EFFECTS OF BENEFICIAL DIAZOTROPHS ON GROWTH OF SEEDLINGS AND TISSUE-CULTURED PLANTLETS OF OIL PALM (*Elaeis guineensis* Jacq.)

By

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LIST OF ABBREVIATIONS

1.	ARA	Acetylene Reduction Assay
2.	ANOVA	Analysis of Variance
3.	BTB	Bromothymol Blue
4.	BNF	Biological Nitrogen Fixation
5.	cfu	Colony Forming Unit
6.	C_2H_2	Acetylene
7.	C_2H_4	Ethylene
8.	CRD	Completely Randomized Design
9.	D	Day
10.	E	Endophytes
11.	GC	Gas Chromatography
12.	g	Gram
13.	h	Hours
14.	H	Hydrogen
15.	ha	Hactre
16.	HMDS	Hexamethyldisilazane
17.	HPLC	High Performance Liquid Chromatography
18.	IAA	Indole-3-Acetic Acid
19.	K	Potassium
20.	kg	Kilogram
21.	L	Litre
22.	LSD	Least Significant Difference

23.	L-Tryp	L-tryptophan
24.	mg	Milligram
25.	Mg	Magnesium
26.	mL	Millilitre
27.	MOP	Muriate of Potash
28.	MS	Murashige and Skoog
29.	nd	Not Detected
30.	$N_{(inorganic)}$	Inorganic nitrogen
31.	N_2	Nitrogen Gas
32.	NAA	Naphthaleneacetic Acid
33.	Nfb	Nitrogen-Free Semi-solid Medium
34.	OD	Optical Density
35.	pp	Page
36.	P	Phosphorus
37.	PGPR	Plant Growth Promoting Rhizobacteria
38.	R	Rhizosphere
39.	SEM	Scanning Electron Microscope
40.	spp.	Species
41.	TSP	Triple Superphosphate

LIST OF SYMBOLS

1.	%	Percent
2.	<	Less than
3.	>	More than
4.	^{o}C	Degree Celsius
5.	µ	Micro

KESAN DIAZOTROF BERFAEDAH TERHADAP PERTUMBUHAN ANAK POKOK DAN PLANTLET TISU KULTUR KELAPA SAWIT

(*Elaeis guineensis* Jacq.)

ABSTRAK

Sektor pertanian di Malaysia sedang bergerak ke arah amalan persekitaran lestari. Proses pengikatan nitrogen secara biologi (BNF) boleh digunakan untuk mengurangkan penggunaan baja N tak organik. Proses pengikatan nitrogen secara biologi tersebut berlaku apabila diazotrof diinokulasi ke tanaman pertanian dan mewujudkan hubungan berfaedah secara simbiotik atau tak simbiotik dengan tanaman perumah. Interaksi di antara tanaman dan diazotrof juga akan mempengaruhi penghasilan fitohormon (indole-3-acetic acid) (IAA). Amnya, IAA dikeluarkan oleh bakteria akan menambah berat bahagian atas dan akar serta bilangan akar lateral tanaman perumah. Justeru, kombinasi kedua-dua pengikatan nitrogen secara biologi dan penghasilan fitohormon oleh diazotrof akhirnya akan meningkatkan hasil tanaman perumah. Oleh itu, diazotrof berfaedah mempunyai potensi untuk diperkenalkan kepada tisu kultur kelapa sawit di dalam keadaan *in vitro* dan rumah tumbuhan. Diazotrof akan membentuk interaksi positif dengan tanaman perumah dan seterusnya penggunaan baja N boleh dikurangkan. Oleh itu, tiga eksperimen telah dijalankan dengan objektif berikut; 1) untuk mengenalpasti kebolehan kultur bakteria mengikat N_2 dan menghasilkan IAA di bawah keadaan bebas dan asosiatif, 2) untuk memerhati kesan diazotrof pada pertumbuhan tanaman perumah dan 3) untuk memerhati pengaruh diazotrof dan kombinasi kepekatan N tak organik ($N_{(tak\ organik)}$) yang berbeza pada pertumbuhan anak pokok kelapa sawit di rumah tumbuhan. Diazotrof yang diuji ialah *Herbaspirillum seropedicae*, Z78 dan bakteria pencilan tempatan *Microbacterium* sp., (E7), *Acetobacter pasteurianus* (E9) dan *Microbacterium testaceum* (E14). Keputusan menunjukkan *H. seropedicae*, Z78

dan bakteria pencilan tempatan, E7 dan E14 bebas menghasilkan kepekatan IAA yang tinggi pada 10.1, 11.5 and 8.9 µg mL^{-1}, masing-masing, berbanding kawalan (1.9 x 10^{-4} µg mL^{-1}) selepas 60 jam pengeraman. Kesemua isolat juga mempamerkan kebolehan mengikat N_2 yang tinggi sehingga 5.0 x 10^{-9} µmol C_2H_4 cfu^{-1} h^{-1} dalam keadaan separa aerobik. Diazotrof yang diuji juga telah menggalakkan perkembangan akar pucuk tisu kultur kelapa sawit pada D_{60} dan D_{90}. Inokula tersebut juga menunjukkan pengaruh positif terhadap pertumbuhan plantlet kelapa sawit (berat, tinggi dan jumlah kandungan protein dalam daun) akibat penghasilan IAA yang tinggi sehingga 0.6 µg mL^{-1} pada D_{60} dan 0.2 µg mL^{-1} pada D_{90}. Pengaruh berfaedah diazotrof terhadap tanaman perumah adalah akibat keberkesanan kolonisasi Z78 pada permukaan akar. Kesan diazotrof dalam kombinasi kepekatan $N_{(tak\ organik)}$ yang berbeza terhadap pertumbuhan anak kelapa sawit juga telah diuji di rumah tumbuhan. Keputusan menunjukkan Z78, E7 dan E14 berpotensi meningkatkan pertumbuhan anak pokok kelapa sawit jika dibekalkan dengan kepekatan $N_{(tak\ organik)}$ yang minima tanpa mencatatkan kekurangan berat tanaman dan isipadu akar. Aktiviti penurunan asetilena telah dikesan untuk tanaman yang telah diinokulasi dengan Z78, E7 dan E14 di mana telah dikombinasikan dengan 25% $N_{(tak\ organik)}$ pada 2.98 x 10^{-3}, 5.06 x 10^{-3} and 2.46 x 10^{-3} µmol C_2H_4 g^{-1} fresh weight h^{-1}, masing-masing. Dapat disimpulkan bahawa fitohormon (IAA) yang dihasilkan oleh diazotrof mampu meningkatkan pertumbuhan plantlet tisu kultur kelapa sawit dalam keadaan in vitro. Dapat juga diperhatikan bahawa inokulasi diazotrof (Z78, E7 dan E14) dengan kombinasi 25% kepekatan $N_{(tak\ organik)}$ adalah diperlukan untuk pertumbuhan anak pokok kelapa sawit yang lebih baik berbanding kawalan.

