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Influence of Various Combinations of Diazotrophs and Chemical N Fertilizer on Plant Growth and N_2 Fixation Capacity of Oil Palm Seedlings (*Elaeis guineensis* Jacq.)

O. Noor Ai'shah[1], H.G. Amir[1,*], Chan Lai Keng[1] and A.R. Othman[2]

[1]*School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia*
[2]*School of Distance Education, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia*

**Corresponding author. Email: amirhg@usm.my*

Abstract

The Biological Nitrogen Fixation (BNF) process, which involves diazotrophic microorganisms, is an important agricultural practice for improving crop yield and environmental quality. Although diazotrophs have a positive interaction with the host plants and can reduce the need for N fertilizer, BNF alone cannot meet the N requirement for maximizing host plant yield. Thus, a combination of plant N_2 fixation and the response to small amounts of nitrogen starter should be considered. The study was conducted to observe the effects of diazotrophs on growth of oil palm seedlings and to determine influence of four strains of diazotrophs and different concentrations of nitrogen source on oil palm seedlings. The tested diazotrophs were *Herbaspirillum seropedicae* (Z78) and the locally isolated *Microbacterium* sp. (E7 and E14) and *Acetobacter* sp. (E9). The host plants were inoculated with the diazotroph strains, and also fertilized with different N fertilizer concentrations (0, 25, 50 and 100%). The seedlings were harvested after 360 days of growth and measured for plant dry weight, root density, nutrient accumulation and total leaf chlorophyll and protein content. Acetylene Reduction Assay (ARA) was performed to quantify nitrogenase activity of the diazotrophic microorganisms. The results successfully showed that strains Z78, E7 and E14 could potentially enhance growth of oil palm seedlings if supplied together with low levels of N fertilizer.

Keywords: diazotrophs, Biological N_2 Fixation (BNF), minimal amount of N fertilizer, plant growth

Introduction

As much as 30% of the fertilizer used for agricultural crops is nitrogen. Producing these fertilizers is energy intensive, and the applied product is quickly depleted (Kim and Rees, 1994). Reducing dependency on chemical fertilizers for agricultural production would reduce the cost and the negative impacts on the economy and the environment. One approach is to inoculate the agricultural crop with diazotrophic microorganisms that fix N_2 in association with the host plants. In this association, the diazotroph would provide the host with fixed N source (Muthukumarasamy et al., 2002; Lery et al., 2008). Co-inoculation of *Glucano-acetobacter diazotrophicus* with other diazotrophs was shown to give yields of sugarcane equivalent to yields obtained from normal plants with the addition of 275 kg N ha^{-1} (Muthukumarasamy et al., 2002, 2006).

However, to achieve the highest N_2 fixation capacity of the plant-microbe relationships requires minimal supply of N source as a starter for the diazotrophs to grow and establish. Furthermore, the diazotrophs may occur more frequently in soils and growth medium with reduced N source (Sevilla, 2001). Mia et al. (2007) showed that inoculation with strain Sp7 or locally isolated *Bacillus* sp.

UPMB10 together with 33% N fertilizer increased fruit yield and stimulated early flowering of banana plants due to efficient N_2 fixation and plant growth enhancing activities. Earlier findings by Sevilla (2001) also showed that sugarcane plants inoculated with wild type *G. diazotrophicus* and supplied with reduced chemical nitrogen fertilizer grew better and had a higher total N content than did plants inoculated with *nif-* mutants or uninoculated plants. In their study, the ARA activity of sugarcane inoculated with *G. diazotrophicus* and treated with 0 to 75 kg N ha^{-1} was higher than that of plants treated with 75 to 150 kg N ha^{-1}. Similarly, Tahir et al. (2009) reported higher nodule numbers in soybean treated with a combined treatment of *Rhizobium* inoculant with 25 kg N ha^{-1} and 90 kg P_2O_5 ha^{-1}. Moreover, they found that a substantial increase in nodulation had directly affected growth enhancement, seed production and nutrient uptake of the host plants due to higher N_2 fixation potential.

