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Evidence of Diazotrophic Symbionts in the Leguminous Cover Crop *Mucuna bracteata*

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ABSTRACT

New studies point to an increasing number of identified bacteria that can nodulate and fix N_2 in legumes which do not belong to the original genus of *Rhizobium* and the *rhizobial phylogenetic* lineages. This study was conducted to isolate and identify diazotrophic microsymbionts from the root nodules of *Mucuna bracteata* (an important cover crop for oil palm) based on nitrogenase gene (*nifH*) isolation and partial 16S rDNA sequence analysis. The findings of this study indicated that the isolated microsymbionts could nodulate and promote N_2-fixation activity in *M. bracteata*. These also contributed to enhanced plant growth in terms of leaf protein and chlorophyll content, as well as in the biomass of whole plants and nodules. Additionally, *nifH* gene fragments were successfully amplified at ~380 bp from eight of the isolates (USM accessions A11, B4, B9, B12, B19, C1, C4 and C8) using *nifH*3 primers, while the remaining isolates (namely, USM accessions B14, B15, B20, C2 and C9) were successfully amplified at various sizes (~550, 650, 350, 450, and 900 bp, respectively) using *nifH*4 primers. The partial 16S rDNA sequencing revealed that the diazotrophic microsymbionts were not only from the traditional *Alphaproteobacteria* class *(Brevundimonas sp.*), but also from the *Betaproteobacteria* class (*Achromobacter* sp. and *Burkholderia sp.*) and the *Gammaproteobacteria* class (*Stenotrophomonas* sp.). Five non-rhizobial isolates were obtained and identified as *Bacillus* sp. from the root nodules of *M. bracteata.* The findings indicate the diversity of potentially-beneficial diazotrophic microsymbionts active in this emerging legume species.

Keywords: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Mucuna bracteata, Diazotrophic microsymbionts

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INTRODUCTION

Rhizobia can infect the roots of leguminous plants, leading to the formation of nodules

wherein nitrogen (N_2) fixation takes place. This symbiosis plays a very important role in agriculture as it can relieve the requirements for nitrogenous fertilizers during the growth of leguminous crops. The term 'rhizobia' has been used for all the bacteria that are able to produce nodules and fix atmospheric nitrogen in legumes (Brewin, 2004; Cheng, 2008). Traditionally, rhizobia were exclusively members of the *Rhizobiaceae* family in the *Alphaproteobacteria* class of bacteria, which includes the genera *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium* (Sprent, 2001; Sawada *et al*., 2003).

New studies, however, have shown the ability of many diazotrophs to nodulate and fix N_2 in legumes which do not belong to *Rhizobium* in the *Alphaproteobacteria* class (Willems, 2006). These diazotrophs include other species within the *Alphaproteobacteria* class (e.g., *Methylobacterium* and *Devosia*), as well as *Burkholderia* and *Ralstonia* in the *Betaproteobacteria* class. Numerous species of *Betaproteobacteria* have recently been isolated from the root nodules of leguminous plants (Chen *et al*., 2005). For example, the strains of *Burkholderia* have been isolated from a variety of legumes such as *Mimosa* spp. (Chen *et al*., 2005; Pandey *et al*., 2005; Elliott *et al*., 2007b) and two papilionoid species (*Macroptilium atropurpureum* and *Cyclopia* spp.) (Elliott *et al*., 2007a). In addition, *Stenotrophomonas maltophilia* (in the *Gammaproteobacteria* class) has also been shown to nodulate legumes (Kan *et al*., 2007). However, more knowledge is needed regarding the diversity and N_2-fixing ability of these bacteria in emerging leguminous cover crops.

The current taxonomy has revealed a wide diversity of diazotrophic microsymbionts that are able to form N_2-fixing symbioses with legume roots in a manner that is similar to rhizobia at the genus, species and intraspecies level. However, relatively little information is available regarding diazotrophic species associated with the leguminous cover crop *Mucuna bracteata*, an emerging cover crop for plantation production in tropical Asia. Thus, the objectives of this study were: 1) to determine indigenous microsymbiont strains which could further promote symbiotic N_2-fixation activities for *M. bracteata*, and 2) to verify the identity of the isolated microsymbionts from *M. bracteata,* based on their partial 16S rDNA sequences.

