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N_2 Fixation, Nutrient Accumulation and Plant Growth Promotion by Rhizobacteria in Association with Oil Palm Seedlings

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Abstract: An experiment was conducted in undrained poly bags under glasshouse conditions to quantify the N_2 fixing capacity (^{15}N isotope dilution method) of plant growth promoting rhizobacteria (*Azospirillum* and *Bacillus* spp.) in association with oil palm seedlings. Effects of inoculation on nutrient uptake and plant growth promotion will also be observed. The experiment was arranged in a randomized complete block design with five replications and harvested at 390 days after planting. The treatments involved were: 1) killed *Azospirillum brasilense* (Sp 7), 2) killed Sp 7; + inorganic-N_i, 3) Sp 7, 4) *A. lipoferum* (CCM 3863), 5) locally isolated rhizobacteria UPMB 10, and 6) UPMB 13 inoculation. Results showed that inoculation of the rhizobacteria could contribute up to 20-50% of the total nitrogen requirement of the host plant through N_2 fixation process. Besides that, the inoculation process had also stimulated accumulation of nutrient and plant growth (tops and roots) comparable to the control with full inorganic nitrogen (N_i) fertilization after 390 days of growth.

Key words: Biofertilizer, bioenhancer, *Elaeis guineensis*, ^{15}N dilution, rhizobacteria

Introduction

Recently there has been considerable interest in diazotrophic rhizobacteria such as *Acetobacter diazotrophicus*, *Herbaspirillum* spp., *Azoarcus* spp. and *Azospirillum* spp. which colonize the exterior and interior of sugarcane, rice and palm trees (Reis *et al*., 2000). The rhizobacteria have been reported as being important for establishment and growth of the host through associative N_2 fixation (biofertilizer) and plant growth enhancement effects (bioenhancer)(Tsimilli *et al*., 2000). The biological N_2 fixation process provides an opportunity to reduce application of synthetic nitrogenous fertilizer, save cost and potentially increase crop production (Cocking, 2000). Elimination or substantial reduction of N fertilizer is considered as a key factor in the development of environmentally friendly agricultural system (Reis *et al*., 2000). Findings by Malik *et al*. (1997) have suggested that rhizobacterial-rice association may acquired nearly 70% of the N requirement from atmospheric N_2, thus conserving soil fertility by reducing the plants reliance on soil N. Exploitation of this associative N_2 fixing rhizobacteria on oil palm seedlings can potentially make the seedling production be more sustainable and profitable (Dobereiner and Baldani, 1998; Shamsuddin *et al*., 2000). Thus, this experiment was conducted: 1) to estimate the total amount of N_2 fixed by *Azospirillum* spp. and locally isolated rhizobacteria in association with oil palm seedlings, 2) to observe effects of inoculation on the accumulation of essential nutrients in plant tissues and 3) to observe effects of rhizobacterial inoculation on growth and development (tops and roots) of the host plants.

Materials and methods

An experiment was conducted in undrained polybag (to prevent leached out of applied ^{15}N solution and fertilizer) with Selangor series soil (*Typic Sulfic Tropaquept*, pH 4.2) at 8 kg/polybag and was planted with newly germinated oil palm seed. The soil was maintained at an appropriate field capacity (28% moisture) daily. N-free fertilizer was applied monthly in the form of P_2O_5, K_2O and MgO (reformulated based on the equivalent rate of NPK fertilizer required) (Foo and Mat, 1995) (Table 1). The ^{15}N labeled solution enriched with 10%^{15}N atom excess (a.e.) (14.92 g $(^{15}NH_4)_2.SO_4$)/6 L distilled water) was applied for labeling the soils for N_2 fixation analyses by ^{15}N isotope dilution method. The ^{15}N solution was applied at 100 mL/polybag for all of the inoculation treatments, which is equivalent to 0.053 g N/polybag (20 kg N ha^{-1}) at 130 and 260 days after planting (Hashim and Zaharah, 1994).