Thus, in this study the effects of diazotrophs and different concentrations of N on growth and development of oil palm seedlings were tested under glasshouse conditions. The significance of this study was that the inoculation combined with the addition of a minimal amount of starter N, may enhance growth of the host plant, indicating the environmental benefit of these diazotrophs with reduced N inputs. The objectives of the study were: 1) to observe the effects of a combination of diazotrophs and different levels of N on growth of oil palm seedlings, and 2) to determine the influence of different levels of N on N_2 fixation capacity of diazotrophs in association with oil palm seedlings.

Materials and Methods

Germinated oil palm seeds (DxP provided by Pusat Perkhidmatan Penyelidikan Pertanian Tun Razak PPPTR, Jengka, Malaysia) were planted in polybags (12.6 x 25 cm) containing 1 kg of planting medium (3:1, soil:sand). After four months of growth, the seedlings were transferred to larger polybags (21.4 x 27 cm) containing 8 kg of the same planting medium and maintained under glasshouse conditions for 360 days. The inoculation treatments were: 1) + *Herbaspirillum seropedicae* (Z78) (ATCC 35893), and locally isolated diazotrophs; 2) + *Microbacterium* sp. (E7); 3) + *Acetobacter* sp. (E9); 4) + *Microbacterium* sp. (E14); and 5) Control (uninoculated). Each of the inoculation treatments were combined with four different concentrations of N at 0, 25, 50 and 100%, respectively. The experiment was laid out in a Completely Randomized Design (CRD) with six replications for each treatment combination. The bacterial inoculants were grown on N-limited medium (Okon et al., 1977). The seedlings were inoculated with respective diazotrophs tested (10^7-10^8 cfu mL^{-1}) (10 mL inocula per seedling) at monthly intervals. The inoculum application rate was increased to 20 mL per seedling after four months of growth. During the first month of growth, a liquid fertilizer solution (1.1 µg mL^{-1} NH_4Cl and 0.46 g mL^{-1} KH_2PO_4 for P and K respectively) was applied to each polybag (10 mL bag^{-1}) based on basal fertilizer requirements for oil palm seedlings (NPK Yellow 15:15:6:4 for 7 g 100^{-1} seedlings) (Foo and Mat 1995). In the subsequent months, complete fertilization of the seedlings with N (urea), P (Triple Super Phosphate) and K (Muriate of Potash) supplied was equivalent to 7 g $palm^{-1}$ of NPK yellow 15:15:6:4 at month 3, 14 g $palm^{-1}$ (NPK yellow 15:15:6:4) at months 4-7, 21 g $palm^{-1}$ (NPK yellow 15:15:6:4) at months 8-10, and 28 g $palm^{-1}$ (NPK yellow 15:15:6:4) at months 11-12. Various concentrations of N at 0, 25, 50 and 100% were prepared through the calculation of total N proportions in full fertilization of seedlings by urea without reduction of P and K.

Growth Observation

Inoculated oil palm seedlings were monitored for plant height, number of leaves formed and total leaf chlorophyll and protein content. Nitrogenase enzyme activity using Acetylene Reduction Assay (ARA), nutrient accumulation (N, P, K, Ca and Mg) in plant tissue, plant dry weight, and root density were measured at the end of the experiment (D_{360}).

Total Leaf Chlorophyll and Protein Content

Leaf greenness of each seedling was recorded using a portable leaf-chlorophyll meter (MINOLTA^{TM} SPAD-502) (Neufeld et al., 2006). The youngest fully expanded leaves were selected /

marked to measure chlorophyll content at D_{360}. The actual leaf chlorophyll content was determined based on the standard curve of SPAD values and total leaf chlorophyll content of the seedlings (mg chlorophyll mg^{-1} leaf fresh weight) (Amir et al., 2001). Total leaf protein content was determined using Lowry's colorimetric assay (Lowry et al., 1951). Protein content of each sample was determined based on the protein assay standard curve (mg BSA mL^{-1} protein).

