolution has not occured or detected in the root nodules of certain leguminous plants due to the expression of uptake hydrogenase (Hup) activity by the N_2-fixing bacteria (Layzell and Hunt, 1990; Curtis *et al.,* 2004). Hydrogenase activity has been found in a wide variety of microorganisms: eukaryotes, archaea and bacteria, such as rhizobial strains. The hydrogenase is frequently found in several rhizobia and it appears to be common in *Bradyrhizobium* sp. than in *Rhizobium* sp. strains. The expression of hydrogenase in some *Bradyrhizobium* sp. strains is controlled by the host plant (van Berkum, 1990).

This hydrogenase enzyme will allow the rhizobia to recycle some or all the generated H_2 from the nitrogenase reaction in the N_2 fixation process (Baginsky *et al*., 2002). The rhizobial strains will induce hydrogenase activity that catalyzes the reversible oxidation of molecular H_2 in the legume nodules. The reaction which involved hydrogenase catalysis is as follows, where X is an electron acceptor, usually oxygen (Giller, 2001):

$$H_2 + X \xrightarrow{\text{hydrogenase}} 2HX$$

In N_2 fixing bacteria, the interaction between H_2 production and oxidation is very important. *Alcaligenes* sp., *Azotobacter* sp., *Bradyrhizobium* sp. and *Pseudomonas* sp. are examples of aerobic bacteria that use O_2 as a final electrons acceptor derived from H_2 production. Consequently, the bacteria will obtain energy through this oxidative phosphorylation (Ruiz-Argueso *et al.,* 2001).

2.7 Qubit System (N_2 Fixation Laboratory Package)

Qubit Systems Inc., Ontario, Canada is a N_2 fixation laboratory package designed to measure N_2 fixation activity in plants *via* H_2 Evolution. Hence, the determination of H_2 evolution provides a measurement of nitrogenase activity that occurr in plant incorporated with various rhizobia (Hunt and Layzell, 1993; Curtis *et al.,* 2004). Therefore, assessment of rhizobial effectiveness on inoculated plant can be shown.

Since nitrogenase can reduce both N_2 and H^+, hence it is useful to measure the relationship between H_2 released and N_2 fixed. Thus, to determine total nitrogenase activity in reducing N_2 fixed for ammonia production in legumes, total H_2 evolution must be measured first. Consequently, a total of maximum H_2 produced by the legume was measured in the absence of N_2, for example, by replacing with $Ar:O_2$ atmosphere as an indicator. In this situation, all electrons for nitrogenase activity will be used to reduce H^+ to H_2 gas (Layzell and Hunt, 1990). Thus, by measuring the maximum total of H_2 produced in air and $Ar:O_2$, the N_2 fixation could be estimated through this gas exchange method.

Measurement of nitrogenase in N_2 fixation activity *via* H_2 evolution is described as 'gas exchange system' (Hunt and Layzell, 1993). The nitrogenase enzyme activities of N_2-fixing plant systems can be estimated *via* gas exchange analysis system through

the equipped H_2 sensor under *in vivo* condition. Assessment of N_2 fixation activities *via* H_2 production is easy and offers minimal disturbance to the plant during the analysis. Thus, this is a better assay in quantification of the N_2 fixation process in leguminous plant based on the H_2 Evolution. The great advantage of measuring nitrogenase activity by H_2 evolution is that measurements can be made with least disturbance of the plant tissue.

2.8 N_2-fixing Bacterial Symbiosis in Legume

Two important properties of N_2-fixing organisms are that they are able to carry out the N_2-fixing mechanism and they are all prokaryotes. The term 'rhizobia' has been used for all bacteria that are able to produce nodules and fix atmospheric N_2 in symbiotic relationship with legume (Brewin, 2004; Kalita *et al.,* 2006). Biological N_2 fixation (BNF) is carried out by the legume-bacteria symbiosis and contributes a lot of benefits in agricultural systems. For instance, this symbiosis provides the required N input toward agricultural sustainability. These bacteria fix atmospheric N_2 into ammonia and provide the nitrogen requirement of the host plants (Giller 2001). Therefore, this symbiotic relationship can reduce the need for inorganic nitrogenous fertilizer which can be expensive and cause environmental problems. However, effective biological N_2 fixation process varies with different microsymbionts or rhizobial species, environmental factors and crop management practices (Tothill, 1985; Sprent and James, 2007). The legume microsymbionts or rhizobia have a lot of beneficial effect on host plants such as the ability to induce systematic resistance to host plant pathogens and as biological control of soil borne pathogens (Seuk Bae *et al.,* 2000). These pathogens may suppress the plant growth by infecting the roots, limiting nutrient uptake and later causing disease or mortality of the plant. Examples of plant pathogens include

Macrophomima phaseolina, Rhizoctonia solani, Fusarium solani, and *F. oxysporum* (Sheikh *et al.,* 2006).