MATERIALS AND METHODS

Isolation of Diazotrophic Microsymbionts from Root Nodules

The nodulated roots of mature *M. bracteata* plants were collected randomly from Taiping Rubber Plantation, Perak, Malaysia. Then, fresh nodules from the roots were detached and preserved in universal bottles containing desiccant (silica gel) and cotton wool for later analysis. The colonies of microsymbionts were isolated from the nodules via a standard laboratory methodology described in Somasegaran and Hoben (1985). Purity of the strains was ensured by single colony isolation, observation of colony morphology on Yeast

Extract Mannitol Agar (YEMA) containing Bromothymol Blue (BTB) and Red Congo (RC) indicators (Yang *et al*., 2008), and by Gram staining (Vincent, 1970, 1982; Somasegaran & Hoben, 1985). The colonies of the pure cultures were maintained on YEMA slants at 4°C and also stored for later use in 15% (v/v) glycerol at -20°C.

Nodulation and N_2-fixation Screening of Diazotrophic Microsymbionts in M. bracteata

The isolated microsymbionts were cultured in YEM broth (YEMB) and shaken at 100 rpm for 3 days for fast growers and 5 days for slow growers, respectively (Vargas-Ayala *et al*., 2000). Simultaneously, the seeds of *M. bracteata* were surface-sterilized using 95% (v/v) ethanol, 0.1% (v/v) mercuric chloride ($HgCl_2$) solution, washed 5 times with sterile distilled water and germinated aseptically for 3-4 days in the dark (Somasegaran & Hoben, 1985). The seedlings were sown in pots containing 1 kg sterilized sand (to allow maximal air flow through the roots for H_2 evolution analysis) and were inoculated with 5 ml (10^9 ml^{-1}) of the respective isolates at D_0, D_{20} and D_{40}.

The experiment was laid out in a completely randomised design (CRD) with each treatment consisting of four replicates. The respective treatments for the plants consisted of: (1) Control 1 (uninoculated plants receiving fertilizer containing N (0.05 M KNO_3)); (2) control 2 plants (uninoculated plants receiving N-free fertilizer); and (3) inoculated plants (inoculated with locally isolated microsymbionts and received N-free fertilizer). The plants were maintained in the green house for 65 days of growth (D_{65}) before harvesting. Controls 1 and 2 were included to determine the effects of fertilizer compared to the inoculated microsymbionts. The seedlings were watered daily with N-free nutrient solution as recommended by Hunt and Layzell (1993). A week prior to the harvest day, H_2 evolution tests were conducted to measure the N_2-fixation activity of the inoculated host plants by using a gas flow system fabricated by Qubit Systems (Logger Pro 3.2; Kingston, Ontario, Canada) (Hunt & Layzell, 1993; Curtis *et al*., 2004). The system includes an AC gas pump, a gas bag containing $Ar:O_2$ (80:20), a flow meter, a desiccator column filled with fresh magnesium perchlorate ($Mg(ClO_4)_2$), a hydrogen gas sensor and a Vernier LabPro interface (Beaverton, Oregon, USA). Pots with the inoculated plants were sealed properly and attached to the gas exchange system. Air was pumped through the pot and was controlled by the flow meter. H_2 production was detected continuously by the H_2 sensor in the system which is linked to the computer. $Ar:O_2$ was used as the indicator to measure total electron flux through nitrogenase in the H_2 evolution rate assay. The N_2 fixation rate was calculated from the rate of H_2 evolution as described by Qubit Systems (Layzell *et al*., 1984, 1989; Hunt & Layzell, 1993; Moloney *et al*., 1994; Curtis *et al*., 2004). At harvest (D_{65}), the plants were analyzed for total length, number of leaves, leaf chlorophyll and protein contents (Lowry *et al*., 1951), number of nodules, dry weight of nodules

and plant biomass (Houngnandan *et al*., 2001). The data were statistically analyzed via one way Analysis of Variance (ANOVA) using SPSS V 15.0 software. The Tukey procedure, $p<0.05$ was chosen to test the significant differences between the means (Colman & Pulford, 2006).

DNA Extraction

Genomic DNA was extracted from the bacterial cultures grown in Luria-Bertani broth. For this study, i-genomic CTB DNA extraction mini kits from iNtRON Biotechnology (Seongnam, South Korea) were used essentially to extract the DNA. The extracted genomic DNA was quantified at $OD_{260/280}$ via UV spectrophotometer (GeneQuant pro; Amersham Biosciences/ GE Healthcare, Uppsala, Sweden).

