ISSN: 1394 7990
Malaysian Journal of Soil Science Vol. 6 (Special Ed.): 75 - 82 (2002) Malaysian Society of Soil Science

N_2 Fixation, Plant Growth Enhancement and Root-Surface Colonization by Rhizobacteria in Association with Oil Palm Plantlets Under *in vitro* Conditions

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ABSTRACT

Association of the N_2 fixing plant growth promoting rhizobacteria (PGPR) with various non-leguminous crops has reinforced the importance of biological nitrogen fixation (BNF) and plant growth stimulation effects. This concept was demonstrated through a laboratory experiment using tissue cultured oil palm plantlets. Under *in vitro* conditions, sterilized tissue cultured oil palm plantlets were grown in test tubes. Tests were conducted to observe the ability of selected rhizobacteria to fix N_2, promote plant growth, enhance essential nutrient uptake and colonize roots of the associated host plants. Results from the experiment show that *Azospirillum* spp. (Sp 7) could contribute up to 66% of the host plant N requirement (% Ndfa), while locally isolated *Bacillus* spp. (UPMB 13) recorded up to 55% Ndfa at D_{56}. The inoculation (especially Sp 7 and UPMB 13) also caused a significant increase in total N and higher leaf chlorophyll content of the host plants. The rhizobacteria tested especially CCM 3863 had enhanced primary root numbers and length compared to the control ($+N_i$). All of the inocula tested successfully colonized the root-surface and benefited the host plants.

Keywords: ***Elaeis guineensis*, rhizobacteria, N_2 fixation, growth enhancer, root colonization, oil palm plantlets**

INTRODUCTION

Elimination or substantial reduction of N fertilizer applications through Biological Nitrogen Fixation is considered a key factor in developing an agricultural sector which is more sustainable and environmental friendly (Reis *et al.* 2000). A major challenge for the development of sustainable agriculture is to use diazotrophic rhizobacteria, capable of assimilating N_2 from the atmosphere. The process, which involves the association of non-leguminous crops with different free-living rhizobacteria such as *Azospirillum* spp. and *Paenibacillus* spp., is becoming increasingly important. The rhizobacteria has been reported to be essential in the establishment and growth of the host through N_2 fixation process and plant growth enhancement (Tsimilli *et al.* 2000). *Azospirillum* has been reported to colonize the

rhizosphere of oil palm and coconut palm (Dobereiner and Baldani 1998) and is also able to fix biological nitrogen (De Carvalho *et al.* 1996; Dobereiner 1997; George 1990). Applications of the rhizobacteria inoculum to economically important crop e.g. tissue cultured oil palm (at seedling stage) can potentially reduce fertilizer cost, especially N. The organisms also enhance root growth, which will eventually help in the field establishment of these seedlings.

Thus the experiment was conducted using oil palm plantlets under axenic conditions in order to: 1) to estimate the total amount of N_2 fixed by *Azospirillum* spp. and locally isolated *Bacillus* spp., 2) observe effects of rhizobacterial inoculation on the growth and development (tops and roots) of the hosts and 3) to determine the ability of the rhizobacteria to colonize the root-surface.

MATERIALS AND METHODS

The experiment was conducted under *in vitro* condition with one sterile plantlet (clone IMP 209; 3 month old provided by Malaysian Palm Oil Board (MPOB)) each in a large test tube, containing N-free MS liquid media (pH 4.2) (Murashige and Skoog 1962). Prior to the establishment of the plantlets in an N-free medium, the plantlets were serially washed twice aseptically with 10 mL N-free MS medium. This was done to wash off any remaining chemicals including N and hormones applied in the previous growth medium. The setup was then kept in a test tube containing N-free MS medium in a temperature controlled growth chamber for a week. The washing procedure was again repeated thrice and the plantlets were finally transferred into a new large test tube (2.5 x 25 cm) containing 8 mL of N-free MS medium, 1 mL ^{15}N fertilizer solution and inoculated with 1 mL of the respective inoculum. The planting medium (MS medium) was labeled with ^{15}N for quantification of N_2 fixation process (^{15}N isotope dilution method) by adding $(^{15}NH_4)_2\ SO_4$ (10%^{15}N a.e.) which was filter sterilized through 0.2 mm Millipore‰ syringe filter. A weight of 0.117g $(^{15}NH_4)_2SO_4$ (10%^{15}N a.e.) equivalent to 250 mg N/tube was dissolved in 100 mL sterile distilled water and 1 mL of the filter sterilized solution was applied by micropipette to each test tube including the control inoculated with killed Sp 7 (Sp 7 k). Dilution of ^{15}N in the planting system by $^{14}N_2$ from the atmosphere indicated the N_2 fixation process of the associated inocula with the host and was analysed by emission spectrometer (NOI-6PC). The setup was kept in a controlled temperature growth chamber with 12 hours of both light and dark period at 28°C ± 0.1°C and 20°C ± 0.1°C during the light and dark period, respectively. The experiment was laid out in a completely randomized design with seven replicates and harvested after 56 days of growth (D_{56}). The treatments involved in this experiment were as follows: 1) Control 1 (+ killed Sp 7; autoclaved at 121°C for 20 minutes (Sp 7 k) - a non-fixing reference (Baldani *et al.* 2000); 2) Control 2 (+ inorganic nitrogen (+N_i)); 3) *Azospirillum brasilense* (Sp 7) inoculation; 4) *A. lipoferum* (CCM 3863) inoculation; 5) locally isolated *Bacillus* spp. (UPMB 10) inoculation; and 6) UPMB 13 inoculation. The observations involved were: 1) proportion of N_2 derived from atmosphere (% Ndfa) and total amount of N_2 fixed (mg N plant^{-1}) by the inocula – estimated on the