Acetylene Reduction Assay (ARA)

Root samples were incubated at room temperature in an airtight vessel (1050 mL) containing 600 mL of sterile distilled water for 6 hours, at which time water was drained and replaced with nitrogen gas N_2:O_2 (70:30) and the incubation continued overnight. The next day, the nitrogen gas was removed and replaced with 945 mL of Ar:O_2 (80:20) (90% volume of the vessels) and 105 mL of acetylene (99% purity) (10% volume of the vessel) (Abrantes et al., 1976; Baldani and Baldani, 2005). The samples were incubated for 6 hours, and then 0.5 mL of the gas mixture was sampled and assayed for ethylene. The assay was conducted with a G-3000 Hitachi gas chromatograph fitted with a Carboxen 1004 micro packed, 2 m x 1/16 inch (0.16 cm) stainless steel column and flame ionization detector (FID). Nitrogen was used as the carrier gas at a flow rate of 42 mL min^{-1}, with the temperature maintained at 165°C (column) and 230°C (injector and detector), respectively (Azlin et al., 2005).

Analysis of N, P, K, Ca and Mg Content in Plant Tissue and Soil pH

Total nitrogen (N) and phosphorus (P) concentrations in the plant tissues were measured with the Kjedahl method and the phosphomolybdate method, respectively (Rowell, 1994). Potassium (K), calcium (Ca) and magnesium (Mg) contents in the plant tissues were determined by using an atomic absorption spectrophotometer (Perkin Elmer A Analyst 100) (Rowell, 1994). At day harvest (D_{360}) the soil pH of each polybag was determined via suspending air-dried soil in distilled water and measuring pH after 15 minutes of shaking (Rowell, 1994).

Statistical Analysis

A generalized linear model (GLM) univariate analysis of variance was performed to test the significance of differences between means using Least Significant Difference (LSD) test, P<0.05. The statistical analysis software (SPSS V. 15) was used for data analysis of this experiment.

Results

Plant Growth Observation

Most of the inoculation treatments resulted in greater plant height (up to 31% greater compared to uninoculated +100% N) and leaf formation (up to 27% greater compared to uninoculated +100% N) than the uninoculated treatments at all concentrations of N supplied (0, 25, 50 and 100%). The seedlings inoculated with Z78, E7 and E9 (+25% N) exhibited the highest average plant height compared to combination of uninoculated treatments (Table 1). The addition of 25% N had the same effects on leaf formation as the addition of 100% N in most inoculation treatments (Table 1). Similarly, better plant growth observations were recorded compared to combinations of Z78, E7, E9 and uninoculated plants at 0% N. The interaction of inoculation treatments and N application was statistically significant with respect to plant height and number of leaves formed per seedling at the day of harvest (P<0.05) (Table 1).

Results of root dry weight and density showed positive effects of inoculation and minimal N combination on the host plants (Table 1). Compared to the uninoculated control, bacterial inoculation increased root dry weight and density up to 264% and 50%, respectively. Inoculation of Z78 with 25% N gave the greatest root biomass. The uninoculated treatment combined with different N concentrations, combinations of E14+100% N, E9+100 and 50% N, E7+100 and 50% N, showed no effects in root biomass. Combination of E7+25% N and 0% N and Z78 and E14 with 25% N increased root density. For uninoculated treatments, the 0 and 25% N had the lowest values for both parameters. Higher root density was accompanied by more root hair formation. More primary roots were initiated in inoculated plants (Z78, E7, E9 and E14) supplied with little or no N (0 and 25%), than in those with higher levels of N. Root density was comparatively low at 50 and 100% of N (Table 1). Treatments of inoculated plants (Z78, E7, E9 and E14) supplied with 50 and 100% N produced stubby (shorter and thicker) roots.

The highest top dry weight of the host plants was recorded for E7+25% N, followed by the combinations of Z78+25% N or 0% N, E9+25% N or 0% N, and E7+0% N. The uninoculated treatment supplied with 100, 50, 25 and 0% N, E9+100% N, E7+100% N and Z78+100% N had the lowest top dry weight of the host plants (Table 1). Thus, inoculation of Z78 and E7 with limited N (25%) had significantly higher plant dry weight than the other combination treatments. In addition, the uninoculated treatment combined with 0 and 25% N showed low response on plant dry weight. Inoculation, N application, and the interaction between inoculation and N were statistically significant for plant dry weight and root density (Table 1).

