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The Effectiveness of Biological Nitrogen Fixation Bacteria on the Growth of oil Palm Seedlings.

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Abstract: *The potential of biological nitrogen fixation (BNF) bacteria in converting atmospheric nitrogen into usable form for plants has been studied in the nursery to meet the nitrogen requirement in oil palm seedlings. In this interaction, the bacteria provide the host plants with fixed nitrogen. A study was conducted to determine the effectiveness of selected BNF bacteria on the growth of oil palm seedlings. A total of 30ml of 48-hour old isolates coded as M3-12, M3-13 and M3-14 with concentration of 10^{12} cfuml^{-1} was drenched separately onto 4-week old oil palm seedlings. The height, stem girth and chlorophyll value of the seedlings were measured at monthly interval for 6 months. The dry matter yield of the roots and shoots was weighted, and the colonization of bacteria in the roots were observed at the end of the study. Results showed that oil palm seedlings grown with BNF bacteria are significantly taller, bigger in girth size and have higher chlorophyll value compared to control seedlings. The dry weight of shoots and roots of the inoculated seedlings were also significantly higher than control. The cross section images of inoculated roots showed massive colonization of BNF bacteria within the root tissues showing the sign of endophyte characteristics.*

Keywords*: Biological Nitrogen Fixation, Endophytic bacteria*

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I. Introduction

Inorganic N fertilizer is the most widely used input in agriculture and it is a major factor that limits crop growth and yield (Zakry, 2008). Based on recommendations by Corley and Tinker (2003), a total of 1.0 kg N from Urea is required to fertilize a mature oil palm per year. With an average price of urea (46% N) at RM1475/tonne, the cost of N input to fertilize one hectare plantation with capacity of 148 palms would be RM475. According to Tarmizi & Mohd Tayeb (2006) a total of 4.2 kg ammonium sulphate is required to meet the demands for one oil palm stand per year. It means the total cost for N input per hectare of oil palm plantation is RM621. Over the years, the requirement of these N inputs has increased due to many reasons.One of main factor is leaching. Nutrient losses through leaching are generally higher in humid than dry climate regions and it can greatly increase in area with agricultural activities(Havlin *et al*., 1999). Rapid losses of N through leaching would further increase the usage of N fertilizer in the plantation. With a relatively high market price of inorganic N input, the operational cost for oil palm plantation would continuously increase in the future.

Environmental pollution due to excessive application of inorganic N in agriculture also need to be addressed. Continuous application of N fertilizer in agricultural land may contribute to acidification which leads to nutrients leach and a decrease in crop growth. Leaching and runoff will lead to greenhouse effects, acid rain, and contamination of ground water affecting water quality for biodiversity (Mark *et al*., 2011).

Therefore, there is a need to reduce the use of inorganic N in agriculture. The use of biological nitrogen fixation (BNF) bacteria can be exploited as one of the potential alternatives to reduce the use of inorganic fertilizers on agricultural crops. The bacteria benefit the plants through nitrogen fixation activities. Atmospheric nitrogen cannot be directly absorbed by plants without being assimilated by the available nitrogen fixing bacteria in the rhizosphere (Santi *et al*., 2012). Nitrogen (N_2)that is naturally present in the atmosphere is first converted by the bacteria into ammonia (NH_3) and then nitrates (NO_3). The final form of N contains organic compounds that can be easily absorbed by the plants. The process is catalysed by oxygen-sensitive enzyme known as nitrogenase that is primarily produced by the BNF bacteria (Bhattacharjee *et al*., 2008). It has been proven in many studies that the introduction of BNF bacteria has successfully promote root induction and morphological growth of plants (Baset Mia *et al.*, 2010; Azlin *et al.*, 2009; Azlin *et al*., 2007; Amir *et al.*, 2001; Blomme, 2000).

Therefore, this study was conducted with the aim to determine the effectiveness of biological nitrogen fixation bacteria on growth of oil palm seedlings.