In the early years of soil microbiology studies, only two types of bacteria were considered to be involved in N_2 fixation activity inside root nodules of legumes: fast and slow-growing types (Nutman, 1987; Sprent, 2008). These bacteria were subsequently distinguished and given different names. The fast growers are known as *Rhizobium* sp. (Buchanan *et al.,* 1974), while the slow grower is known as *Bradyrhizobium* sp. (Jordan, 1982, 1984; Holt *et al.,* 1994; Brenner *et al.,* 2005). Over the years, the situation has changed and the legume-nodulating bacteria known as rhizobia, form a group of soil bacteria belonging to the α-subclass of Proteobacteria. These N_2-fixing bacterial symbiont include the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* (Young and Haukka, 1996; Sprent, 2001; Sawada *et al.,* 2003). Bacteria from this family are able to form N_2-fixing symbiosis with bean and legume plants. The nodule formation on host plant roots is evidence of this advantageous symbiotic relationship with the rhizobia.

2.9 *Rhizobium* sp.

In the early studies on nodulation, bacteria that were able to nodulate legumes were all classified as *Rhizobium* sp. Six species of *Rhizobium* were initially named as *R. leguminoserum*, *R. lupini*, *R. meliloti*, *R. phaseoli*, *R. trifolii* and *Bradyrhizobium japonicum.* The rhizobia without species names were characterized based on their plant host, such as *Rhizobium* spp. (lotus) which produces nodules on lotus roots and *Rhizobium* spp. (cowpea) which nodulates cowpea (Broughton, 1982). The taxonomy of *Rhizobium* sp. is as follows:

Kingdom	:	Bacteria
Class	:	Proteobacteria
Sub-Class	:	Alpha proteobacteria
Order	:	Rhizobiales
Family	:	Rhizobiaceae
Genus	:	*Rhizobium* sp.

Microsymbionts that form N_2-fixing association with legumes have traditionally been known as rhizobia and placed in the genus *Rhizobium.* These bacteria are Gram-negative, fast-growing, rod-shaped, non-sporeforming, aerobic and motile. In addition, their size range is 0.5-0.9 μm wide and 1.2-3.0 μm long. The older cells of these strains have polymeric-*β*-hydroxybutyric acid and are highly frectile when observed under phase-contrast microscope. They grow fast on solid medium of Yeast Extract Mannitol Agar (YEMA) within 2-3 days and produce abundant growth. If Bromothymol blue (BTB) is added as a pH indicator, a yellow colour will result from the acidic effects from the growth of *Rhizobium* (Jordan, 1982, 1984; Vincent, 1982; Holt *et al.,* 1994; Brenner *et al.,* 2005).

In the early years of research in legume-rhizobial interactions, these genus have been divided into two groups: fast-growing and slow-growing bacteria. A rapid grower rhizobia is identified as *Rhizobium* sp. while a slow grower rhizobia is recognized as *Bradyrhizobium* sp. (Giller, 2001; Brenner *et al.,* 2005). *Rhizobium* sp. is the most well known species of bacteria that acts as primary symbiotic fixer of N_2. These bacteria can infect the roots of leguminous plants, leading to the formation of lumps or nodules where the N_2 fixation takes place (Brewin, 2004, Sprent, 2008).

2.10 *Bradyrhizobium* sp.

The taxonomy of *Bradyhizobium* sp. is as follows:

Kingdom	:	Bacteria
Class	:	Proteobacteria
Sub-Class	:	Alpha proteobacteria
Order	:	Rhizobiales
Family	:	Bradyrhizobiaceae
Genus	:	*Bradyhizobium* sp.