ABSTRACT

The agricultural sector in Malaysia is moving towards environmentally sustainable practices. Biological Nitrogen Fixation (BNF) process can be adopted to minimize the usage of inorganic N fertilizer. The biological nitrogen fixation process occurred when the diazotrophs are inoculated to the agricultural crop and establish beneficial symbiotic or non-symbiotic relationships with the host plants. The interaction between plant and diazotrophs would also influence synthesis of phytohormone (indole-3-acetic acid). Generally, IAA excreted by the bacteria would increase top and root biomass and lateral root numbers of the host plants. Thus, combination of both BNF and synthesis of phytohormones of the diazotrophs would finally improve yield of the host plants. Therefore, the beneficial diazotrophs have the potential to be introduced to tissue cultured oil palm under *in vitro* and glasshouse conditions. The diazotrophs would create positive interaction with the host plants and consequently the utilization of N fertilizer could be reduced. Thus, three experiments were carried out with the following objectives; 1) to determine the ability of the bacterial culture to fix N_2 and to excrete IAA under free-living and associative conditions, 2) to observe the effects of diazotrophs on growth of the host plant and 3) to observe influences of diazotrophs and different concentrations of inorganic N ($N_{(inorganic)}$) on growth of oil palm seedlings under glasshouse conditions. The diazotrophs tested were *Herbaspirillum seropedicae*, Z78 and locally isolated, *Microbacterium* sp., (E7), *Acetobacter pasteurianus* (E9) and *Microbacterium testaceum* (E14). The results showed that free living *H. seropedicae*, Z78 and locally isolated, E7 and E14 produced higher concentration of IAA at 10.1, 11.5 and 8.9 µg mL^{-1}, respectively, compared to control (1.9×10^{-4} µg mL^{-1}) after 60 h of incubation. These isolates also exhibited higher N_2 fixation capability up to 5.0×10^{-9} µmol C_2H_4 cfu^{-1} h^{-1} under microaerophilic conditions. The diazotrophs tested had also

influenced root development of tissue cultured oil palm shoots at D_{60} and D_{90}. The inocula also revealed positive influences on growth of oil palm plantlets (biomass, height and total leaf protein content) due to higher production of plant growth regulator (IAA) of up to 0.6 µg mL^{-1} at D_{60} and 0.2 µg mL^{-1} at D_{90}. Beneficial influences of the diazotrophs on the host plants were also due to effective colonization of Z78 on root surfaces. Effects of diazotrophs in combination with different concentrations of $N_{(inorganic)}$ on growth of oil palm seedlings were also tested under glasshouse conditions. The results indicated that Z78, E7 and E14 could potentially enhance growth of oil palm seedlings if supplied with low concentration of $N_{(inorganic)}$ with no losses of plant biomass and root density recorded. The acetylene reduction activity was detected for plants inoculated with Z78, E7 and E14 which were combined with 25% $N_{(inorganic)}$ at 2.98 x 10^{-3}, 5.06 x 10^{-3} and 2.46 x 10^{-3} µmol C_2H_4 g^{-1} fresh weight h^{-1}, respectively. It is concluded that phytohormone (IAA) excreted by the diazotrophs could enhance growth of tissue cultured oil palm plantlets under in vitro conditions. It was also noticed that inoculation of diazotrophs (Z78, E7 and E14) in combination with 25% $N_{(inorganic)}$ improved growth of oil palm seedlings compared to the Control.

CHAPTER ONE

INTRODUCTION

The Malaysian oil palm industry, one of the world's most highly organized agricultural sectors, has grown significantly over the past five decades, as described by Basiron (2007). Total land area under oil palm cultivation has increased from 54,000 hectares in 1960 to over 4 million hectares in 2005, representing a compound annual growth rate of over 10 percent. Moreover, production has increased from 94,000 tonnes in 1960 to 15 million tonnes in 2005, or by almost 160 times within 45 years, representing a compound annual growth rate of almost 12 percent (Basiron, 2007). This growth shows the industry's success and contribution to world food resources; however, over-dependence on the primary commodity has resulted in the use of large quantities of chemical fertilizers in order to sustain higher yields, posing environmental and economic concerns (Basiron, 2007). As highlighted by Tarmizi and Mohd Tayeb (2006), the recommended rate of N fertilizer for oil palm is 4.2 kg ammonium sulfate $palm^{-1}$ $year^{-1}$

Nitrogen fertilizer is well known as the most expensive nutrient input which is highly required for economic crop cultivation including oil palm. The cost of urea fertilizer as for December 2008 is 223 USD $tonne^{-1}$ (Yara International ASA, 2008). For this reason, low cost alternative N input for plant growth could be used such as biological nitrogen fixation (BNF). BNF is a process of converting atmospheric nitrogen gas (N_2) to ammonia (NH_4) by diazotrophic bacteria. This process can be the most viable N source to replace N fertilizers required by the agricultural crops (Carvalho *et al.,* 2008; Lery *et al.,* 2008). The BNF concept can be implemented as a

key factor in the development of environmental friendly agricultural practices for oil palm.