TABLE 1
N concentration and content, percentage of ^{15}N atom excess (a.e.), N_2 derived from atmosphere (% Ndfa) and N_2 fixed (mg N plant^{-1}) of inoculated oil palm plantlets at D_{56}

Treatments	N (%)	N content (g plant^{-1})	%^{15}N a.e.	% Ndfa**	N_2 fixed** (mg N plant^{-1})
Sp 7 k (control)	3.19b	5.49c	3.59a		
+ N_i (control)	4.81a	10.92a	0.319d		
Sp 7	3.38b	6.62b	1.218c	66.07a	4.38a
CCM 3863	2.98b	5.69bc	2.094b	41.67b	2.37b
UPMB 10	3.4b	5.98bc	1.918bc	46.57b	2.79ab
UPMB 13	3.36b	6.45b	1.631bc	54.56ab	3.52ab

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

dilution of ^{15}N added (Amir *et al.* 2001); 2) accumulation and concentration of N (Bremner 1996); 3) total dry matter accumulation; 4) root length; 5) primary and secondary root numbers; 6) leaf chlorophyll content (Amir *et al.* 2001); and 7) root colonization. Triplicates of root samples (root-hair zones) at D_{56} were subjected to Scanning Electron Microscope (JEOL™ 6400; Molla *et al.* 2001) to observe the colonization of rhizobacteria on roots of the plantlets. All of the observation data were statistically analyzed by Statistical Analyses System (SAS). Following the analyses of variance procedure, differences among treatment means were determined using the Student Newman Keuls tests (SNK) comparison methods.

RESULTS AND DISCUSSION

Inoculation of *A. brasilense* (Sp 7) resulted in the accumulation of fixed N_2 (66.07% Ndfa; 4.38 mg N_2 fixed), whereas inoculation of CCM 3863 showed lower N_2 fixation capacity (41.67% Ndfa; 2.37 mg N_2 fixed). Meanwhile, inoculation of locally isolated *Bacillus* spp. (UPMB 10 and 13) accumulated up to 46.57 and 54.56% Ndfa (2.79 and 3.52 mg N_2 fixed), respectively (Table 1). The results also strengthened the earlier findings (Amir *et al.* 2001), which showed 40% Ndfa of N_2 fixation capacity of *Azospirillum* in association with oil palm seedlings under pot conditions. Similar response of bacterial inoculation on N_2 fixation ability for rice under sterile conditions has been shown by Malik *et al.* (1997) in that nearly 70% of the plant N was derived from fixation by *Azospirillum* spp. Reis *et al.* (2000) showed that association of *Herbaspirillum seropedicae* and *Azospirillum brasilense* with rice seedlings grown in monoaxenic agar cultures could contribute up to 54 and 32% N from fixation process, respectively. The association also caused a significant increase in dry weight and total N accumulation of the host plant. Earlier findings by Saad *et al.* (1999) have highlighted the fact that inoculation of *Azospirillum* on sweet potato (*Ipomea batatas*) can reduce fertilizer application and consequently reduce the production cost through substitution of a substantial

TABLE 2
Effects of rhizobacteria inoculation on plant growth and development of oil palm plantlets at D_{56}

Treatments	Chlorophyll content	Primary root no.	Secondary root no.	Primary root length	Top dry wt. (g)
Sp 7 k (control)	0.36b	2b	45b	12.8b	172a
+ N_i (control)	0.45a	3ab	86a	14.7b	227a
Sp 7	0.45a	3ab	76a	13.7b	196a
CCM 3863	0.45a	4a	75a	20.8a	191a
UPMB 10	0.46a	3ab	79a	14.3b	176a
UPMB 13	0.46a	3ab	50b	13.8b	192a

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

amount (67%) of N required by the host plant. It was concluded that *Azospirillum* is a potential biofertilizer for sweet potato grown on sandy soils. This highlights the important role of the rhizobacteria as a highly potential N_2 fixer (biofertilizer) for sustainable oil palm plantlets production. According to Cocking (2000), applications of biological nitrogen fixation concept into the major non-leguminous crops of the world would be one of the most significant contributions that biotechnology could make to agriculture. It has also been recognized for many years that it is also a major technical and research challenge.