PCR Amplification and Sequencing of nifH *Gene Fragments*

In order to amplify *nifH* gene fragments, two sets of *nifH* degenerate oligonucleotides were used: 1) *nifH*3 forward primer (5'-TAY GGN AAR GGN GGN ATN GGN AA-3') with nifH3 reverse primer (5'-GCR AAN CCN CCR CAN ACN ACR TC-3') (Choo *et al*., 2003); and 2) *nifH*4 forward primer (5'-TAY GGI AAR GGI GGI ATI GGI AA-3') with *nifH*4 reverse primer (5'-GCR AAI CCI CCR CAI AG ACR-3'). Primer *nifH*4 was designed based on primer *nifH*3 by replacing the degenerate nucleotide N with I to increase the accuracy of the primer. A 50 µl sample of the PCR reaction mixture was prepared and it contained the template genomic DNA (80 ng μl^{-1}) in 10x PCR buffer, 1.5 mM $MgCl_2$, 25 pmoles of each primer, 0.2 mM of each dNTP and 1U of Taq DNA polymerase. PCR amplifications were carried out with a Bio-Rad thermocycler (Hercules, California, USA) in the following conditions and with slight modifications (Choo *et al*., 2003): an initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s each, annealing at 45°C for 30 s, extension at 72°C for 30 s and a final extension step at 72°C for 10 min. Meanwhile, amplification of the *nifH* PCR products was analyzed by electrophoresis in 2.0% (w/v) agarose gel and visualized via Bio-Rad UV transilluminator after staining gels with ethidium bromide. The initial sequencing of *nifH* PCR products was performed by Macrogen Laboratories (Seoul, South Korea). The sequences were then analysed using the nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTn) programme of the National Centre for Biotechnological Information (NCBI) to determine presence of the *nifH* gene.

Cloning and Analysis of Partial 16S rDNA Sequences

The partial sequences of 16S rDNA were amplified from the genomic DNA of the isolates using the forward primer UP2 5'-GGG CCC CCG YCA ATT CCT TTG ART TT-3' and the reverse primer URP 5'-GTG CCA GCM GCC GCG GTA A-3', as described by Bavykin *et al*. (2004). The PCR mixture consisting of 0.5 µl genomic DNA, 5 µl 10xPCR buffer, 3 µl 25mM $MgCl_2$, 2 µl 25pmol forward primer, 2 µl 25pmol reverse

primer, 1 μl 10mM dNTP, and 0.25 U Taq DNA polymerase was brought to a final volume of 50 μl with deionized distilled H_2O. The PCR profile conditions were as follows: an initial denaturation at 95°C for 5 min, 30 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 35 s, and final extension at 72°C for 10 min. The PCR products were separated by electrophoresis in 2.0% agarose gel. The 16S rDNA sequences obtained from PCR amplification were then ready for purification and cloning.

To accomplish this, the amplified PCR products of the partial 16S rDNA sequences were purified with Promega PCR Purification Kit (Madison, Wisconsin, USA) and cloned with *E. coli* JM109. The purified PCR products were ligated into pGEM-T Easy Vector Systems (Promega, USA) containing 1 μl 50ng μl^{-1} pGEM-T vector, 5 μl 2X rapid ligation buffer, 1 μl 3U μl^{-1} T4 DNA ligase, with a final volume of 10 μl and incubated overnight at 4°C. The ligation products were transformed into *E. coli* JM109 and cultured on Luria-Bertani agar media containing ampicillin (100 μg ml^{-1}), IPTG (0.1 M) and X-Gal (50 μg ml^{-1}). This was followed by achieving plasmid extraction from the white colonies using Wizard Plus SV Minipreps DNA Purification System (Promega, USA). The digestion of recombinant plasmid pGEM-T was verified using restriction enzymes *Not*1 and *EcoR*1 to confirm the ligation of partial 16S rDNA fragments before sequencing by Macrogen Laboratories. In order to identify each isolated strain, the closest genetic match was compared to those in the GenBank database in NCBI via nucleotide-nucleotide BLASTn programme.

RESULTS

Isolation and Identification of Microsymbionts

A total of thirteen microsymbionts were successfully isolated from the root nodules of *M. bracteata.* The isolates were grouped as Gram negative and positive strains. The results also showed that the isolated microsymbionts were 1.2-3.0 (length) x 0.5-0.9 (width) μm in size and varied in shape such as rod, short-rod, curved, straight and coccobacilli shapes, when observed under microscope. These isolates were differentiated by their growth rate into either fast-growing (3 days) or slow-growing (5 days) bacteria. The fast-growing bacteria (isolates USM-A11, USM-B9, USM-B12, USM-B20, USM-C4 and USM-C9) were observed as acid producers, while the slow growers (isolates USM-B4, USM-B14, USM-B15, USM-B19, USM-C1, USM-C2 and USM-C8) were alkaline producers, based on the changes of pH in YEMA incorporated with BTB. Most of the strains failed to absorb the red colour from RC.