N, P, K, Ca and Mg Accumulation and Total Leaf Chlorophyll and Protein Content

All inoculation treatments accumulated more N than did any of the uninoculated treatments (control). Inoculation with Z78 and E7 produced the highest N accumulation in plant tissues; due to N_2 fixation capacity of the inocula (Table 2). The plants inoculated with Z78 and E7 and supplied with 25% N accumulated significantly more P (Table 2). Plants inoculated with E9 and supplied with 100% N had the highest potassium (K) accumulation. Inoculation of E9 supplemented with 50% N and the uninoculated + 100% N control also had elevated K accumulation. Similar results were obtained for plants inoculated with Z78+100% N, E7+25 and 100% N and E9+25% N. Most of the combination treatments had significantly higher calcium (Ca) accumulation. The highest Ca accumulation was recorded for the combinations of Z78+100% N, E14+25% N,

Table 1 Effects of diazotrophic inoculants and different concentrations of N on growth of oil palm seedlings at D_{360}.

Inoculation	N	Plant dry weight		Plant height	Leaf formation/seedlings	Root density
		Top	Root			
	(%)	(----------- g ----------)		(cm)		(cm^3)
Z78 (*Herbaspirillum seropedicae*)	0	39.0fgh	26.3f	59.2abcdef	10a	277.0fghi
	25	43.0gh	37.9g	70.9i	13de	315.2kl
	50	20.5abc	28.8ef	58.7abcde	12abcd	257.2cdefg
	100	23.4abcd	16.8abcde	53.7ab	12abcde	253.5cdef
E7 (*Microbacterium* sp.)	0	37.8efgh	20.4def	63.2cdefghi	12abcde	303.4ijk
	25	47.8h	26.0f	70.5hi	14e	334.1l
	50	23.1abcd	14.4abcd	63.6defghi	12abcd	273.8efgh
	100	21.1abc	16.4abcde	59.1abcdef	12abcde	280.5fghij
E9 (*Acetobacter* sp.)	0	38.5fgh	19.8cdef	69.3ghi	13bcde	282.4fghij
	25	39.3fgh	19.4bcdef	72.1i	13cde	300.8hijk
	50	24.7bcd	11.8abc	68.1fghi	11ab	259.1defg
	100	18.9abc	11.5ab	61.3bcdefg	11abcd	246.4bcde
E14 (*Microbacterium* sp.)	0	28.6cdef	19.7cdef	65.0efghi	11abcd	283.6ghij
	25	34.2defg	24.3ef	69.0ghi	13de	309.3jkl
	50	26.4cde	19.8cdef	60.5bcdefg	10a	259.9defg
	100	24.3bcd	13.3abcd	61.6bcdefgh	11abcd	244.6bcd
Uninoculated	0	11.6a	9.7a	51.2a	10a	185.8a
	25	14.0ab	10.4b	51.4a	10a	222.3b
	50	18.7abc	13.5abcd	54.3abc	11abc	229.0bc
	100	21.8abc	11.4ab	55.2abcd	11abc	241.2bcd
Significance[1/]	Inoculation	*	*	*	*	*
	N	*	*	*	*	*
	Inoculation × N	*	*	*	*	*

[1/] * Significant at P < 0.05; means followed by the same letter within one parameter at four different levels of N for each treatment do not significantly differ at P < 0.05.

Table 2 Nutrient uptake and soil pH of oil palm seedlings inoculated with diazotrophs and supplied with different concentrations of N at D_{360}.