Within the scope of soil microbiology, *Bradyrhizobium* sp. is a well known species of slow growing rhizobia such as *B. japonicum* (Brenner *et al.,* 2005), *B. elkanii* (Kuykendall *et al.,* 1992) and *B. liaoningense* (Xu *et al.,* 1995). *Bradyrhizobium* sp. was the first rhizobial genus to be highlighted in addition to *Rhizobium* sp. (Jordan, 1982). Additionally, based on Giller (2001), it was designated to classify a 'slow-growing strain' of rhizobia. *Bradyrhizobium* sp. also will infect the roots of legume plants and produce nodules which provide N_2 fixation activity like *Rhizobium* sp.. *B. japonicum* and *B. elkanii* are the most effective bradyrhizobia species that have been used as commercial inoculants for soybean especially in Brazil (Giongo *et al.,* 2008).

This slow-growing strain takes 4-7 days to grow on YEMA medium. In contra to *Rhizobium* sp., *Bradyrhizobium* sp. will increase the pH of the medium and turn the green colour of BTB dye to blue colour (alkaline) on solid medium of YEMA. These strains are also Gram negative, non-sporeforming, with 0.5-0.9 μm width and 1.2-3.0 μm long. *Bradyrhizobium* are various in shapes based on species, including coccobacilli and rod-shaped (Brenner *et al.,* 2005).

2.11 Isolation of Rhizobia

Rhizobia can be found in both free-living forms in soil and in the root nodules of legumes in a symbiotic relationship with the host plant. In legumes, N_2 fixation occurs only in the root nodules but not in the free-living condition. Thus, to isolate the rhizobia from nodule, the outer part of the nodule must be chemically sterilized before crushing it to obtain the nodule slurry or extract. The extract containing the concentrated rhizobia is then streaked onto an agar culture medium. Yeast extract mannitol (YEM) medium is a suitable medium for isolation of rhizobia from nodules. This medium contains sources of fixed nitrogen (in this case yeast extract), carbon, energy (mannitol) and some mineral salts (Vincent, 1970; Somasegaran and Hoben, 1985).

Bromothymol blue (BTB) dye was added to YEM agar as a pH indicator to observe the pH changes in bacterial growth medium qualitatively (Somasegaran and Hoben, 1985; Ojo, 2001). The pH of the medium reflects different types of rhizobial strains that occupy root nodules of *M. bracteata.* Almost all of the endosymbionts produced a blue colour (alkaline reaction) on YEMA+BTB while others showed yellow colour (acidic reaction). Based on Vincent (1982), fast-growers dropped the pH medium sufficiently to change BTB to yellow colour while the slow growers changed the indicator (BTB) in the direction of alkalinity.

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF N_2-FIXING MICROSYMBIONTS FROM *M. bracteata* NODULES AND SCREENING FOR SYMBIOTIC ASSOCIATION

3.1 Introduction

The establishment and management of vegetative ground covers is an important aspect of oil palm and rubber cultivation (Giller, 2001). The most important functions of these ground covers are to enrich soil fertility, compete against noxious weeds and protect the soil from erosion (Alley *et al.,* 1999; Nicholls *et al.,* 2001; Mendham *et al.,* 2004). Legume cover crops such as *Calapogonium mucunoides*, *Centrosema pubescens* and *Pueraria javanica* are the conventional ground covers that have been widely planted in Malaysia (Pushparajah, 1977; Wahab, 1997). However, *M. bracteata* is a new leguminous cover plant which was introduced and adapted in local oil palm and rubber plantations in the early 1990s. It produces high biomass which decomposes slowly and increases the fertility of the surface soil, subsequently giving benefits for the immature oil palm or rubber plants. Mathews (1998) reported that the biomass production of *M. bracteata* could exceed that of other conventional legume cover crops.

Earlier observations on plant growth showed that *Mucuna* had poor establishment in some farmers' fields. It was happened due to lack of symbiotic effectiveness of bacteria with *Mucuna* (Houngnandan *et al.,* 2001). When a legume is established in a new environment, it may fail to produce effective nodules with indigenous rhizobia or diazotrophic microsymbionts in the soil (Bogino *et al.,* 2008). Thus, failure of nodulation may be avoided by inoculating the plants with isolates for which the legume is already adapted to and from existing collections of rhizobia known to be suitable for the legumes (Gerald, 1987). 'Rhizobia' are symbiotic bacteria

(microsymbionts) which develop nodules on roots of legume host plants, thus enabling the bacteria to fix N_2 to ammonia and enhance plant growth (Giller, 2001; Alberton *et al.,* 2006; Bogino *et al.,* 2008). Nevertheless, the efficiency of biological N_2 fixation varies with crop and bacterial inocula, environment and crop management (Tothill, 1985; Sprent and James, 2007).