Two bacterial cultures of *Azospirillum* spp. were used for inoculation; *A. brasilense* (Sp 7) and *A. lipoferum* (CCM 3863), which were obtained from Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA), Brazil and Czechoslovakian Collection of Microorganism, Republic of Czechoslovakia. Two locally isolated rhizobacteria (*Bacillus* spp. UPMB 10 and *Bacillus* spp UPMB 13)

Table 1: Basal fertilizer rate based on recommended fertilizer applications for oil palm seedlings

		Reformulated fertilizer rate (g/seedling)			
Days	Actual fertilizer	$CO(NH_2)_2$**	P_2O_5	K_2O	MgO
150	NPK 7 g/palm	1.05	1.05	0.42	0.28
180-270	NPK 14 g/palm	2.10	2.10	0.84	0.56
300-390	NPK 21 g/palm	2.52	2.52	3.57	0.42

**Supplied only to the control treatment (Sp 7 k +N_i)

provided by Soil Microbiology Laboratory, Universiti Putra Malaysia were also tested. The strains were sub-cultured in 100 mL nutrient medium/flask and shaken continuously for 48 h (150 rpm, 28°C). The treatments applied in this experiment were as follows: 1) Control 1 (+ killed Sp 7, non-sterile soil (nss); autoclaved at 121°C for 20 minutes (Sp 7 k nss) - a non-fixing reference)(Malik *et al*., 1987; Dobbelaere *et al*., 1999), 2) Control 2 (+ killed Sp 7 nss, + inorganic-N (Sp 7 k +N_i)), 3) *Azospirillum brasilense* (Sp 7) inoculation; nss, 4) *A. lipoferum* (CCM 3863) inoculation; nss, 5) UPMB 10 inoculation; nss and 6) UPMB 13 inoculation; nss, arranged in a randomized complete block design with five replications. Inoculation of the seedlings with respective treatment was carried out after the plants emerged and at two monthly interval with 20 mL inoculum (approx. 10^8 cfu mL^{-1}) per polybag.

The observations undertaken at harvest (D_{390}) were; rates of N_2 fixation by the inocula tested, uptake of essential nutrient (N, P, K, Ca and Mg) and top and root growth of the inoculated host plants. At harvest (D_{390}), the plant samples were prepared for total N and ^{15}N analysis by semi-micro Kjeldahl methods (Bremner, 1996). Dilution of the ^{15}N was analyzed by emission spectrometer (NOI-6PC). The ^{15}N abundance found in the plant tissue was corrected for the $\%^{15}Na.e.$ present in the atmosphere (0.3663 % ^{15}N a.e.). The proportion of N derived from the atmosphere (%Ndfa) and total N_2 fixation were calculated using the following equation:

$$\%Ndfa = \frac{1 - \%^{15}Na.e._{\text{fixing system}}}{\%^{15}Na.e._{\text{non-fixing system}}} \times 100$$

$$N_2 \text{ fixed (mg)} = [(\%Ndfa)(\text{total N content})/100] \times 1000$$

An analysis of P was conducted by Technicon autoanalyzer (2nd Ed.), while for K, Ca and Mg were analyzed by Atomic Absorption Spectrophotometer (Perkin Elmer 5100 PC).

Results and Discussion

The ^{15}N dilution technique has been used widely for quantification of biologically fixed nitrogen in legumes (Chalk, 1985) and associative nitrogen fixation in grasses

Table 2: Percentage of N_2 derived from atmosphere (%Ndfa) and N_2 fixed (mg N $plant^{-1}$) of inoculated oil palm seedlings at D_{390} in Selangor series soil

Treatments	$\%^{15}N$ a.e.	%Ndfa*	N_2 fixed* (mg N $plant^{-1}$)
Sp 7 k (nss)	0.79b	-	-
Sp 7 k + N_i	0.07d	-	-
Sp 7	0.54c	31.6a	537a
CCM 3863	0.53c	32.9a	523a
UPMB 10	0.57c	27.8a	478a
UPMB 13	0.60c	24.1a	351ab

Means with the same letters are not statistically significant at 5% level
*Estimated based on Sp 7 k (nss) reference plants

Table 3: Effects of rhizobacteria inoculation on uptake of N, P, K, Ca and Mg of oil palm seedlings at D_{390} in Selangor series soil

	(g $plant^{-1}$)				
Treatments	N	P	K	Ca	Mg
Sp 7 k (nss)	1.20c	0.49b	2.59b	0.36b	0.55a
Sp 7 k + N_i	5.41a	0.62a	3.43a	0.38a	0.62a
Sp 7	1.70b	0.56ab	3.32a	0.37a	0.60a
CCM 3863	1.59b	0.55ab	3.18a	0.37a	0.57a
UPMB 10	1.72b	0.52ab	3.10a	0.39a	0.59a
UPMB 13	1.46bc	0.47b	2.52b	0.34b	0.55a

Means with the same letters are not statistically significant at 5% level

Table 4: Effects of rhizobacteria inoculation on plant growth and development of oil palm seedlings at D_{390} in Selangor series soil