Inoculation	N	Nutrient uptake					Soil pH
		N	P	K	Ca	Mg	
	(%)	(--------------------------------		%		---)	
Z78 (*Herbaspirillum seropedicae*)	0	0.5	8.1ab	7.2bc	1.8cd	0.4b	6.0
	25	11.3	11.0c	11.1ef	2.1i	0.8i	5.5
	50	16.7	8.7bc	9.0d	1.7bc	0.6d	5.2
	100	28.9	9.9bc	13.6gh	3.2m	1.2o	4.1
E7 (*Microbacterium* sp.)	0	0.5	5.6a	5.5a	1.8cd	0.4b	5.8
	25	9.9	10.1c	13.7gh	2.0h	0.8hi	5.6
	50	14.0	8.9bc	11.6b	1.5a	0.5c	5.0
	100	25.6	9.6bc	12.5fgh	2.4j	0.9k	4.2
E9 (*Acetobacter* sp.)	0	0.4	8.2ab	9.5de	1.6a	0.4a	5.4
	25	3.2	9.2bc	12.1fg	1.7d	0.7e	5.4
	50	10.3	9.3bc	14.1h	2.6k	1.1n	5.2
	100	24.3	9.2bc	18.5i	1.9fg	0.9j	4.1
E14 (*Microbacterium* sp.)	0	0.5	8.4abc	7.9bcd	2.0gh	0.7ef	5.6
	25	5.1	9.2bc	13.8gh	2.7l	0.9l	5.6
	50	10.9	8.8bc	11.9f	1.7b	0.7fg	5.1
	100	24.1	10.4bc	12.3fg	1.9ef	1.0m	3.9
Uninoculated	0	0.3	7.5ab	4.6a	1.7b	0.4a	6.2
	25	1.9	8.5bc	6.2ab	1.8de	0.5bc	5.9
	50	9.8	8.7bc	8.3cd	1.8de	0.7gh	5.2
	100	21.6	9.4bc	14.0h	1.8de	0.9j	4.5
Significance[1/]	Inoculation	*	ns	*	*	*	ns
	N	*	*	*	*	*	*
	Inoculation × N	ns	*	*	*	*	ns

[1/] * Significant at $P < 0.05$; ns - not significant; means followed by the same letter within one parameter at four different levels of N for each treatment do not significantly differ at $P < 0.05$.

E9+50% N, E7+100% N and Z78+25% N (Table 2). The highest magnesium (Mg) accumulation was shown in the combination treatments of Z78+100% N, while the lowest value of Mg accumulation was recorded for plants inoculated with E9 and uninoculated plants + 0% N (Table 2). Inoculation of E7 and E14 combined with 100% N also resulted in more Mg accumulation than at any other levels of N (Table 2). There were no significant interactions between inoculation and applied N with respect to nitrogen (N) accumulation in plant tissues. The average pH values measured in the soil are presented in Table 2. The pHs were within the acidic range. The pH increased inversely with the concentrations of N applied to the oil palm seedlings.

The total leaf chlorophyll and protein contents were significantly influenced by the combination treatments (Table 3). Inoculation of Z78 supplemented with 100, 50 and 25% N, E14+100 and 50% N, E7+100 and 50% N and uninoculated+100% N, all had elevated levels of leaf chlorophyll. The differences were highly significant from the combinations of Z78, E7, E9 and uninoculated plants at 0% N (Table 3). The chlorophyll content of plants inoculated with Z78, E9 and E14 and supplied with low amounts of N was similar to the treatment with 50 and 100% N. Thus, the inoculations successfully stimulated chlorophyll synthesis in leaf tissues of oil palm seedlings. In addition, total leaf protein content was highly increased in inoculated combination treatments (100% N) followed by 50, 25 and 0% N, and the uninoculated (control) plants; plants inoculated with Z78 supplemented with 100% N showed the highest (Table 3). Inoculations of Z78+50, 25 and 0% N, E7+100 and 50% N, E9+100 and 50% N and E14+100 and 50% N and uninoculated+100% N were statistically similar to one another and significantly better than treatments with E7 or E9 with 0% N and uninoculated+25 and 0% N. Inoculation and N interaction were significant for total leaf chlorophyll and protein content (Table 3).