Generally, every healthy soil may contain enough numbers of diazotrophs to fix N_2 through BNF process. The diazotrophs, which are also referred as plant growth promoting rhizobacteria (PGPR), have been reported to enhance growth and yield of several non-leguminous crops (Dalton and Kramer, 2006; Chong-Min *et al*., 2005). Chi *et al*. (2005) suggested that the plant-diazotrophs interaction has potential values for future exploitation of sustainable agricultural practices for cereal crops. The diazotrophs have been identified as *Azospirillum* spp, *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans* and *Glucanoacetobacter diazotrophicus* (Steenhoudt and Venderlyden, 2000; James *et al.,* 2002; Ladha and Reddy, 2003). The diazotrophs are isolated from plant tissues of roots, stem and leaves and are able to produce plant growth regulators (indole-3-acetic acid, gibberellins and cytokinins) (Park *et al.,* 2005; Cavalcante and Dobereiner 1988). The diazotrophs may enter the roots and colonize the vascular systems of roots and stem and thus be able to fix substantial amounts of N_2 (Boddey *et al.,* 2003).

The potential of implementing BNF concept has been recently demonstrated for improving the growth of non-leguminous crops such as sugarcane (Oliveira *et al.,* 2006; Govindarajan *et al.,* 2006; 2008; Munoz-Rojas and Caballero-Mellado, 2003), rice (Jha *et al.,* 2009; Senthilkumar *et al.,* 2008; Rodrigues *et al.,* 2008; Muthukumarasamy *et al.,* 2007), grass (Reinhold-Hurek *et al.,* 2005; Hurek *et al.,* 2002), banana (Weber *et al.,* 2007; Mia *et al.,* 2005) and oil palm seedlings (Amir *et al*., 1999; 2001; 2003; Azlin *et al.*, 2007; Carvalho *et al.,* 2008). Consequently, a decreasing dependency on chemical

fertilizer through BNF inputs for agricultural production is needed in order to reduce cost and negative impact of extensive crop fertilization practices to the economy and environment.

However, the N_2 fixing capacity of the associative diazotrophs is often very low, generally around 30-40% and even lower in some cases. This amount is lower compared to N derived from inorganic fertilizer and cannot provide the total N needs of the host plants for optimal growth (Cong *et al.,* 2009; Muthukumarasamy *et al.,* 2002). Therefore, addition of small doses of N fertilizer as a starter N in combination with the diazotrophs is required to accelerate higher N_2 fixation activities and higher yield for the host plants. Thus, minimal concentration of starter N should be applied together with the diazotrophs for optimum BNF activities in agricultural practices to sustain crop production as well as to manage soil health and biodiversity.

Previous investigations have established that the inoculation of diazotrophs to commercially important crops such as rice, sugarcane and wheat can reduce N fertilizer input (Matthews *et al.,* 2001; Nath *et al.,* 2002; Govindarajan *et al.,* 2006, 2008). Nath *et al.* (2002) report that inoculation of *A. lipoferum* and *A. brasilense* to grasses and rice plants supplemented with minimal amounts of inorganic N fertilizer has significantly influenced the growth and biomass of the host plants compared to the control (supplied with 100% inorganic N fertilizer). Similarly, low input of N in breeding and subsequent cropping of *G. diazotrophicus* inoculated sugarcane is primarily responsible for the development of high yielding Brazilian sugarcane varieties (Baldani *et al.,* 2002). In a separate experiment, inoculation of *Burkholderia* MG43 in sugarcane exhibits greater biomass of the host compared to the control supplied with increasing concentrations of

N fertilizer (from half to full recommended rate). Thus, it saves the cost by approximately 140 kg ha^{-1} N fertilizer (Govindarajan *et al*., 2006). Therefore, the population of diazotrophs and BNF activity appears to be influenced by minimal nitrogen input. Consequently, it is crucial to discover the interaction effects of different concentrations of inorganic N ($N_{(inorganic)}$) with diazotrophs to the host plants. Thus, the objectives of the study are as follows:

a) To determine the ability of diazotrophs to fix N_2 and produce plant growth regulator (IAA) (Chapter 4)

b) To observe the potential effects of diazotrophs on the growth of tissue-cultured oil palm shoots (Chapter 5)

c) To observe the ability of diazotrophs to colonize the root surface of newly initiated roots of tissue-cultured oil palm shoots (Chapter 5)

d) To observe the effects of diazotrophs in combination with different concentrations of $N_{(inorganic)}$ on the development of oil palm seedlings under glasshouse conditions (Chapter 6)

CHAPTER TWO

LITERATURE REVIEW

2.1 Plant Growth Promoting Rhizobacteria (PGPR)

Rhizosphere is an important niche that supports the growth of a broad range of microorganisms. The bacteria live and benefit the host plants without causing any apparent harm to the external and internal structures (Kuklinsky-Sobral *et al.,* 2005; Pedraza *et al.,* 2009). The presence of plant growth promoting rhizobacteria PGPR and the possibility of a significant increase in plant growth and yield under nutrient limiting conditions have been discussed for many years especially for rice, sugarcane, maize and wheat crop. The invading bacteria benefit the acquired host with a marked increase in plant growth, vigor and yield (Bhattacharjee *et al.,* 2008; Jha *et al*., 2009). PGPR promotes plant growth in two different ways, (1) directly affecting the metabolism of the host by providing substances that are usually in short supply or (2) improving plants tolerance to stress such as drought, high salinity, metal toxicity and pesticide load.

These bacteria are capable of fixing atmospheric nitrogen, producing phytohormones such as auxin, gibberellin, cytokinin, ethylene and solubilizing phosphorus and iron (Kennedy *et al.,* 2004; Mantelin and Touraine, 2004; Barea *et al.,* 2005; Bashan and de Bashan, 2005; Tarkka *et al.,* 2008). The mechanisms mentioned above may contribute to plant growth improvement compared to uninoculated control. Another group of PGPR plays a role as biocontrol agent, which indirectly promotes plant development by preventing the deleterious effects of phytopathogenic

microorganisms (bacteria, fungi and viruses). The bacteria produces substances that harm or inhibit competing microorganisms by reducing the availability of iron to pathogens or by increasing its resistance towards pathogen infection (Bashan and de Bashan, 2005; Pinon *et al*., 2002).