Treatments	Root dry wt. (g)	Root vol. (cm^3)	Top dry wt. (g)	Chlorophyll content
Sp 7 k (nss)	68.6bc	325b	112b	0.34c
Sp 7 k + N_i	55.1c	335b	163a	0.57a
Sp 7	89.1a	436a	117b	0.44b
CCM 3863	80.3ab	388ab	118b	0.44b
UPMB 10	93.7a	460a	114b	0.41b
UPMB 13	65.2bc	380ab	118b	0.40b

Means with the same letters are not statistically significant at 5% level

(Rennie, 1980; Boddey *et al*., 1983; Giller *et al*., 1986). This experiment has proven that association of rhizobacteria (*Azospirillum* and locally isolated *Bacillus* spp.) with oil palm seedlings could successfully contribute fixed N_2 for the host plants (20-30%Ndfa; 300-500 mg N $plant^{-1}$; 0.50-0.60%^{15}N a.e) (Table 2). Similar studies using ^{15}N isotope dilution which was conducted by Urquiaga *et al*. (1992) and Boddey *et al*. (1995) have also shown that inoculated brazilian sugar cane with *Azospirillum* can fix substantial amount of N_2 up to 70% of their N-requirement (150 kg N fixed ha^{-1} $year^{-1}$). A number of tropical forage grasses including *Brachiaria humidicola*, *B. decumbens*, *Paspalum notatum* and *Panicum maximum* have shown relatively high N_2 fixation rates by the associated N_2 fixer and may derive up to 40% of their N-needs (Boddey and Knowles, 1987). Malik *et al*. (1997) have also reported the N_2 fixation ability of *Azoarcus* in association with kallar grass and contributed 26% of its N content from fixation. Another report by Dobereiner (1997) has shown that associative diazotrophic microorganisms could contribute at least 20-40% of the plant N requirement of several non-leguminous crops through N_2 fixation process. According to Dobereiner (1997), development of N_2 fixation process

on oil palm would increase the net bio-energy yield by eliminating the large fossil energy input inherent in the use of N fertilizer. The technology hopefully will reduce input cost, increase the energy balance and diminish the negative environmental consequences on the use of excessive N fertilizer.

The inoculation process also showed significant effects on total uptake of N, P and K of the host plants (Table 3). Increment in N content was related to the N_2 fixation process by the inocula tested (Sp 7, CCM 3863 and UPMB 10). Highly accumulation of P and K was also shown in the inoculated host plants and could be related indirectly to the inoculation effects through enhanced essential nutrient uptake by stimulation of root growth and development of the host plants (root dry weight and volume) (Table 4). Inoculation of Sp 7 and UPMB 10 had enhanced root dry weight (37%), volume (41%) and chlorophyll content of the host plants compared to the control (Sp 7 k). This is in agreement with earlier findings by Lin *et al.* (1983), Rai and Hunt (1993) and Bashan and Holguin (1997), that *Azospirillum brasilense* inoculation could improve ion uptake and contributed to significant elevation of plant growth. Saad *et al.* (1999) have shown that, inoculated sweet potato (*Ipomea batatas*) with *Azospirillum* produced similar or higher root yield, vigorously vegetative growth, and higher N content in the roots and leaves compared to uninoculated plants given normal rate of N fertilizer.

Bashan (1998) reported that inoculation of *A. brasilense* and *A. lipoferum* 1842 would increase the root hair formation and produced more lateral roots of wheat. Similar response of inoculation on root growth and development of soybean was also highlighted by Molla *et al.* (2001), where single or co-inoculation of Sp 7 with *Bradyrhizobium* (UPMR 48) had significantly stimulated higher root dry weight, root volume, specific root length, total root length and shoot dry weight of the host plant compared to the control (without any inoculation). It was proposed earlier by Bashan *et al.* (1990) that enhancement of mineral uptake by plants should result in an increased accumulation of both dry matter and minerals in the stem and leaves of the plants. Positive response of the inoculation process in promoting plant growth was related to the fixation capacity of the inocula tested.

The experiment indicated that the rhizobacterial strains especially Sp 7, CCM 3863 and UPMB 10 are potential biofertilizer with an average of 30% Ndfa (500 mg N $plant^{-1}$). The inoculation process had also stimulated essential nutrient accumulation especially N, P and K and considered as a bioenhancer. As a PGPR the inocula had enhanced root dry weight, volume and chlorophyll content of the host plants. These strains are suitable and could be recommended for oil palm seedlings production and more in-depth studies are necessary to observe the efficiency of the inoculum on immature and mature oil palm in the field.

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