II. Material and methods

2.1 Screening for Biological Nitrogen Fixation (BNF) Bacteria

Eight isolates of nitrogen fixation bacteria (M3-12, M3-13, M3-14, M3-15, M3-16, M3-17, M2-12, and M2-14) were screened for theirability to fix atmospheric nitrogen via *in-vitro* technique. The isolateswere previously isolated from healthy oil palm roots and maintained in Microbial Culture Collection of Beneficial Microbes Laboratory, FGV R&D Sdn Bhd, Malaysia. Each isolate was cultured on nutrient agar (NA) and incubated for 24 hours. One loop of the 24-hour old culture was inoculated into N-free broth (composition for 1L broth: 10g glucose, 0.41g KH_2PO_4, 0.52g K_2HPO_4, 0.05g Na_2SO_4, 0.2g $CaCl_2$, 0.1g $MgSO_4.H_2O$, 0.005g $FeSO_4.H_2O$, 0.0025g $Na_2MoO_4.H_2O$). All flasks were covered with sponge for aeration priorshake at 150 rpm for 48 hours. The level of nitrogen content from each flask was determined after 48 hours. The analysis used to quantify the level of fixed nitrogen was based on Kjeldahl in house method (AOAC Official Method 981.10)conducted byUnipeq Sdn Bhd, Malaysia. Isolates that fixed the highest content of nitrogen were selected to be tested in the nursery trial.

2.2 Preparation of Planting Materials

A total of 320 oil palm germinated seeds from variety Dura × Pisifera (D X P Yangambi) were used in this study. The germinated seeds were obtained from FGV Agri Services Sdn Bhd, Pusat Penyelidikan Pertanian Tun Razak, Jengka Pahang, Malaysia. The seeds then were planted in plug tray filled with peat moss (Base Subtrate; Klasmann) as planting medium (**Table 1**) and watered regularly.

Table 1: Composition of Peat Moss Medium-Base Substrate 2 Medium Basic (Klasmann)

Composition	White peat
pH (H_2O)	6.0
Fertilization (g/l)	None
Extra Trace Elements	Included
Wetting Agent	Hydro S
Structure	Medium

2.3 Preparation of BNF Bacteria Inoculum for Nursery Trial

The BNF isolates (M3-12, M3-13 and M3-14) that fixed high level of nitrogen content through*in-vitro* technique were sub-cultured onto NA and incubated at 28°C±2 for 24 hours. Then, one loop of the 24-hour old cultures was transferred into 100 mL Nutrient broth (NB) and shaken at 150 rpm for 48 hours. The final concentration of each isolate was adjusted to 10^{12}CFUmL^{-1}.

2.4 Inoculation of BNF Bacteria into Oil Palm Seedlings

The oil palm seedlings were inoculated with BNF bacteria for the first time at the age of four (4) weeks old. 30 mL bacterial suspension with concentration of 10^{12} CFUmL^{-1} was drenched into every seedling. The oil palm seedlings were raised in the nursery until they reached three (3) months old before ready to be transplanted into main nursery polybag. Second inoculation was carried out right after the seedlings were transplanted into main nursery. Another 30 mL of BNF bacterial suspension with similarconcentration was drenched into every seedling. Sterilized distilled water (SDW) was used as control.

2.5 Effect of BNF on Plant Vigour

The height, girth and chlorophyll value of all seedlings were measured every week during the first two (2) months in the pre-nursery. Then, the parameters were continually measured at monthly intervals for six (6) months. Chlorophyll value was measured by using SPAD meter (SPAD- 502 Minolta). Six months after transplanted into the main nursery, all seedlings were destructed to determine the dry weight of roots and shoots of the seedlings.

2.6 Preparation of Roots Sample for Field Emission Scanning Electron Microscopy (FESEM)

During the destructive sampling, one of each inoculated and un-inoculated seedlings were randomly selected to undergo FESEM to observe the colonization of introduced BNF bacteria within the root tissues. The process was conducted as described by Puentea *et al*., (2009) with some modifications. The root samples were cross sectioned and cut into 0.5 to 1.5 cm long before leaving itovernight in 1% (v/v) glutaraldehyde. The roots were then rinsed with phosphate buffer and dehydrated for 20 minutes in the respective ethanol concentrations; 30%, 40%, 50%, 60%, 70%, 80%, 90 and 100%. The samples were then dried and coated with gold before viewing under field emission scanning electron microscope.