The most common genera of PGPR are *Azospirillum, Azotobacter, Bacillus* and *Pseudomonas.* Some of these genera include endophytic species as well. The best-characterized endophytic bacteria include *Azoarcus* spp, *Gluconacetobacter diazotrophicus,* and *Herbaspirillum seropedicae* (Fuentes-Ramirez and Caballero-Mellado, 2005). These microorganisms have potential in promoting plant growth in association with different agricultural crops (Caballero-Mellado *et al.,* 2003). Additionally, the endophytes are ubiquitous, locally and systematically colonizing and influencing plant health by disease suppression, degradation of contaminants and promoting plant growth (Pedraza *et al.,* 2009; Pedraza, 2008; Sturz *et al.,* 2000). Similarly, Sevilla *et al.* (2001), show growth enhancement of sugarcane owing to effective colonization of bacterial inoculum (*Acetobacter diazotrophicus*). Therefore, PGPR has been proposed as microbial inoculants which can promote plant growth, enhance crop yield, diminish pollution caused by the chemical fertilizers and thus, create environmentally friendly agricultural system (Fuentes-Ramirez and Caballero-Mellado, 2005). *Azospirillum* is one of the most studied PGPR that associates with plants, which has been demonstrated using micropropagation system as well as glasshouse experiment or under field conditions (Oliveira *et al.,* 2009; Dobbelaere *et al*., 2003; Lucy *et al*., 2004). The endophytic bacteria such as *Herbaspirillum* spp*, Klebsiella pneumonia* and *Burkholderia* have received increasing attention in recent

years owing to their associations with important crops and have the potential to enhance plant growth (Pedraza *et al.,* 2009; Dong *et al*., 2003; Verma *et al*., 2001). Inoculation of sugarcane with *G. diazotrophicus* has influenced higher plant biomass increment and better nutrient and water uptake of the host plants after 60 days of growth (Muthukumarasamy *et al*., 2006).

2.2 Diazotrophic Rhizobacteria

Diazotrophic rhizobacteria were firstly isolated in the early sixties. However their biological nitrogen fixation (BNF) contribution to the sustainable agriculture system is still unknown, although their positive effect on plant growth and productivity is well documented. In other words, advance studies are essential to complete the understanding and exploitation of diazotrophic organisms as a natural nitrogen source (Cocking, 2003; Kennedy *et al.,* 2004).

The diazotrophs are always referred to as microorganisms that are capable of converting atmospheric N into NH_3 which can be used by plants. The diazotroph consists of two major groups which are (1) exophytic (diazotrophs that remain outside the host plant) and (2) endophytic (diazotrophs that are able to colonize the inner tissues of host plant) (Shenoy *et al.,* 2001). Most of non-endophytic diazotrophs, such as *Azospirillum* are colonizing the root surfaces, particularly on the root hair and root elongation zone, or within disrupted epidermis outer cortical. In contrast, endophytic diazotrophs, such as *Azotobacter* spp.*, Azoarcus* spp., *Herbaspirillum* spp. and certain strains of *Azospirillum brasilense* tend to colonize the root cortex, and may even penetrate the endodermis to colonize the stele. From there, the endophytes may be

subsequently translocated to aerial parts of the plants. Additionally, they do not live within healthy host cells, instead they colonize the apoplast, intercellular space, xylem vessels and lignified xylem parenchyma as well as dead cells (James, 2000). Similar finding is shown by Zakria *et al.* (2007) that, *Herbaspirillum* sp. strain B501 gfp1 has successfully colonized the intercellular spaces of the root epidermis and at the junctions of the lateral roots of cultivated rice. Higher bacterial populations are observed and show significant amounts of $^{15}N_2$ detected in the infected plants. This evidence supports the interior colonization within the plant tissues which can promote higher nitrogenase activity and excretion of plant growth promoting substances, which, in return, benefits the host plants (Bhattacharjee *et al.,* 2008).

Many diazotrophs are associated with the roots of plants where they exchange fixed nitrogen for the products of photosynthesis. Fractions of N derived from amino acids are taken up by the plants to synthesize enzymes and proteins. Both components are mainly involved in photosynthesis that subsequently exported to reproductive and storage organ (seeds, bulbs or trunk) (Hirel *et al.,* 2007; Philippot and Germon, 2005). Plants associated with N_2 fixers, can grow in highly acidic soils and swamps area, and can be successfully used for soil remediation (Martinez-Romero, 2006; Koponen *et al.,* 2003). The free living diazotrophic rhizobacteria such as *Azospirillum* spp and *Bacillus* spp. are able to colonize the exterior and interior roots of host plants. In this association, the rhizobacteria has been reported to be very essential for the establishment and growth of the host plants through N_2 fixation activities and phytohormones production (Tsimilli-Michael *et al*., 2000). Magalhaes *et al.* (1979) report that higher frequency of *Azospirillum* colonies (10^5 to 10^7 cells g^{-1} root tissues) occurrs during the grain filling

stage of maize plants. During this stage, the nitrogenase enzyme activity of the host plants is usually much higher.

The endophytic microorganisms can multiply and spread inside the host plants without causing any damage to the host plants or conferring any ecological threat (Bhattacharjee *et al.,* 2008; Quispel, 1992). Their unique characteristic and recent findings on their contribution of fixed nitrogen to host (grass), have made endophytes lifestyle very interesting (Hurek *et al*., 2002; Sevilla *et al*., 2001). Gyaneshwar *et al*. (2002) report that *H. seropedicae* has colonized the interior of roots and aerial parts of rice cultivars grown under axenic and field conditions. Further analysis of GUS-stained material and transmission electron microscopy (TEM) has elucidated the colonization pattern of *H. seropedicae* within root tissues of rice seedlings. *H. seropedicae* aggressively enters primary roots of rice, invades and colonizes them. The invasion process involves large number of cells attached and multiplied within the cracks at the lateral root junctions and moves deeply into the primary roots *via* the intercellular spaces (James *et al.,* 2002). The diazotrophs which are present in these areas are *Azospirillum* spp. and *Herbaspirillum* spp. as well as other unidentified microorganisms (Nobrega *et al*., 2004). This discovery leads to a suggestion that endophytic diazotrophs can serve as an alternative strategy to avoid pollution from extensive usage of inorganic fertilizer.