Observation of N_2-fixation, Nodulation and Plant Growth

The isolates that showed positive symbiotic N_2-fixation activities based on H_2 evolution in the *M. bracteata* host plants were (in order of highest to lowest rate): USM-A11, USM-C1, USM-B19, USM-C2, USM-C9, USM-B14, USM-B20, USM-B15, USM-B4, USM-C4 and USM-B9; these and their

respective rates are presented in Fig.1. The N_2-fixation rates ranged from 24.1 to 78.5 μmol N_2 h^{-1} g^{-1} nodule dry weight. In this process, the nitrogenase enzyme catalyzes the reduction of N_2 into NH_3 and involves a successive allocation of electrons, together with evolution of H_2. For isolates USM-B12 and USM-C8, no fixation of N_2 was detected although both isolates could enhance plant growth. This non-detection was probably due to the presence of hydrogenase enzyme (Hup^+) uptake, which can recapture the H_2 evolved within the nodule.

Most of the isolates successfully developed diverse nodule shapes on *M. bracteata* roots, such as ovoid, cylindrical, lobed and irregular. The nodules were in various shades of black, dark-brown and reddish-brown. The isolated microsymbionts successfully infected the roots and nodulated the host plants, which then allowed the N_2-fixing process to supply the N source required for plant growth, as shown in the growth parameters listed in Table 1. Effective N_2-fixation activity by the microsymbionts (especially USM-B9, USM-B14, USM-B19, USM-B20 and USM-C2) could be suggested as the reason for the increases in leaf protein and chlorophyll content and in the biomass of the whole plants and nodules. The results derived for the leaf protein content indicated that the plants inoculated with isolates USM-B4 and USM-C2 recorded the highest protein content (57.91-59.88 mg BSA ml^{-1} protein) and had a significantly different effect compared with Control-2 (Table 1). Thus, the inoculation process with these potential isolates was important in fixing N_2 and in increasing the leaf protein content. In addition, the results for the leaf chlorophyll showed that the plants inoculated with isolates USM-B9, USM-B12, USM-B15, USM-B19 and USM-B20 produced higher leaf chlorophyll content as compared to Control 2. Similar results were also

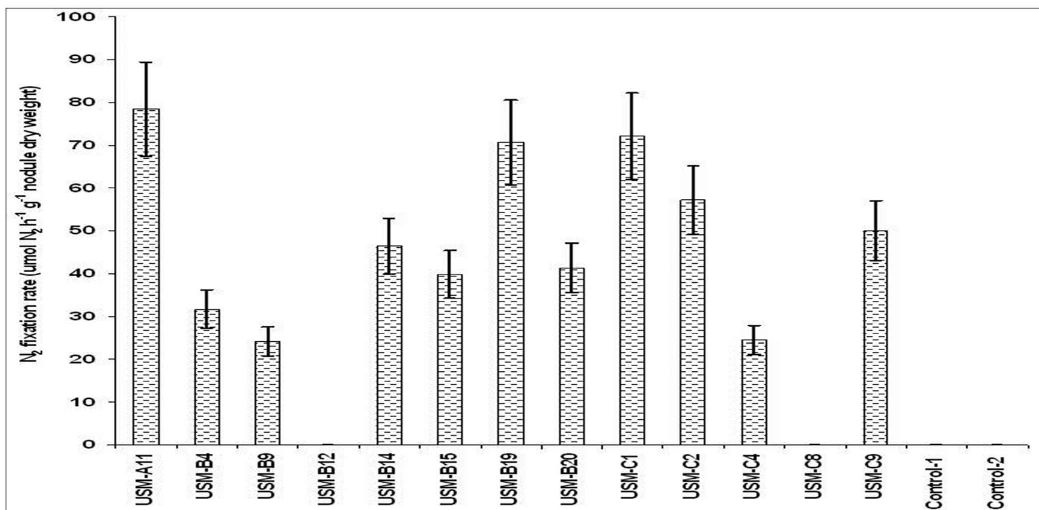

Fig.1: The influence of diazotrophic microsymbionts on the N_2 fixation rate of Mucuna bracteata at D_{60}

TABLE 1
Bacterial identification and influence of diazotrophic microsymbionts on the growth of *Mucuna bracteata* on the day of harvest (D_{65})