2.7 Identification of BNF bacteria

Identification of isolates M3-12, M3-13 and M3-14 were conducted by scraping their colonies from the agar media surface and lysed in cell lysis buffer solution (1% SDS, 0.4M NaCl, 0.4M EDTA, 0.5M Tris-HCL; pH 8.0, 10 µg mL^{-1} of proteinase K). DNA was isolated from lysate with standard 25:24:1 phenol-chloroform-isoamyl alcohol method and was precipitated with isopropanol and then washed with 70% ethanol. DNA pellet was dissolved in SDW. Polymerase chain reaction (PCR) was carried out using TERRA PCR Direct Polymerase Mix (Takara, Japan) according to manufacturer's protocol to amplify DNA barcode marker. Forward primer 27F(5'-AGAGTTTGATCMTGGCTCAG-3')andreverseprimer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used to amplify all hypervariable regions of 16s rRNA gene. PCR fragment was purified with QIAquick PCR Purification kit (QIAGEN, Germany) and then ligated in pGEM-T Easy Vector System II (Promega) according to manufacturer's protocol and cloned in *E. coli*. Plasmid vectors carrying insert were extracted from *E. coli* using Wizard® Plus SV Minipreps DNA Purification System (Promega, USA) according to manufacturer's protocol. Sanger nucleotide sequencing was performed bi-directionally inward from the two ends of the vectors's backbone flanking the insert using specific universal primers at First Base Laboratories Sdn Bhd (Seri Kembangan, Selangor). Nucleotide sequence was analyzed at SIRIM and then searched against Genbank database of the National Center of Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) programme (Altschul *et al*.,1990). Species identification was determined according to the following acceptance criteria: highest 'Max Score', 'Query cover' ≥98% and 'Identity' ≥95%. Additional isolates from other sources were obtained from homologous sequences from Genbank for comparison. A phylogenetic tree was generated through neighbour-joining algorithm with supported by bootstrap analysis using 1000 replication by MEGA 6.0 (Hall, 2013).

2.8 Statistical analysis

The study was conducted in randomized completely block design (RCBD) with four replications, whereby each replicate consisting 20 oil palm seedlings. All the data were analyzed by ANOVA using SAS version 9.3. The means were compared using Tukey (H≤0.05).

III. Result

3.1 Total Nitrogen Content

Total Nitrogen Content of eight (8) BNF bacteria isolates (M3-12, M3-13, M3-14, M3-15, M3-16, M3-17, M2-12, and M2-14) was determined by grown in N-free broth medium. After 48 hours, results shown that isolate M3-12 has fixed the highest amount of nitrogen at 160.0 mg L^{-1}, followed by M3-13 at 46.7 mg L^{-1} and M3-14 at 40.0 mg L^{-1} (**Figure 1**). M3-16 produced the least amount of nitrogen at 5 mg $L^{-1.}$ Therefore, the best three isolates M3-12, M3-13 and M3-14 were selected to be tested in the nursery trial.

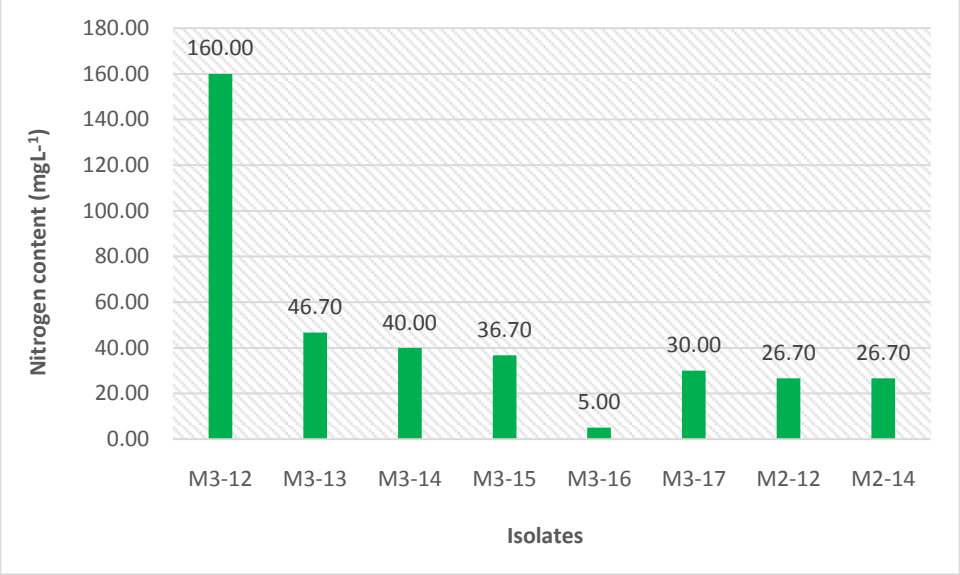

Figure 1. Total Nitrogen Content fixed by biological nitrogen fixation (BNF) bacteria after 48 hours fermented in N-free broth media.