Nitrogen Fixation Activity

The inoculation treatment showed associative N_2 fixation activities. Nitrogenase activity was recorded at 2.98 x 10^{-3} µmol C_2H_4 fresh weight^{-1} h^{-1}, 5.06 x 10^{-3} µmol C_2H_4 fresh weight^{-1} h^{-1}, and 2.46 x 10^{-3} µmol C_2H_4 fresh weight^{-1} h^{-1} for plants inoculated with Z78, E14 and E7 combined with 25% N, respectively (Table 3). Nitrogenase activity was also detected in the uninoculated treatment supplied with 25% N at 2.98 x 10^{-4} µmol C_2H_4 fresh weight^{-1} h^{-1}, which is probably due to indigenous rhizobacteria. Bacterial inoculation combined with 0% N yielded less nitrogenase activity than 25% N application. No nitrogenase activity was detected in plants supplied with 50 and 100% N (Table 3). Thus, there was greater N_2 fixing potential by Z78, E7 and E14 if the soil contained some N (0 and 25% N).

Discussion

Significant interaction effects were identified between bacterial inoculations and the levels of N applied. Plants inoculated with Z78, E7 and E14 had higher root dry weight and density and more young root hair formation (secondary and tertiary roots). These additional root hairs provided spaces for the bacteria to colonize the roots. The bacteria presumably colonize the epidermis and intercellular spaces of the root cortex, and maximizing these spaces increases the nutrient availability for bacterial growth. As the bacteria penetrate the plant cells, they stimulate cell division and expansion, therefore inducing more root hairs (Zakria et al., 2007). Thus, the inoculations promoted more lateral roots through effective bacterial colonization. Increased root and top dry weight of inoculated seedlings was expected given earlier work with bacterial inocula in field nurseries (Amir et al., 2005).

The results indicated that addition of 0 and 25% N together with inoculation using Z78, E7 and E14 produced significantly higher values for root dry weight and density (Table 1). The significant interaction was due to biological N_2 fixation and phytohormone production in the root-bacteria association. The increased formation of root hairs and lateral roots presumably allowed greater N_2 fixing activity, and this may explain why nitrogenase activity was detected in all inoculated roots combined with 0 and 25% N. The effects of inoculation combined with a small amount of N as a starter, stimulated top dry weight and the production of more leaves per plant. Similar results were reported by Suman et al. (2008) in sugarcane with respect to higher root biomass and root proliferation due to low N input providing more sites for diazotrophic bacterial colonization and for their entry into the plant through cracks at root initiation sites. The starter N application enables the establishment of the microbial population on roots and stimulates nitrogenase activity (Caliskan et al., 2008). A positive correlation of nitrogenase activity with higher population size of *G. diazotrophicus* and *Herbaspirillum* sp. in roots and stems of host plants indicates that these parameters describe an important role in nutrient uptake and plant growth performance (Suman et al., 2008). The ARA analysis recorded positive results of nitrogenase activity for oil palm seedlings on the day of harvest (D_{360}). Nitrogenase activity was detected from plants inoculated with Z78, E14 and E7 (+25% N). The application of starter N increased biological N_2 fixation and reduced the demand for N. Application of a small amount of N (0 and 25% N) also improved growth as previously noted by Osborne and Riedell (2006), Caliskan et al. (2008) and Coelho et al. (2009). Thus, such studies illustrate the importance of N starter in early plant growth, as young plants need to be physiologically active in order to make best use of any diazotrophic inoculants applied.

Another reason that better plant growth might result from plant-bacteria associations is the production of plant growth regulating substances. The diazotrophs produce phytohormones such as indole-3-acetic acid (IAA). The phytohormones increase root hair production, thereby improving nutrient uptake from the soil (Martinez-Morales et al., 2003). Diazotrophs such as *Azospirillum*, *Herbaspirillum* and *Bacillus* stimulate plant growth through excreting plant growth hormones (Kloepper, 1989; Lambrecht et al., 2000; Asghar et al., 2002; Azlin et al., 2007, 2009). In the presence

Table 3 Total leaf chlorophyll and protein contents and nitrogenase activity of oil palm seedlings inoculated with diazotrophs and supplied with different concentrations of N at D_{360}.