Identified Bacteria				Plant Growth Parameters			
					Leaf	Dry Weight	
Class	Genus	Isolate/ Treatment	Similarity of identification (%)	Leaf Protein Content (mg BSA/ ml protein)	Chlorophyll Content (chlorophyll/ mg leaf fresh weight)	Whole Plant (g)*	Nodules (mg)*
Alphaproteobacteria							
	Brevundimonas sp.	USM-B4	100	57.91 b	0.21 a	1.84 ab	160.20 c
	Brevundimonas sp.	USM-C2	99	59.88 b	0.27 a	3.04 ab	85.85 abc
Betaproteobacteria							
	Achromobacter sp.	USM-B9	98	26.62 ab	1.08 c	3.95 b	142.50 bc
	Achromobacter sp.	USM-C8	100	41.24 ab	0.24 a	1.97 ab	46.20 ab
	Burkholderia sp.	USM-B15	100	17.92 ab	0.97 c	2.37 ab	93.00 abc
	Burkholderia sp.	USM-B20	99	27.98 ab	1.12 c	3.91 b	173.75 c
	Burkholderia sp.	USM-C9	100	18.63 ab	0.88 bc	1.68 ab	111.11 abc
Gammaproteobacteria							
	Stenotrophomonas sp.	USM-B14	99	17.38 ab	0.24 a	3.78 b	129.57 bc
Bacilli							
	Bacillus sp.	USM-A11	100	34.34 ab	0.17 a	2.72 ab	80.38 abc
	Bacillus sp.	USM-B12	99	40.97 ab	1.06 c	2.23 ab	168.75 c
	Bacillus sp.	USM-B19	99	22.76 ab	1.06 c	3.05 ab	127.25 bc
	Bacillus sp.	USM-C1	99	37.33 ab	0.25 a	1.31 ab	56.85 abc
	Bacillus sp.	USM-C4	99	28.30 ab	0.79 bc	2.91 ab	157.00 c
Control							
	+ N, - microsymbiont	Control-1		31.35 ab	1.10 c	2.44 ab	25.00 a
	- N, - microsymbiont	Control-2		6.87 a	0.54 ab	1.05 a	21.75 a

Note: Bacteria were identified by matching 16S rDNA sequences with those in BLASTn (Basic Local Alignment Search Tool) online database, National Center for Biotechnology Information (NCBI).Values for growth parameters are the means of four replications; for each growth parameter, values with the same letter(s) are not statistically significant at the Tukey probability of $p<0.05$. * Data were transformed to Log10 before being analyzed with SPSS V. 15.

recorded for the control plants i.e. Control-1 (+nitrogen, -inoculum), suggesting the ability of the inoculated microsymbionts in providing the host plant with fixed N_2 was equivalent to the plants receiving nitrogen fertilizer (Table 1).

Plants inoculated with USM-B9, USM-B14 and USM-B20 grew well and showed vigorous growth. Thus, totals of the plant dry weight were higher and showed a significant effect compared to the plants in Control 2 (Table 1). The experiment indicated that the plants inoculated with USM-B20 recorded the highest mean value for nodule dry weight at 173.75 mg plant^{-1}; a similar high nodule dry weight was also recorded for the plants inoculated with isolates USM-B4, USM-B12 and USM-C4 (Table 1). In addition, *M. bracteata* treated with isolate USM-B20 recorded the highest

mean values for the plant dry weight and chlorophyll content. Therefore, N_2 that was fixed by the microsymbionts in the root nodules provided an N source for *M. bracteata* that showed an increase in plant growth.