3.2 Effect of BNF on Plant Vigour

Eight (8) weeks after the first inoculation, seedlings which were inoculated with BNF bacteria generally have better growth performance as compared to un-inoculated seedlings (**Table 2).** The height of seedlings inoculated with isolates M3-12, M3-13 and M3-14 were 12.6cm, 12.6cm and 12.3cm respectively while control is only 11.3cm. The girth size of inoculated seedlings was also bigger at 0.67cm (M3-12), 0.66cm (M3-13), and 0.63cm (M3-14) respectively than control at 0.61cm. Inoculated seedlings with BNF bacteria also show chlorophyll values that significantly higher compared to the control (**Figure 2**).

Table 2: Plant vigour assessment oftreated oil palm seedlings with BNF bacteria, 8 weeks after the first inoculation.

Treatment	Plant height (cm)	Stem girth (cm)	Chlorophyll value (Spad Meter)
M3-12	12.60 a	0.67 a	60.20 a
M3-13	12.60 a	0.66 a	58.40 a
M3-14	12.30 a	0.63 b	58.00 a
Control (No bacteria)	11.30 b	0.61 c	35.40 b

Means with the same letters between treatments are not significantly different according to the least significant different (Tukey) test at H≤0.05

Figure *2*. *Oil palm seedlings treated with three BNF bacteria (M3-12, M3-13 and M3-14) have greener pigment as compared* to control seedlings.

During transplanting the oil palm seedling (3 months old) to the main nursery, booster inoculation was applied by drenching 30 mL of BNF bacterial suspension at concentration of 10^{12} CFU mL^{-1}. Chlorophyll value, height and girth of the stem were recorded a month later. As expected, the chlorophyll value of the inoculated seedlings was significantly higher than control seedlings at month one and two (M1 and M2) after the booster inoculation.However, there was no significant difference in chlorophyll value at month 3 after booster inoculation compared to the control. Results also show that the height and girth size of the inoculated seedlings are significantly higher than control seedlings towards the end of the trial (**Table 3**). It shows that the introduced bacteria has successfully stimulates the growth of the seedlings.

Table 3: Plant vigour assessment of oil palm seedlings after booster inoculation.

Treatment/Isolate	Month after booster inoculation								
	Chlorophyll value (Spad Meter)			Plant height (cm)			Stem girth (cm)		
	M1	M2	M3	M1	M2	M3	M1	M2	M3
M3-12	48.6a	60.5a	62.7a	31.9a	46.8ab	60.3a	1.12a	2.07a	2.88a
M3-13	48.2a	60.0a	62.1a	31.5a	47.3a	60.7a	1.09a	2.13a	2.86a
M3-14	47.9a	59.5ab	62.6a	30.8a	45.1b	57.5b	1.01b	2.04a	2.79a
Control	38.8b	57.3b	62.2a	23.7b	38.9c	51.2c	0.89c	1.68b	2.45b

Means with the same letters between treatments are not significantly different according to the least significant different (Tukey) test at $p \leq 0.05$

All seedlings were destroyed at the age of 6-months old to determine the dry weight of shoots and roots of the seedlings. Prior to that, some roots of both inoculated and control seedlings were sampled and cross sectioned for viewing under scanning electron microscope. **Figure 3** shows the colonization of BNF bacteria in the inoculated oil palm roots (a) while (b) shows no bacterial colonization within root tissues of control seedling.

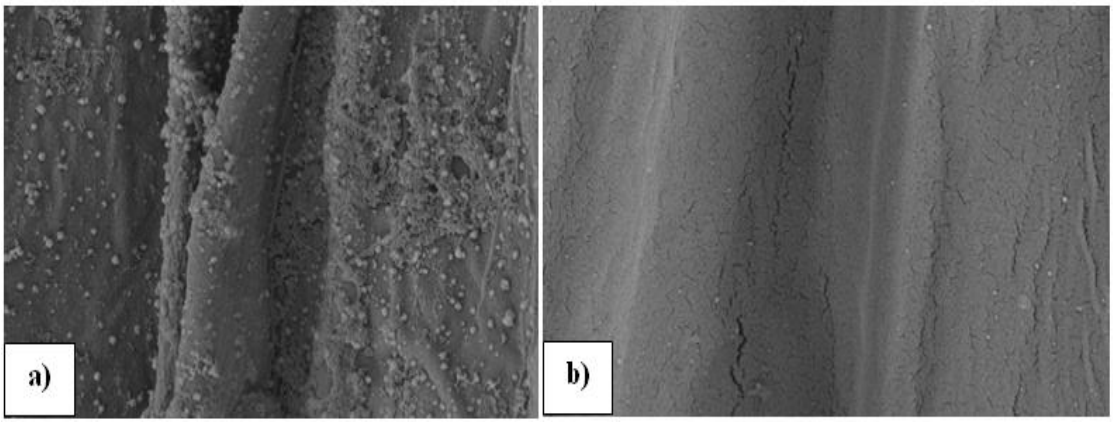

Figure 3. FESEM pictograph of oil palm root's crossed sectioned (a) inoculated oil palm seedling which has colonized by endophytic BNF bacteria and (b) control.