2.3 Production of Phytohormones (IAA)

Plant growth regulators are involved in growth and development of cells, tissues, organs and in fact the entire plants. These compounds are active in plants and

the synthesis is extremely regulated. Plants not only produce phytohormones but also numerous plant-associated bacteria, both beneficial and harmful (Fuentes-Ramirez and Caballero-Mellado, 2005). The ability to synthesize phytohormones is widely distributed among plant-bacteria interactions. Almost 80% of the rhizobacteria are able to produce common auxin such as indole-3-acetic acid (IAA) (Costacurta and Vanderleyden, 1995; Patten and Glick, 1996).

Indole-3-acetic acid (IAA) is one of the common physiologically active auxins. IAA is common product of L-Tryptophan metabolism by several microorganisms including PGPR which may exert pronounced effects on plant growth. Among the PGPR species, *Azospirillum* is well known for its ability to excrete auxins (Fuentes-Ramirez and Caballero-Mellado, 2005). Three types of phytohormones can be detected in the culture supernatant of *Azospirillum* which includes auxins and small amount of gibberellins and cytokinins (Lambrecht *et al*., 2000). Findings by Barea and Brown (1974) showed the ability of *Azotobacter paspali* to excrete IAA into the culture medium. Sarwar and Kremer (1995) reported that all the rhizosphere isolates that belong to genera *Enterobacter, Xanthomonas, Pseudomonas* and *Agrobacterium* are more efficient producers of auxin.

The phytohormone will bring morphological and physiological changes of the inoculated plant roots, which leads to plant-bacteria interaction (Malhotra and Srivasta, 2009; Pedraza, 2008). The involvement of IAA in the interaction of *Azospirillum brasilense* and roots of *Panicum miliaceum* has been emphasized by Harari *et al.* (1988). It is essential to elucidate the existing of several pathways for IAA synthesis by

the bacteria (Vandeputte *et al*., 2005; Lambrecht *et al.,* 2000). Three main pathways are known for the conversion of tryptophan into IAA: the indole-3-pyruvic acid (IPyA) pathway, the indole-3-acitemide (IAM) pathway, and the tryptamine TAM pathway (Costacurta and Vanderleyden, 1995; Lambrecht *et al*., 2000). The existence of an additional tryptophan independent pathway is suggested when no exogenous tryptophan is added. About 90% of the IAA is formed in the tryptophan independent pathway (Lambrecht *et al*., 2000). Tryptophan (Trp) is generally regarded as a precursor of IAA as the addition of these amino acids into cultures of *Azospirillum* results in higher production of IAA and other related compounds (Lee *et al*., 2004; Asghar *et al*., 2002; Glickmann and Dessaux, 1995). The IAA production rate of *Rhodopseudomonas* KL9 strain is much higher (14.5-32.7 μg mL^{-1}) than *Pseudomonas putida* GR12-2 (supplemented with tryptophan as a precursor of IAA), which enhances 35% to 50% root elongation in canola seeds (Patten and Glick, 2002). A relatively high percentage of epiphytic bacteria isolated from pear leaf and fruit surfaces have the ability to produce indole-3-acetic acid (IAA) in culture media supplemented with tryptophan. Rae-Hyun and Song, (2007) reveal that *Rhodopseudomonas* strain KL9 produces up to 5.56 mM min^{-1} mg^{-1} protein of indole-3-acetic acid (IAA). It might be one of the mechanisms for plant growth enhancement of tomato. The germination rates of the tomato seed, total length, and dry mass of germinated tomato seedling increase by 30.2%, 71.1%, and 270.8%, respectively, compared with of those 7 days after inoculation of the uninoculated control.

There are several methods to identify indolic compounds derivatives from the bacteria. Chromatography techniques such as high performance liquid chromatography

(HPLC) and gas chromatography-mass spectrometry (GC-MS) have been used to visualize IAA (Glickmann and Dessaux, 1995). These techniques are based on physiochemical properties of the indolic compound that can be obtained from the extraction of the bacterial culture supernatants and concentration of the extracts prior to separation (Manulis *et al.,* 1991). Such methods, however, are time-consuming and cannot be used as routine assays.

Other methods that have been used to determine indole-3-acetic acid (IAA) production are based on colorimetric technique derived by Salkowski (Salkowski, 1885; Gordon and Weber, 1951). This method is preferable because it is simple, fast, cheap and convenient to handle, allowing daily analysis of numerous bacterial supernatants (Somers *et al.,* 2005; Asghar *et al.,* 2002; Patten and Glick, 2002). It proved to be useful for screening bacterial mutants affected in auxin synthesis (Zimmer *et al.,* 1991).

2.4 Biological Nitrogen Fixation Process

Nitrogen, derived from atmosphere is present almost as inert nitrogen (N_2) molecules, which are not directly available to plants need. Some microorganisms called diazotrophs can utilize atmospheric N_2 to produce nitrogenous compounds to be used in their own cells. This process called biological nitrogen fixation (BNF), which requires a great deal of energy. Therefore, free living microorganisms that perform the reaction such as *Azotobacter*, generally fix little nitrogen each year (< 9.07 kg acre^{-1}) due to limited food energy. In contrast, *Rhizobia* that nodulates legume roots, receives much energy from the host plants. Thus, leguminous plant can fix much more nitrogen per year (up to 45.4 kg acre^{-1}) to meet the needs of the *Rhizobia* itself and host legume

plant. This is why well nodulated legumes do not often respond to the addition of nitrogen fertilizer (Eckert, 2007; Philippot and Germon, 2005)

Dinitrogen fixation, the biocatalytic conversion of gaseous nitrogen (N_2) to ammonium, is an exclusive property of prokaryotes. The enzymes which are responsible for this reaction are nitrogenases (Martinez-Romero, 2006). Nitrogen fixation is the most important way for N_2 to enter biological systems and therefore a key step in nitrogen cycle. The nitrogen cycle begins with the conversion of inorganic atmospheric nitrogen (N_2) into organic compounds, which makes up 78 percent of the atmospheric gases, into compounds containing ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-). The reaction is mediated by an oxygen sensitive enzyme nitrogenase and requires 16 moles of ATP as energy sources for each mole of N_2 reduced (Giller, 2001). However, it is metabolically unavailable directly to higher plants or animals. It is available to some microorganisms through biological nitrogen fixation (BNF) (Saikia and Jain, 2007).