PCR Amplification and Sequencing of nifH Gene

Genomic DNA was successfully extracted from the isolated microsymbionts. The N_2-fixing ability of the potential microsymbionts was verified by the amplification of *nifH* fragments through PCR analysis. Since dinitrogenase reductase enzyme was encoded by the *nifH* gene, this gene was amplified and sequenced in representative isolates. The amplification of *nifH* region with degenerate primers yielded a single band of the expected size (approximately 380 bp) using primer *nifH*3, as suggested by Choo *et al*. (2003). The results indicated that the primer set *nifH*3 was suitable to amplify *nifH* fragments at 380 bp with a few optimized PCR protocols for isolates USM-A11, USM-B4, USM-B9, USM-B12, USM-B19, USM-C1, USM-C4 and USM-C8, respectively. Hence, the nifH fragment (~380 bp) was amplified in *Rhizobium leguminosarum* ATCC 10004 (a positive rhizobia strain) and identified as a nitrogenase iron protein (*nifH*) gene (90% similarity) using the BLASTn programme in NCBI (gene bank accession number FJ263754.1). The primer *nifH*3 was found to be unsuitable for amplifying the *nifH* fragments for the remaining isolates. Therefore, the *nifH* fragments for isolates USM-B14, USM-B15, USM-B20, USM-C2 and USM-C9 were amplified using primer *nifH*4 and exhibited *nifH* fragments at 550 bp, 650bp, 350 bp, 450 bp and 900 bp, respectively. The sequencing results confirmed that the *nifH* fragments of USM-B14 (93% similarity), USM-B20 (100% similarity) and USM-C2 (96% similarity) were nitrogenase iron protein (*nifH*) with the gene bank accession numbers AY787541.1, AJ010288.1 and GU433550.1, respectively, in the NCBI database. Nevertheless, the amplified *nifH* fragments from isolates USM-B15 and USM-C9 failed to show the presence of nitrogenase iron protein (*nifH*) based on the sequencing results.

Cloning and Sequencing of Partial 16S rDNA

Further analysis using partial 16s rDNA sequences was performed to recognize and confirm the identity of the isolated microsymbionts (Table 1). Identification via 16s rDNA sequence analysis is one of the most effective tools for identifying bacteria. In this study, the observation of the root nodules of *M. bracteata* revealed that they contained diazotrophic rhizobial and non-rhizobial microsymbionts. The partial 16S rDNA fragments was succesfully amplified at 450 bp. This fragment was successfully ligated into p-GEMT and cloned with *E. coli* JM109. Based on the BLASTn result in NCBI, the isolates USM-B15, USM-B20 and USM-C9 were identified as *Burkholderia* sp. with 100% similarity, the isolates USM-B4 and USM-C2 as *Brevundimonas* sp. with

99-100% similarity, the isolates USM-B9 and USM-C8 as *Achromobacter* sp. with 98-100% similarity, the isolate USM-B14 as *Stenotrophomonas* sp. with 99% similarity, and the isolates USM-A11, USM-B12, USM-B19, USM-C1 and USM-C4 as *Bacillus* sp. with 99-100% similarity (Table 1). Among the identified strains, several microsymbionts were determined as *Betaproteobacteria* (*Burkholderia* sp. and *Achromobacter* sp.). The identified diazotrophic microsymbionts obtained from *Alphaproteobacteria* was *Brevundimonas* sp. and this was *Stenotrophomonas* sp. from *Gammaproteobacteria*. These findings are similar to those in other reports, in which the bacteria outside the family of *Rhizobiaceae* and *Alphaproteobacteria* have been observed to produce nodules in legumes (Chen *et al*., 2005; Pandey *et al*., 2005; Elliott *et al.,* 2007b).

DISCUSSION

The recovered nodules from *M. bracteata* in this study were seen as being active in N_2-fixation. This was because their internal colouration was pink-red, showing that the root nodule bacteria were able to produce effective nodulation and N_2-fixation activity (Somasegaran & Hoben, 1985; Ojo, 2001). The microsymbionts showed diversity in classification and in response to the tests carried out. The isolates that produced blue colour on YEMA containing BTB were alkaline producers, while some isolates produced yellow colour that showed them to be acid producers. The changes of pH on YEMA were detected by incorporating BTB as a pH indicator in agar medium for the rhizobia. The fast growing isolates lowered the pH of the YEMA + BTB causing the agar to turn yellow within 3 days. In addition, these fast growing rhizobia had a mean generation time of 24 hours (Keyser *et al*., 1982; Anand & Dogra, 1991). In contrast, the slow growing isolates increased the pH and turned the media to blue within 5 days. Meanwhile, RC was added into YEMA to distinguish between the rhizobia and contaminants (Vincent, 1982). From this study, many of the isolates from *M. bracteata* failed to absorb the red colour from RC and this therefore indicated that the strains could be qualified as the rhizobial species. Additionally, most of the alkaline-tolerant strains were recognized from this identification. Consequently, further experiments could allow for the identification of these isolated microsymbionts in terms of their genetic identity and characteristics.