Inoculation	N (%)	Total leaf: Chlorophyll content (mg mg^{-1})	Total leaf: Protein content (mg mL^{-1})	Nitrogenase activity[2/] (µmol g^{-1} h^{-1})
Z78 (*Herbaspirillum seropedicae*)	0	0.39bcde	45.4defg	8.01×10^{-4}
	25	0.45fgh	51.5fgh	2.98×10^{-3}
	50	0.46gh	53.0ghi	nd
	100	0.46gh	60.3i	nd
E7 (*Microbacterium* sp.)	0	0.38bcd	40.3bcd	nd
	25	0.43defg	44.2def	2.46×10^{-3}
	50	0.45fgh	46.3defgh	nd
	100	0.48h	54.2hi	nd
E9 (*Acetobacter* sp.)	0	0.37bc	35.4bc	5.93×10^{-4}
	25	0.41cdef	40.2bcd	nd
	50	0.43defg	44.7defg	nd
	100	0.44efgh	50.0efgh	nd
E14 (*Microbacterium* sp.)	0	0.41cdef	39.3bcd	5.50×10^{-4}
	25	0.43defg	42.3cde	5.06×10^{-3}
	50	0.45fgh	44.7defg	nd
	100	0.47gh	52.8fghi	nd
Uninoculated	0	0.26a	24.3a	nd
	25	0.35b	32.5ab	2.89×10^{-4}
	50	0.41cdef	40.6bcd	nd
	100	0.45fgh	49.4efgh	nd
Significance[1/]	Inoculation	*	*	
	N	*	*	
	Inoculation × N	*	*	

[1/] * Significant at $P < 0.05$; means followed by the same letter within one parameter at four different levels of N for each treatment do not significantly differ at $P < 0.05$.
[2/] nd = not detected.

of 25% N, the inoculated plants (+Z78, E7 and E9) best stimulated plant growth (top and root dry weight, plant height and number of leaf formations per plant) relative to uninoculated plants supplied with 100% N. Thus, the diazotrophs can be considered for use as a biofertilizer alternative to chemical fertilizers for increasing soil fertility and crop production. Similar conclusions were drawn by Cong et al. (2009) on the relative effects of inoculants (*Pseudomonas* sp. and *Bacillus* sp.) and N fertilizer on yield of rice. Their study indicated that inoculants significantly improved the efficiency of N uptake and could save up to 43 kg of N fertilizer ha^{-1} together with increasing rice yield by 270 kg ha^{-1}. Moreover, since bacterial inoculants produce plant hormones (IAA) that promote root branching and root hair development, this can increase root exudation. Azlin et al. (2007) showed that the plant-bacteria association enhances root fresh weight, chlorophyll and the number of primary and secondary roots of tissue-cultured oil palm plantlets due to IAA production. The plant association provides good physiological conditions for bacterial survival.

Application of N at high rate might result in reduced plant growth and N_2 fixation activity. High N input influences diazotroph populations which, in turn, affects growth and N_2 fixation activity, as seen in sorghum (Coelho et al., 2009). In their study, Coelho et al. (2009) observed that there was a significant reduction in the abundance of the *nifH* gene in rhizosphere soil when high levels of nitrogen (e.g., 120 kg N ha^{-1}) were applied to the crops. Similar results were observed in this study where the nitrogenase enzyme activity of plant-diazotroph associations was influenced by the application of high N amounts (50 and 100%). The inoculations were less effective in promoting root growth when 50 and 100% N was applied. This reduction could be caused by deleterious effects of inorganic nitrogen to plant roots (Medeiros et al., 2006). An oversupply of N has also been shown to soften plant tissue and reduce microbial colonization of plant roots (Chen, 2008). Thus, applying the full amount of a chemical fertilizer can in some cases result in the reduction of diazotroph populations, crop susceptibility to disease attack, acidification or alkalization of the soil, reduction in soil fertility, and eventually, irreversible damage to the overall plant system (Vessey, 2003; Chen, 2008). This might be related to the pH level of the soil sample. In this study, soil pH of fully applied N fertilizer (100% N) was highly acidic recorded compared to 0 and 25% of N. Soil acidity often limits the production on nitrogen fixation. Acid stress may affect any stage of plant-microbe interactions including the survival and multiplication bacteria in the soil. Furthermore this could also affect root infection and inhibit the growth of the host plant (Lindstrom et al., 1985; Maccio et al., 2002). This evidence was confirmed with results of growth parameters obtained from this study. Lower density and dry weight of roots resulted in lower plant height and number of leaf formation. These effects subsequently produced lower total biomass in plant.