In the early studies on nodulation, there are two classes of bacteria involved in N_2 fixation activity inside root nodules of legumes; these are fast- and slow-growing types. The fast growers are within the *Rhizobium* genus (Buchanan *et al.,* 1974), while the slow growers are in the *Bradyrhizobium* genus (Jordan, 1982; Holt *et al.,* 1994). Then, in later years, the number of identified genera that produce nodules in legumes increased. These genera were exclusively members of the family Rhizobiaceae in the alpha class of proteobacteria which include the genera: *Allorhizobium*, *Azorhizobium*, *Mesorhizobium* and *Sinorhizobium* (Sprent, 2001; Sawada *et al.,* 2003). In addition, *Rhizobium* and *Bradyrhizobium* are also included in this family (Brenner *et al.,* 2005).

In previous study, isolated rhizobial strains from *Mucuna* sp. consisted of *Rhizobium* sp. and *Bradyrhizobium* sp. (Ojo, 2001). However, relatively little information exists concerning the diazotrophs or N_2 fixing microsymbiont associated with *M. bracteata,* an emerging cover crop for plantation production in tropical Asia. Thus, this experiment was carried out to isolate, identify and characterize the symbiotic N_2-fixing microsymbionts from root nodules of *M. bracteata.* At the end of this study, the most efficient microsymbiont for *M. bracteata* that can increase biological N_2-fixing ability and enhancing plant growth in Malaysian conditions would be chosen.

3.2 Materials and Methods

3.2.1 Isolation

3.2.1.1 Collecting nodules from the field

Nodules of established *M. bracteata* plants growing as cover crop for young oil palm trees were collected randomly at field location A, B and C at Taiping Oil Palm Plantation Sdn. Bhd., Perak. At field location A, the cover crop, *M. bracteata* were planted in 2004, while at locations B and C the cover crop were planted in 2003. First, a circle was made around each plant and the roots were carefully removed. Fresh nodules from the roots were then detached and placed in tightly sealed preservation vials. The nodule preservation vials contained desiccant (silica gel) at the bottom plus a layer of cotton wool, all of which had previously been autoclaved. This method is able to preserve the nodules at ambient room temperature for 6-12 months (Vincent, 1970; 1982; Somasegaran and Hoben, 1985).

3.2.1.2 Isolation of microsymbionts from nodules

The nodules were rinsed in 95% (v/v) ethanol for 5 s to remove waxy materials and trapped air. Then, nodules were transferred into 0.1% (w/v) solution of mercuric chloride ($HgCl_2$) for 5 min for sterilization, followed by five changes of sterile distilled water (Vincent, 1970). The sterile nodules were crushed by using sterile blunt-tipped forceps to get the nodule suspension or slurry. A loopful of the nodule suspension was streaked on Yeast Extract Mannitol Agar (YEMA) (**3.2.1.2.1**), added with 0.5% (v/v) bromothymol blue (BTB) as an indicator dye (**3.2.1.2.2**) and incubated at 28°C for 3-10 days (Vincent, 1970; Somasegaran and Hoben, 1985).

3.2.1.2.1 Yeast Extract Mannitol Broth (YEMB) and Yeast Extract Mannitol Agar (YEMA)

The ingredients for YEMB are based on Somasegaran and Hoben (1985) (**Table 3.1**). For YEMA, 15 g of agar was added to 1 L of YEMB and autoclaved. After autoclaving, the medium was poured into Petri dishes and allowed to solidify at room temperature (27 ±1 ^{o}C).

Table 3.1: Ingredients (g/L) for Yeast Mannitol Broth (YEMB) and Yeast Mannitol Agar (YEMA) preparation.

Item	g/L
Mannitol	10.0
KH_2PO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
Yeast extract	0.5
pH	6.8±0.2

3.2.1.2.2 Bromothymol blue (BTB)

Bromothymol Blue dye solution was prepared by mixing 0.5g of BTB powder with 100 ml of 95% (v/v) ethanol. Then, for 1 L medium, 5 ml of BTB solution was added (Somasegaran and Hoben, 1985).