In the nodulation screening, all the 13 isolates were used to inoculate *M. bracteata.* This experiment was conducted under N-free conditions (except for the Control 1) as the main goal was to observe the ability of these microsymbionts to enhance the N_2-fixation activities in *M. bracteata.* The results of this study indicated that the nodulation and N_2-fixation activity increased plant growth parameters, such as plant biomass, protein and chlorophyll contents as compared to the Control-1 (+N, -microsymbiont) and Control-2 (-N, -microsymbiont). These microsymbionts successfully infected the roots and nodulated the host plants, thus allowing the N_2-fixation process to supply the N source required for plant growth**.**

This type of effective symbiotic relationship may explain why this legume has widely been used as a resource in the agricultural ecosystems. However, these benefits extend beyond the plant itself. The production of higher plant biomass and protein content is advantageous to the soil in terms of providing more decomposing organic matter, especially N nutrients, to immature crops in fields and plantations. Additionally, the vigorous growth of *M. bracteata* forms a thick leafy canopy close to the soil surface and consequently reduces soil temperature, leading to higher microbial activity and enrichment of the nutrient status of the soil (Zhao *et al.*, 1997; Mathews, 1998; Graham, 2008). Moreover, this type of symbiotic relationship provides a great compensation as it is not hazardous to the environment (Appunu & Dhar, 2008).

The N_2-fixing ability of the potential isolated microsymbionts was confirmed through the PCR analysis by amplifying the *nifH* fragment and sequencing in the representative strains. This gene is a key enzyme in N_2-fixation activity and is known as nitrogenase enzyme. In part of the nitrogenase enzyme region (nif gene), there is a *nifH* gene which is involved in encoding dinitrogenase reductase. Thus, this *nifH* PCR amplification and sequence analysis were undertaken to evaluate the diversity among the N_2-fixing microsymbionts in the root nodules of *M. bracteata.* The amplification of the *nifH* region with degenerate primers yielded a single band of the expected size using primers *nifH3* and *nifH,* as suggested by Choo *et al*. (2003). The results indicated that these primer sets were suitable to amplify the *nifH* fragments at several particular sizes. Thus, these molecular methods, based on the PCR detection of the *nifH* marker gene, have been successfully applied to describe the diazotroph populations in the nodules of *M. bracteata.*

The 16S rDNA sequence analysis is an effective tool to be used in identifying bacteria. The observation of the root nodules of *M. bracteata* revealed that they contained diazotrophic rhizobia and non-rhizobia based on the sequence analysis of partial 16S rDNA. From this experiment, several microsymbionts were identified as beta-class proteobacteria: *Burkholderia* sp. and *Achromobacter* sp. As for the alpha-class of proteobacteria, the identified bacterium was *Brevundimonas* sp.*,* while *Stenotrophomonas* sp. was from the gamma-class proteobacteria. Five isolated microsymbionts were identified as *Bacillus* sp. and this particular species is a non-rhizobial microsymbiont. This group of non-rhizobial microsymbionts was Gram-positive bacteria, as was detected in the Gram staining screening. These are Gram-positive and non-rhizobial strains but they may also be found co-existing with rhizobia in the root nodules of legumes.

In the recent years, the bacteria which do not belong to the Rhizobiaceae in *Alphaproteobacteria* were isolated. These bacteria are able to produce nodules and fix atmospheric N_2 (Willems, 2006; Chen *et al*., 2005; Sprent, 2008). These new nodulating bacteria have been identified

through 16S rDNA and are distinct from the Rhizobiaceae in the phylogenetic observation. For instance, *Brevundimonas, Devosia, Methylobacterium, Ochrobactrum* and *Phyllobacterium* from the *Alphaproteobacteria* class are also capable of nodulating and fixing N_2 in legumes.