Inoculation with Z78, E7 and E14 supplemented with 50 and 100% N increased the biosynthesization of leaf chlorophyll and protein. Previously, chlorophyll content was found to increase in oil palm seedlings inoculated with *A. brasilense* (Sp7) and locally isolated *Bacillus* spp. (UPMB10) (Amir et al., 2003; Azlin et al., 2009). Beneficial effects of PGPR on host plant chlorophyll levels have been shown in other studies (Sharma et al., 2003; Lucy et al., 2004). Overall, the positive effect in the combination treatments of N and diazotrophs on promoting higher chlorophyll and protein content might be due to extensive root development and higher root density of the inoculated plants, facilitating nutrient and water uptake. Biological nitrogen fixation by the inoculants could provide additional N and enable higher plant chlorophyll and protein content.

Diazotrophs can significantly increase leaf N content, nutrient uptake and crop yield, as shown in sugarcane (Muthukumarasamy, 1999). However, diazotrophic production of N cannot completely substitute for N fertilizer. In the present study, biological nitrogen fixation activity occurred only when a small amount of N (0 and 25%) was added as a starter. *Azospirillum* induces and promotes plant growth through BNF activity, and as a result enhances nutrient uptake by the host plant (Torres Netto et al., 2005). A significant increase in crude protein concentration in wheat and barley was due to diazotrophic bacterial inoculation (Lisova et al., 1995; Ozturk et al., 2003). The current study found that a combination of diazotrophs and starter N produced higher leaf protein content than found in uninoculated control plants with 0% N. Biological nitrogen fixation from the bacteria-root association could also be responsible for the result observed in this study.

Our study also found a significant relationship between applied N to increasing nitrogen (N) concentration in the inoculated plants. In earlier studies with banana plants inoculated with *Bacillus* sp. UPMB10 (+ 33% N-fertilizer) there was a synergistic effect on N accumulation; this result might be due to higher N_2 fixation capacity of the strain together with minimal N-fertilizer application (Mia et al. 2005). Bacterial inoculation significantly increased top and root dry weight and consequently the total dry weight of plant. In general, higher biomass production resulted in increased N accumulation by the inoculated plants.

Similarly, phosphorus (P), potassium (K) and magnesium (Mg) content in oil palm seedlings all increased when N was supplied (Table 2). Diazotrophs are known to promote plant growth through many mechanisms of action including biological nitrogen fixation, production of auxins, cytokinin and gibberellins, and increasing nutrient uptake by the plants. In addition, nitrogen-fixing bacteria have some nutritional advantages for the plant relative to other bacteria in the soil and root vicinity. Therefore, nitrogen-fixing bacteria may be able to colonize the plant root system more intensively under conditions where soil micro-organisms compete for root excretion which serves as the substrate for microbial growth. The improved colonization increases nutrient uptake and mineral nutrition as well as contributes to the development of the plants (Suman et al., 2008). This may explain the observed inoculation effects on nutrient accumulation (N, P, K, Ca and Mg) by the plants. Furthermore, higher nutrient uptake might be due to increased number of lateral roots formed and the higher root density. Other researchers also report the beneficial effects of plant growth-promoting bacteria in increasing nutrient uptake by non-legume plants such as rice (Cong et al., 2009; Biswas et al., 2000). Without N fertilizer application (i.e., at 0% N), Z78 performed the best in terms of promoting plant growth (e.g., plant biomass, N_2 fixation activity and protein content), indicating its natural ability to fix N and thus enhance growth. However, addition of minimal N fertilizer (25% N) as a starter resulted in better growth performance in the host plant for all parameters studied.

Thus the diazotrophs, especially Z78, E7 and E14, were shown to improve growth of oil palm seedling if supplied with a low amount of N. This study found no loss of plant dry weight or root density even with 25, 50, or 100% reduction of the recommended N. An optimal condition would be a minimal amount of N fertilizer (0 and 25%) and inoculation with the most effective diazotrophs (*Herbaspirillum seropedicae* (Z78) and the locally isolated *Microbacterium* sp. (E7 and E14)).

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