3.2.1.3 Alkalinity and acidity production on YEMA

The alkalinity and acidity production on YEMA were observed based on the pH change in YEMA. The pH change was detected by incorporating 0.5% (v/v) of BTB as pH indicator. Pure colonies of isolates were streaked on YEMA plates and incubated at 28°C for 3-10 days until the colonies appeared (Somasegaran and Hoben, 1985; Brenner *et al.,* 2005).

3.2.1.4 Growth characteristics on different media

A single colony from each plate was subcultured in YEMA which was added with Congo Red dye 1% (v/v) (**3.2.1.4.1**) and Peptone Glucose Agar (PGA) (**3.2.1.4.2**) (Somasegaran and Hoben, 1985, Brenner *et al.,* 2005). The plates were incubated for 3-10 days at 28°C in dark conditions. The fast-growing colonies appeared after 2-3 days of incubation while the slow-growing colonies appeared after 4-10 days. The colony characteristics such as shape, colour, texture and size were examined and recorded.

3.2.1.4.1 Congo red dye

Congo red dye solution was prepared by mixing 0.25g of RC powder with 100 ml 95% (v/v) ethanol. Then, for 1 L medium, 10 ml of RC solution was added (Somasegaran and Hoben, 1985).

3.2.1.4.2 Peptone Glucose Agar (PGA)

The ingredients for PGA were based on Somasegaran and Hoben (1985) (**Table 3.2**). After autoclaving, the medium was poured into Petri dishes and allowed to solidify at room temperature (27 ±1 ^{o}C).

Table 3.2: Ingredients (g/L) for Peptone Glucose Agar (PGA) medium preparation.

Item	g/L
Glucose	5
Peptone	10
Agar	15
pH	6.8±0.2

3.2.1.5 Maintenance of bacterial culture

Freshly isolated microsymbiont cultures were preserved in sterile glycerol stock media with the ratio 1:1. The mixture was poured into sterile 1.5 ml microcentrifuge tubes aseptically and stored at -20 ^{o}C.

3.2.2 Bacterial Identification

The isolated microsymbionts were grown in Yeast Extract Mannitol Broth (YEMB) and shaken at 100rpm for 3 days (fast grower) or 5 days (slow grower), respectively (Somasegaran and Hoben, 1994; Humberto *et al*., 2005). The broth culture was subcultured into YEMA plate and further incubated at 28°C for subsequent analysis.

3.2.2.1 Gram staining

Gram staining method was done to classify the microsymbionts for Gram positive or Gram negative. One drop of distilled water was put on the microscope slide. A few colonies from the pure culture were transferred on to the glass slide with distilled water and mixed gently to make a thin smear. The sample was air-dried, heat-fixed and allowed to cool.

The primary staining was started with crystal violet. The whole smear was flooded with the stain solution for 1 min and rinsed with water. Next, a few drops of iodine solution were applied for 1 min and rinsed with distilled water again. Decolorizing process was performed with 95% (v/v) ethanol for 10 s and rinsed with water respectively. Finally, the samples were counterstained with safranin solution for 1 min followed by a final rinse with water. The sample was observed under light

microscope (Shushan *et al.,* 1981; Somasegaran and Hoben, 1985; Lupwayi *et al*., 1999).

3.2.2.1.1 Endospore staining for Gram positive strain

This procedure was carried out for Gram positive isolates as a confirmation for *Bacillus* strain. Smears of the isolates were prepared using an inoculating loop on the glass slide. Then, the smears were flooded with malachite green solution and heated over a steam bath for 5 min. The slides were then cooled and rinsed with water before being soaked with safranin for 30s. Finally, the slides were rinsed with water and observed under light microscope (Winn *et al.,* 2006).

3.2.2.2 Bright field and Phase contrast microscopy test

One drop of fresh bacterial culture was placed on a microscope slide and covered with a cover slip. The isolates were observed using bright field and phase contrast settings. This procedure was carried out to determine and identify the presence of poly-*β*-hydroxybutyrate which are refractile by phase-contrast microscopy (Brenner *et al.,* 2005).

3.2.2.3 Biochemical tests

All of the isolated microsymbionts were classified and differentiated through biochemical tests according to Buchanan *et al*. (1974), Atlas (1993), Holt *et al*. (1994); Zhao *et al.* (1997) and Brenner *et al.* (2005). These preliminary identification tests were focusing on *Rhizobium* sp. and *Bradyrhizobium* sp., as both strains are well known microsymbiont that are able to produce nodules and fix N_2 in association with legumes. The tests involved were alkalinity and acidity production on YEMA, growth at various