The beta-class of the proteobacterial branch also contains nodulating bacteria such as *Burkholderia, Achromobacter, Cupriavidus* and *Ralstonia* (Willems, 2006). *Burkholderia* have recently been isolated from a variety of legumes (Chen *et al*., 2003a, 2005; Barrett & Parker, 2005, 2006; Elliott *et al*., 2007a, 2007b). The capabilities of these *Betaproteobacteria* in fixing N_2 and nodulating the host plant have been confirmed due to the existence of nodulation genes (*nod*) and *nif* genes which are similar to those of alpha-rhizobia and are located on a symbiotic plasmid (Chen *et al*., 2003b, 2005). From this experiment, *Burkholderia* spp. was found to be an effective inoculant for promoting plant growth. The isolates were able to fix N_2 and contained the *nifH* gene. *Burkholderia vietnamiensis* has previously been shown to be capable of enhancing plant growth, promoting indirect nodulation, serving as an antifungal, and aiding in phosphorus mobilization (Peix *et al*., 2001). Initially in the N_2-fixation studies, *B. vietnamiensis* was the only species identified as a N_2-fixing strain in the genus of *Burkholderia* and found to be associated with rice plants (Gillis *et al*., 1995). Apparently, a number of N_2-fixing *Burkholderia* species have recently been discovered in the natural environment and are associated with certain plants including legumes (Wong-Villarreal & Caballero-Mellado, 2010). *Burkholderia plantarii* was first identified by Azegami *et al*. (1987) and it is considered as plant pathogenic bacteria (Suarez-Moreno *et al*., 2008). However, several *B. plantarii* isolates have been used for rice seeding cultivation (Maeda *et al.,* 2006). Thus, the *Burkholderia* species have potential for agro-biotechnology applications. From this experiment, another beta-class proteobacterial isolate was obtained, i.e. *Achromobacter* sp. Benata *et al*. (2008) also successfully isolated *A. xylosoxidans* from the root nodules of *Prosopis juliflora* from the eastern area of Morocco. Moreover, their analysis of the *nodC* also yielded and revealed that the *Achromobacter* sp. isolates contained approximately 930 bp of the *nodC* gene based on the PCR amplification using appropriate oligonucleotide primers (Benata *et al.,* 2008). Thus, *Achromobacter* sp. has contributed to broadening the new legume-nodulating-bacteria taxonomy. *Stenotrophomonas* sp. [formerly known as *Xanthomonas* sp. (Juhnke & Des Jardin, 1989) and as *Pseudomonas* sp. (Swings *et al*., 1983)] has useful properties in the biological control of soil-borne plant disease, bacterial microflora in soil and in the plant rhizosphere (Lambert *et al*., 1987). Kan *et al*. (2007) isolated *S. maltophilia* from the root nodules of herbaceous legumes grown in Tibet, China, as well as *Rhizobium, Sinorhizobium, Mesorhizobium* and *Phyllobacterium.* Likewise, this strain is also associated with the roots of another

legume plant, *Astragalus bisulcatus* (Di Gregorio *et al*., 2005; Kan *et al*., 2007).

Results from partial 16S rDNA sequence analysis also confirmed that five *Bacillus* sp. microsymbionts were obtained from the root nodules of *M. bracteata.* This non-rhizobial species is considered as endophytic bacteria, which live within the plant tissues and are not harmful to the plant host (Kobayashi & Palumbo, 2000). Meanwhile, the presence of *B. thuringiensis* in legume plants has been shown to absorb nutrients from soil and inhibit soil-borne pathogens and insect pests (Chattopadhyay *et al.,* 2004; Kuklinsky-Sobral *et al*., 2004; Reyes-Ramirez *et al*., 2004; Taghavi *et al*., 2005; Wang *et al*., 2006; Pandey & Maheshwari, 2007), as well as increase overall plant growth (Andrews & Harris, 2000). The inoculation of *Phaseolus vulgaris* L. with a combination of *Bacillus* spp. and *Rhizobium* sp. was shown to promote root nodulation and other beneficial interactions (Karanja *et al*., 2007). Similarly, Bai *et al*. (2002) reported that *B. thuringiensis* could enhance root nodulation and plant growth in soybean when applied as a co-inoculum with *Bradyrhizobium japonicum.*

In addition, Mishra *et al*. (2009) showed that the co-existence of rhizobial and non-rhizobial plant-growth-promoting strains in leguminous plants might improve nodule production and N_2-fixation activity. In bacteria-legume symbioses, enhancement of N_2-fixation in the host plant is the most important factor. Thus, in this experiment, plants inoculated with *Bacillus* spp. (USM-A11, USM-B19 and USM-C1) showed better N_2-fixation, which resulted in increased plant growth, even when it was under N-free conditions. In addition, the *nifH* gene fragments were successfully amplified and studied from each *Bacillus* sp. isolate to aid in understanding the ability of these isolates in enhancing the growth of *M. bracteata*.

CONCLUSION

Various microsymbionts in the present study showed potential to enhance plant growth and N_2-fixation in *M. bracteata*. From partial 16S rDNA sequence analysis, the isolated strains of microsymbionts from the nodules of *M. bracteata* exhibited a species-rich variety. This suggests that the actual diversity of bacteria that can nodulate this legume is higher than expected, and this includes the bacteria outside the *Alphaproteobacteria* class. Such non-rhizobial bacteria deserve further study in terms of their species identify, long-term effects on plant growth, biochemical interactions with other endophytic bacteria, and potential for use in agro-biotechnology applications.

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