The equation below concludes the nitrogen fixation process:

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

Chemically, BNF is essential for the conversion of dinitrogen into ammonia which is catalysed by nitrogenase enzyme. The nitrogenase enzyme consists of two components: the dinitrogenase protein (MoFe protein, NifDK), which contains a molybdenum-iron cofactor, is the site of N_2 reduction; the dinitrogenase reductase

protein (Fe protein, NifH) transfers electrons from an electron donor to the nitrogenase protein (Dalton and Kramer, 2006).

The process of N_2 fixation significantly impacts the environment, and occurs in various ways. Terrestrial N_2 fixation (e.g., natural mechanisms of fixation) were estimated to be 90–130 Tg N per year in 1995 (Galloway *et al*., 1995; Karl *et al*., 2002). However, the total rate of N_2 fixation at this same time period was estimated to be more than twice this pre-industrial rate due to legume cultivation, fertilizer production and energy demands of society. Moreover, the increased N deposition resulting from the higher N inputs in the environment, is believed to be responsible for the loss of biodiversity and plant extinction in ecosystems (Steven *et al.,* 2004).

However, biological N_2 fixation is still the main source of nitrogen in soil and marine environment where the dissolved fixed nitrogen content is extremely low (Staal *et al.,* 2003). Meanwhile, associated plant bacteria are able to provide larger amount of nitrogen to plants compared to free-living diazotrophs (Saikia and Jain, 2007). The interaction which includes the supply of energy by the host plants to the associated bacteria will lead to a significant impact on agriculture. The end products (ammonia) are then metabolized by the plant to meet its nutritional nitrogen needs for the synthesis of proteins, enzymes, nucleic acid and chlorophyll (Saikia and Jain, 2007).

2.4.1 Acetylene Reduction Assay (ARA)

The methods used to measure biological N_2 fixation include the quantification of total nitrogen differences based on Kjeldahl analysis, acetylene reduction assay and ^{15}N incorporation or dilution. Acetylene reduction assay has been used for over 30 years to

measure nitrogenase enzyme activity and as an indicator of N_2 fixation process (Hardy *et al.,* 1968). Nitrogenase enzyme may reduce other substrates which also contains triple bonds such as acetylene gas (C_2H_2), N_2O, cyanide, methyl isocyanide, azide, cyclopropene and diazirine. The reduction of these substrates has been the basis for acetylene reduction assay, which indirectly measures nitrogenase enzyme activity (Giller, 2001; Martinez-Romero, 2006). Among the potential substrates, acetylene is the most useful for analyzing nitrogenase activity.

Dilworth (1996) and Schollhorn and Burris (1966) discovered that acetylene (C_2H_2) is reduced to ethylene (C_2H_4). These findings introduce a quantitave method that has several advantages for N_2 fixation studies. The method consists of supplying 10% acetylene to the atmosphere of the nitrogen-fixing system, followed by certain incubation period. The product of the conversion of ARA from the incubation is then measured by gas chromatography (Azam and Farooq, 2003). The nitrogenase activity is directly expressed as μmole C_2H_4 produced $plant^{-1}$ h^{-1}.

A number of symbiotic and non-symbiotic, endophytic and non-endophytic bacteria have the ability to fix N_2. However, there are two limitations of effective biological N_2 fixation activities. Firstly, it is related to the required sources of energy to break the nitrogen triple bond, N≡N. Thus, only some organisms with highly developed catalytic systems are able to fix the nitrogen. The second limitation is related to the obligatory anaerobic conditions required for N_2 fixation reductive process. In contrast, any nitrogenase activity under aerobic conditions is inhibited by high concentration of oxygen (Magalhaes *et al*., 1978). Accordingly, only those bacteria that live in an

anaerobic environment or those can create this environment will fix the nitrogen. Nevertheless, some bacterial have the ability to protect the nitrogenase enzyme from high oxygen concentration. *H. frisingense* sp. nov is able to reduce acetylene to ethylene with a mean ratio of 130 ηmol C_2H_4 h^{-1} per 10^8 cells at 30°C (Kirchhof *et al*., 2001). Han and New (1998) report that *A. lipoferum* exhibits greater nitrogenase activity than free living *A. brasilense* (in Nfb medium) and in association with wheat roots at 79.9 ηmole C_2H_4 mg^{-1} protein h^{-1}, and 56.0 ηmole C_2H_4 mg^{-1} protein h^{-1}, respectively.

The acetylene reduction assay can determine the N_2 fixing capacity of free living and associative diazotrophs. Results of the acetylene reduction assay by maize-inoculated either alone or with plant hormone (2,4-D) show that carbon substrates and oxygen pressure enhance the nitrogenase activity (ARA). Higher ARA activity is recorded for plants treated with hormone (2,4-D) and inoculated with *Azospirillum*, in a range of 300 to 800 ηmole C_2H_4 h^{-1} g^{-1} dry weight of roots (Saikia and Jain, 2007). Wild rice *Oryza officinalis* inoculated with a homologous *Herbaspirillum* isolate reveales higher ARA activity and $^{15}N_2$ reduction activities (Elbeltagy *et al*., 2001).