These advantages make endophytic BNF bacteria very beneficial to the oil palm seedlings by directly providing the fixed nitrogen to them. This will subsequently increase the uptake of nutrients by the seedlings and increasing the dry matter yield. **Figure 4** shows that generally, the dry weight of shoots and roots of inoculated seedlings are higher than un-inoculated seedlings. The highest dry weight for shoots was recorded on seedlings which were previously inoculated with isolate M3-12 at 29.4g. It was followed by seedlings which were inoculated with isolates M3-14 and M3-13 with average shoots' dry weight at 28.8g and 28.4g respectively. Similar pattern of result was obtained from the roots' dry weight. No significant difference on shoots and roots dry weight among the inoculated seedlings showing the same ability of all isolates to promote growth.

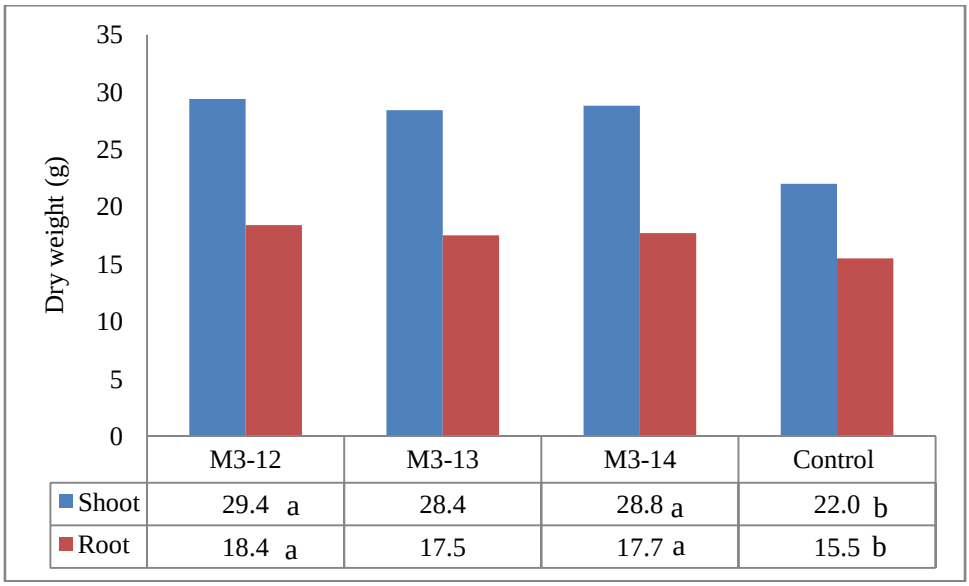

Figure 4. Dry weight of shoots and roots of inoculated and un-inoculated 6-month old oil palm seedlings.

3.3 Identification of BNF bacteria

Three isolates of BNF that produce high nitrogen content were identified through 16S rRNA gene sequence data. The isolates were identified *as Paenibacillus alvei*, *Brevundimonas diminuta* and *Ochrobacterium pseudogrignonense* at 99% similarity with NCBI genebank accession number given in the **Table 4**.

Table 4: 16S gene sequence analysis

Strain	BNF Bacteria	NCBI database match	Percentage of identity (%)
M3-12	*Paenibacillus alvei*	*Paenibacillus alvei* strain AUG 6 (AB377108)	99
M3-13	*Brevundimonas diminuta*	*Brevundimonas diminuta* strain zjs01 (DQ857897)	99
M3-14	*Ochrobacterium pseudogrignonense*	*Ochrobacterium pseudogrignonense* (CP015776)	99

The phylogenetic studies of 16S rRNA gene sequence data of our isolates and their homologous sequences were performed. The generated NJ-based phylogenetic tree shows that BNF bacteria are clustered together according to their species; *Paenibacillus alvei*, *Ochrobactrum pseudogrignonense*and *Brevundimonas diminuta*(**Figure 5**).The first two clades comprise of five BNF genes, while the third clade has two BNF genes that make up their own group of species. The phylogenetic tree reveals a high reliability of closed relationship among the same species of BNF 16s rRNA genes though the support of their high bootstrap value.