The inoculation process also caused a significant increase in total N content of the inoculated host plants. Higher N accumulation in the inoculated host plants was related to the N_2 fixation ability of the inocula especially Sp 7 and UPMB 13 (66 and 55% Ndfa) (Table 1). The leaf chlorophyll content of the inoculated plantlets was also significantly affected by the inoculation treatments and the effects were statistically similar to plants fertilized with full N (+ N_i) (Table 2). These phenomena indicate the ability of the inocula tested to fix N_2 and supply fixed N_2 to the host plants and proved the fact that *Azospirillum* and locally isolated rhizobacteria are good for stimulating growth of oil palm plantlets (bioenhancer). Baldani *et al.* (2000) have shown that inoculation of endophytic diazotrophs (*Herbaspirillum seropedicae* and *Burkholderia* spp.) on rice plants could contribute up to 54% Ndfa (gnotobiotic conditions) and 20% Ndfa (pot experiment) through BNF process. The inoculation process also increased the N content of the rice grains compared to the control. This is in agreement with earlier findings by Rennie (1980) who showed that *Azospirillum* inoculation had a significant effect on total N yield of wheat (best indication for occurrence of N_2 fixation process) compared to the control. The inoculation process had also affected root growth including primary and secondary root formation (especially, Sp 7, CCM 3863 and UPMB 10) and primary root length (especially, CCM 3863; Table 2). Enhancement in root formation was significantly better or similar to the control plants fertilized with N_i. Similar results have been reported that inoculation of *Azospirillum*