2.5 Plant Growth Promotion by Diazotrophs in Association with Non-Leguminous Crops

Azospirillum is one of the most extensively studied genera of diazotrophs. Detail studies on the N_2 fixation activities and plant growth promoting effects of non-leguminous crops have been well documented. The mechanism of plant growth promotion effects goes beyond nitrogen fixation, to include nitrate reduction,

phytohormone production and the enhancement of nutrient uptake by host plants in response to root elongation. The relative contribution of each process is influenced by soil environmental parameters, plant and bacterial growth phases and bacterial interactions (Dalton and Kramer, 2006; Bashan and Holguin, 1998; Okon and Itzigsohn, 1995; Okon and Labandera-Gonzalez, 1994). *Azospirillum* is essentially ubiquitous in tropical ecosystems and is associated with roots of sugarcane, corn, kallar grass and many others. It is found at a density as high as 10^5-10^7 of bacteria per gram soil or root in the tropics. *Azospirillum* has been described as facultative endophyte, which is capable of inhabiting living plant tissues (Dobereiner *et al.,* 1995). It is presumed to enter the plant through cortical wounds and cracks although the entrance into plant tissues may be facilitated by other enzyme activities (pectinolytic) as well (Faure *et al.,* 2001; Ramos *et al.,* 2002). The results of inoculation trials reveal a significant increase in yields up to 60% of agronomically important crops such as pearl millet and wheat (Dalton and Kramer, 2006; Baldani *et al.,* 1983; Dobbelaere *et al.,* 2001).

At a nursery stage experiment of oil palm, it is successfully proven the positive effects of *Azospirillum* inoculation in terms of N_2 fixation and growth enhancement of the host plants. By using isotopic method of ^{15}N%, the nitrogen, derived from the fixation of *Azospirillum* in association with the host plants, is recorded at 40.7%. This association also influences growth and increased the photosynthetic activities of the host plants (Amir *et al.,* 2003). Similar benefits of the diazotroph (locally isolated R12) in association with tissue-cultured oil palm have been shown under *in vitro* conditions. The inoculum influences better shoot growth and highly induced root development of host plant which allows the colonization of R12 compared to the control. The plant-

bacterial association also enhances higher root fresh weight, chlorophyll content and a number of primary and secondary roots of oil palm plantlets (Azlin *et al.,* 2007; 2009).

The association of sugarcane and *Gluconacetobacter* has probably attracted most attention on N_2 fixation activity. This bacterium, originally called *Acetobacter* (now *Gluconacetobacter*) *diazotrophicus*, has a number of features that make it well-suited for its role as a plant-associated diazotrophs. It can use sucrose, which is abundant in sugarcane stem as its sole carbon sources. This bacterium is an aerobe, but is capable of fixing N_2 under microaerobic conditions. Besides, NO_3^- and NH_4^- show little or no inhibition of nitrogenase enzyme activity, thus allowing the bacteria to potentially remain unending source that leaks out fixed N which is not subjected to any inherent down regulation (Dalton and Kramer, 2006).

In the earlier findings, plant associated diazotrophs are assumed to be located primarily in the rhizosphere. In contrast, *Gluconacetobacter* is present in large numbers (up to 10^7 cfu g^{-1} fresh weight) within the root and becomes established in xylem vessels (James *et al.,* 1994). In addition to *Gluconacetobacter,* several other endophyts including *H. seropedicae, H. rubrisubalbicans* and *Burkholderia* sp. have also been discovered within the plant (Boddey *et al.,* 2003). The complexity of BNF in sugarcane is further evidenced by the range of diazotrophic bacteria associated with the host plants which include species of *Azosprillum, Beijerinckia, Azotobacter, Enterobacter, Bacillus* and *Pantoea* (Boddey *et al.,* 2003; Loiret *et al.,* 2004; del Amor *et al.,* 2008).

Substantially, more rhizobacteria are present near the root surfaces than in the bulk soil. This rhizosphere effect is caused by the release of exudates from growing root

tissues and the lysis of cells of older root parts. Bacteria rapidly colonizes growing root tips, which contain more mineral nutrients, simple sugars, organic and amino acids. However, saprophytic fungi is more prevalent on older root parts, where cortical cells being degraded (Lynch and Whipps, 1991). The diazotrophs such as *A. diazotrophicus, Herbaspirillum* spp. and *Azospirillum* spp. are capable of colonizing wheat exterior and interior of sugarcane, rice and oil palm plant (Elbeltagy *et al.,* 2001; Amir *et al.,* 2001; Sevilla *et al.,* 2001; Reis *et al.,* 2000). Recent study has shown that cells of *A. lipoferum* and *A. brasilense* are aggregated to each other and anchored to the root surfaces of primary and secondary roots of oil palm. Furthermore, interior colonization of *A. brasilense* is observed inside the corticle cells of the primary roots. The establishment of these bacteria inoculum associated to host plant could enhance plant growth and N_2 fixation activities (Amir and Lim, 2004; Azlin *et al.,* 2007; 2009).

2.6 Effects of Diazotrophs in Combination with Different Concentrations of N on N_2 Fixation Activities and Plant Growth Enhancement

Biological N_2 fixation by associative diazotrophs is a spontaneous process. However, it could be inhibited due to abundant supply of inorganic N fertilizer. Fuentes-Ramirez *et al.* (1999) found that high supply of N-fertilizer at 11 mM of NH_4NO_3 per plant for every two weeks has reduced the population of *G. diazotrophicus*. The population inhibition of endophytic diazotrophs is also reported in sugarcane fertilized with 300 kg N ha^{-1} (Reis *et al.,* 2000). The association also fails to show any nitrogenase enzyme activity (Reis *et al.,* 2000). The inhibition of *G. diazotrophicus* proliferation by N is particularly associated with NH^+_4 nutrition and has

resulted in morphological changes of the bacteria (Muthukumarasamy *et al.,* 2002). Thus, nitrogen metabolism, especially the molecular organization and regulation of nitrogen fixation bacterium should be studied in order to understand the nature of the process (Cheng, 2008; Schmid *et al.,* 2006).