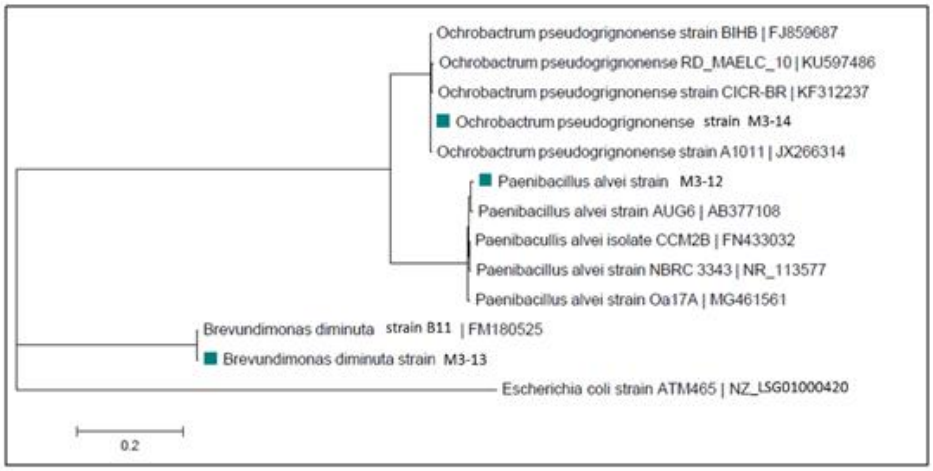

Figure 5: Phylogenetic tree for 16s rRNA of BNF bacteria generated using Neighbour-Joining (NJ) method. The numbers in branches indicate the bootstrap percentage values for 1000 replicates.

IV. Discussion

In this trial, the pre nursery seedlings which were introduced with BNF bacteria shows greener pigment than control seedlings which validate the results of chlorophyll values shows in Table 1. The inoculated seedlings also grown taller and bigger than control. It shows that the BNF bacteria has successfully benefits the seedlings by providingconverted atmospheric nitrogen that can be absorb by the plants. It is proven when the seedlings were solely depend on the bacteria for the nitrogen source as no other nitrogen input was applied. High chlorophyll valuemeasuredon the inoculated seedlings shows the good sign of plant health and it is closely related to photosynthesis activity and N status of the leaves (Ling *et al*., 2011, Sim *et al*., 2015). Lack of nitrogen in leaves can be identified through loss of green pigment as been observed in control seedlings where finally reduce their ability to photosynthesize (Bojovic and Marković, 2009).

As the seedlings enter the main nursery, they were boosted with second inoculation. Based on their height and size of girth, they have better growth performance compared to control seedlings although at the final assessment the chlorophyll value was no significantly different to the control seedlings. Seedlings which were introduced with BNF bacteria were also have significantly higher dry weight for both roots and shoots compared to control seedlings. At the end of the trial, the inoculated seedlings are 10% to 15% higher than the control seedlings and the girth size is 12% to 14% bigger.

All three BNF isolates have shown the same ability to promote the growth of oil palm seedlings.The FESEM pictograph of oil palm root's crossed sectioned also proves that the introduced BNF bacteria were endophytes inhabiting plant organs and established within the internal plant tissues without causing apparent harm to the host and cause any pathogenic symptoms (Baldani *et al*., 1997). The use of endophytic BNF bacteria to fix nitrogen for plant use has greater advantages than using non-endophytes. First, there will be less competition for nutrient within the roots as compared to rhizosphere that is rich with indigenous soil microbes (Gupta *et al*., 2012). Secondly, closed environment within the root system hindered the endophytes against high level of oxygen (O_2) concentration which may affect the production of nitrogenase enzyme which is very sensitive to oxygen (O_2) (Baldani *et al*., 2000). And thirdly, they are well protected from abiotic stresses such as extreme variations of temperature, pH, nutrient and water availability (Rosenblueth & Martinez-Romero, 2006). Therefore, the use of endophytes as plant growth enhancer would be an ideal solution as it can sustain their population in the host plants. This would be a good way to reduce the use of chemical fertilizer that harmful to the environment.

V. Conclusion

The use of endophytic BNF bacteria has successfully enhanced the growth of oil palm seedlings through nitrogen fixation activity. Therefore, this may be a sustainable approach to reduce the use of chemical fertilizer which is not only harmful to the environment but also to consumer. Further study to develop a suitable formulation for such bacteria need to be done so that it can be easily applied in the field and maintain viable.

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