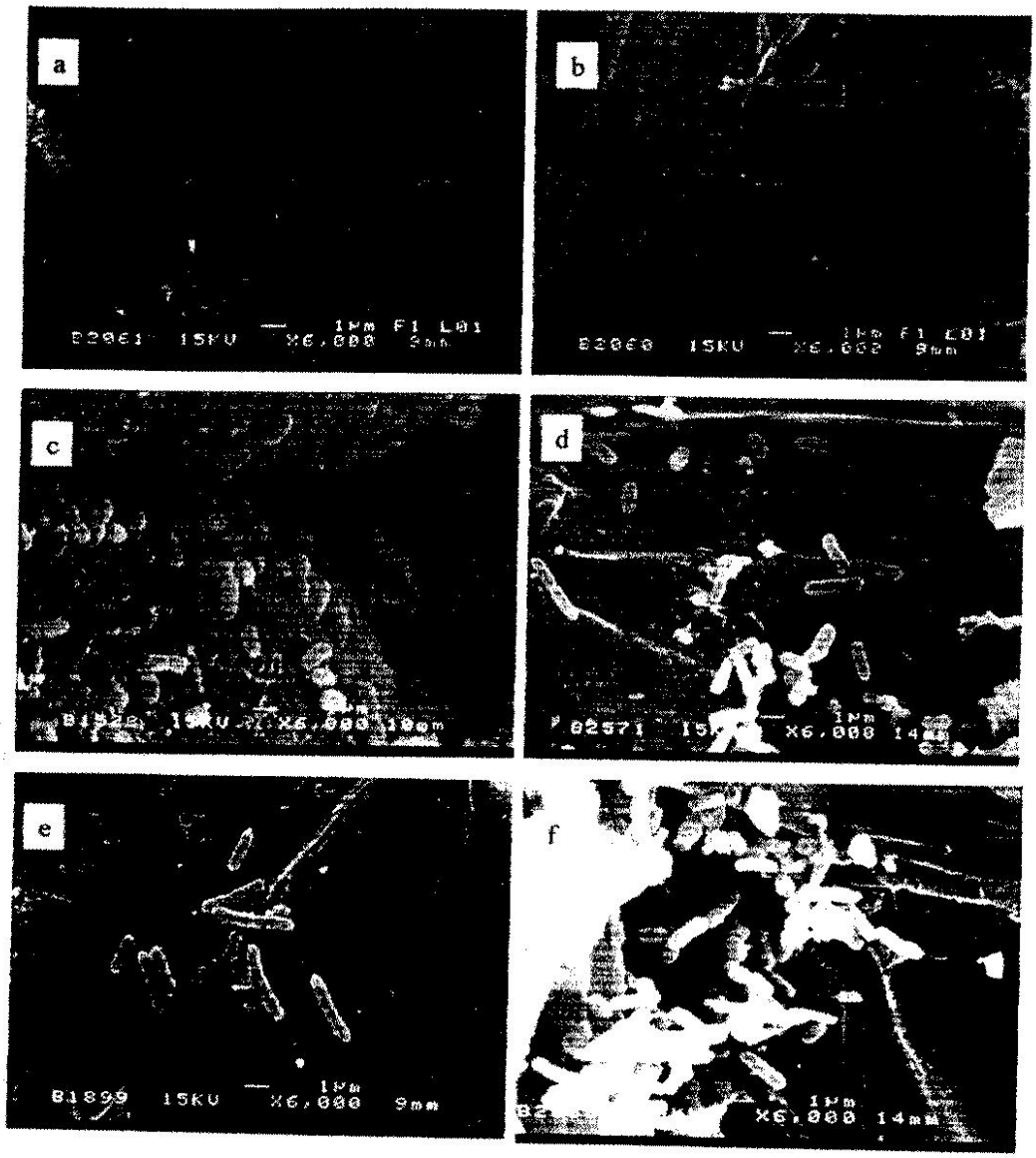

Fig. 1. Colonization of rhizobacterial strain on root surface of oil palm plantlets; (a) + Sp 7 k, (b) +N_2, (c) + Sp 7, (d) + CCM 3863, (e) + UPMB 10 and (f) + UPMB 13

brasilense could significantly improve wheat and soybean growth by increasing shoot and root dry weight and root surface area (Bashan *et al.* 1990). Saubidet and Barneix (1998) have shown that inoculation of different strains of *A. brasilense* to young wheat plants (sterile conditions) successfully colonized both the root surface and interior and stimulated root and shoot growth of the host plants. However, the degree of stimulation was different for the different strains of inoculum tested. The inoculation process had also shown enhanced dry matter production of the plantlets especially Sp 7 compared to the control (+ Sp7 k) (Table 2).

Azospirillum and locally isolated rhizobacteria had colonized roots of oil palm plantlets. The bacterial cells were aggregated to each other and anchored to the root surface by a network of fibrillar material (*Fig. 1*). According to Diamantidis *et al.* (2000), a laccase-like polyphenol oxidase activity (PPO) has been reported

to facilitate the root colonization process of the micro-symbiont. Swimming motility in *Azospirillum* has been suggested to play a role in movement of bacteria towards the plant roots. Swarming across the surface of roots may be important for long-term colonization. Inoculation of *Herbaspirillum* spp., *Burkholderia* spp. and certain strains of *Azospirillum* spp. had colonize roots, stems and leave of maize and penisetum and even C3 plants such as rice and wheat (Dobereiner 1997). Puente *et al.* (1999) have highlighted the occurrence of heavy colonization of *A. brasilense* and *A. halopraeferens* on root surface of axenic mangrove seedlings grown in seawater, which is very important for the development of the host plants. According to Mia *et al.* (1999), *A. brasilense* (Sp 7) formed colonies on the root surface of banana plantlets especially in the root hair and lateral root zone and were also found inter and intra-cellularly. Endophytic colonization of diazotrophs, including certain strains of *Azospirillum*, is important in maximizing the N_2-fixing potential having less competition from indigenous rhizosphere bacteria for carbon substrates, nutrient and protection against high levels of O_2 present on the root surface (Dobereiner *et al.* 1993; Boddey and Dobereiner 1995; Stoltzfus *et al.* 1997).

CONCLUSION

The inocula (Sp 7, CCM 3863, UPMB 10 and UPMB 13) are highly effective biofertilizers and bioenhancer for sustainable production of oil palm plantlets through their ability to fix atmospheric N_2 and stimulate plant growth. The inoculation process also caused a significant increase in total N and higher leaf chlorophyll content of the host plants similar to the control, which was supplied with full inorganic N fertilizer. Besides, enhancement in root growth was statistically similar to the control supplied with full inorganic N_i. All of the inocula tested could successfully colonize the root-surface of oil palm plantlets, benefiting the host plants with their N_2 fixing qualities.

ACKNOWLEDGEMENTS

The authors are indebted to Universiti Sains Malaysia (USM), Universiti Putra Malaysia (UPM), Malaysian Institute for Nuclear Technology Research (MINT), Malaysian Palm Oil Board (MPOB) for technical assistance and advice and Ministry of Science, Technology and the Environment for the research funding under IRPA program.

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