The structural organization and regulation of nitrogen fixation genes are well studied (Pedrosa *et al.,* 2001). *H. seropedicae* fixes N_2 under microaerobic conditions and is tightly regulated by nitrogen compounds both at the level of synthesis and activity. In addition, ammonium causes a rapid and reversible switch off of nitrogenase activity in *H. seropedicae*, *A.brasilense* and *A. lipoferum* (Hartmann *et al.,* 1986; Burris, 1989). Application of nitrogenous compounds such as ammonium or glutamine as sole nitrogen and carbon sources of diazotrophs will completely inhibit N_2 fixation activity through the appearance of a modified dinitrogenase reductase enzyme (switch off). Once the ammonium is used up, the nitrogenase activity is regained and the modified subunit disappeares (switch on) (Elmerich *et al.,* 1992; Hill *et al.*, 1992). However, at 1 to 2 mM of glutamate supply, the cells are still fixing nitrogen even at a reduced rate. The central regulator of nitrogen control is a NifA protein, the *nif*-specific transcriptional activator in response to the levels of fixed nitrogen and oxygen (Souza *et al.,* 1999). In addition, general nitrogen fixing control of the cell is regulated by NtrC, which also controls the expression of the *glnA* gene coding for glutamine synthetase (Cheng, 2008; Souza *et al.,* 2000). Glutamine synthase (GS) is the key enzyme of the high affinity ammonium assimilation pathway. In contrast, γ-*Proteobacteria* such as *K. pneumonia* and *A. vinelandii*, contain NiFL proteins which form an inactivated

complex with NifA protein in the presence of high level of ammonium and oxygen. The NifA-protein is directly inactivated in reponse to the increased level of nitrogen and oxygen in *H. seropedicae* and *A. brasilense* (Schmid *et al.,* 2006).

Nitrogen fertilizer is the most expensive nutrient input that is highly required in economic crops. Nitrogen is essential for better growth and development of plants. Biological N_2 fixation process provides an opportunity to reduce application of synthetic nitrogenous fertilizer, save cost and potentially increase crop production (Cocking, 2000). Furthermore, utilization of diazotrophs as biofertilizer can be considered as a key factor in the development of environmental friendly agricultural system (Reis *et al.,* 2000). However, BNF process alone could not completely fulfill the N requirements of crop plants. Therefore, N fertilizer as a starter N is important to meet early N needs of the crops. However, the rate and formulation of the starter N fertilizer should never exceed from the total amount recommended (Durst and Beegle, 1999). Thus, it is suggested that low N inputs to the Brazilian sugarcane industry might have promoted efficient biological nitrogen fixation in supplying N for the agricultural system (Reis *et al.,* 2000; Medeiros *et al.,* 2006). This may also be true for South African sugarcane industry cultivated by small-scale growers which applies little or no N fertilizer (Hoefsloot *et al.,* 2005).

For optimum plant growth, sufficient and balanced quantity of nutrients must be available. Soil contains natural reserves of plant nutrients, but these reserves are largely in unavailable forms to plants as only a minor portion is released each year through biological activity or chemical processes (Chen, 2008). This release is too slow to

compensate for the removal of nutrients by agricultural production and meet crop requirements. Therefore, fertilizers are designed to supplement the nutrients already available in soil. In addition, microbial inoculants can be used as an economic input to increase crop productivity where inorganic fertilizer doses can be lowered and more nutrients can be harvested from the soil, apart from gaining higher positive effect on microbial biomass (Dutta *et al.,* 2003; Chen, 2008).

Many researchers have demonstrated the beneficial effects of the combined use of chemical fertilizer and microbial inoculants to mitigate the deficiencies. Yanni *et al.* (1997) use one third of the recommended dose of N fertilization in a rice field to produce equivalent grain yield as obtained by the full-recommended dose of fertilizer (144 kg N ha−1). The effects of combined treatment of biofertilizer (a mixture of *B. subtilis, B. pumilis, B. erythropolis*) with 50% chemical fertilizer are observed on growth of lettuce (Young *et al.,* 2003). The results show that there is an increase of 25% yield for the treatment compared to the controlled supplied with full amount of chemical fertilizer. The results indicate that, at least 50% of chemical fertilizer can be saved as multifunctional biofertilizer is used along with chemical fertilizer.

Earlier findings show that by inoculating sugarcane with local isolates of *G. diazotrophicus* and supplied with low N input (75 kg ha^{-1}) has improved plant growth and enhanced diazotrophs population (Suman *et al.,* 2005). In addition, Suman *et al.* (2008) report that the population of *G. diazotrophicus* in the plant tissues is higher at N_{75} than at N_0. However, at N_{50}, the population is much lower than N_{75}. In contrast, it is different for *H. seropedicae*where the population is gradually increased with the

increasing amount of N input. Fuentes-Ramirez *et al.* (1993) also suggest that the population of *H. seropedicae* is not affected by chemical N even up to 300 kg N ha^{-1}. In other findings, *H. seropedicae* has promoted higher biomass of grasses such as *Spartina pectinata, Pennisetum purpureum* and *Mischanthus* spp. when combined with relatively low nitrogen requirements. Low N input provides more sites for bacterial colonization and its entry through cracks developed on the tissues of the host plant. Thus, this study suggests that N input will influence the BNF activity and population size of diazotrophs.

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1 Medium Preparation

3.1.1 Nitrogen free medium

The bacteria were cultured in N-free medium (Eskew *et al*., 1977). The yeast extract was supplied to the medium as a growth starter and did not affect the ability of the diazotrophs to fix N_2. The chemicals were weighed and stirred (**Table 3.1**). The pH of the medium was at 7.2 ± 0.2 by using pH meter (Mettler Toledo 320) prior to autoclave at 15 psi at 121°C for 15 minutes.

Table 3.1: Chemicals for nitrogen free medium (Eskew *et al.,* 1977)

Chemicals	**g L^{-1}**
KH_2PO_4	0.4
K_2HPO_4	0.1
$MgSO_4$	0.2
NaCl	0.1
$CaCl_2$	0.02
$FeCl_3$	0.01
Na_2MoO_4	0.002
Malic Acid	5.0
Yeast extract	0.05
pH	7.2 ± 0.2