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EFFECTS OF *Azospirillum* INOCULATION ON N_2 FIXATION AND GROWTH OF OIL PALM PLANTLETS AT NURSERY STAGE

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*Nitrogen fertilizer is the most expensive nutrient input in oil palm production, with an average total nitrogen fertilizer cost estimated at RM 470 million yr^{-1}. The use of nitrogen fixing bacteria (*e.g. Azospirillum *spp.) as a biofertilizer and bioenhancer can reduce the production cost of this crop. A glasshouse experiment was undertaken to observe the effects of* Azospirillum *inoculation on N_2 fixation, plant growth and photosynthetic rate of the host plant. This experiment was conducted in undrained pots with ^{15}N labelled Selangor series soil and each pot was planted with a two-month-old oil palm plantlet (MPOB clone, P149). Three treatments were applied: 1) control [+ killed inoculum (Sp7)], 2)* Azospirillum brasilense *(Sp7) inoculation and 3)* A. lipoferum *(CCM 3863) inoculation. This experiment was laid out in a randomized complete block design with four replications and harvested four months after planting. Two weeks before harvest, the first fully expanded leaf from each seedling was analysed for light and CO_2 response using a closed system of portable infra-red gas analyser. At harvest, the plantlets were separated into tops and roots, dried, weighed and ground for total nitrogen and ^{15}N analyses. Results showed that* Azospirillum *inoculation contributed up to 40% of the total nitrogen requirement of the oil palm plantlets, stimulated top and root growth by 30% and 60%, respectively and increased the host photosynthetic rates compared to the control.* Azospirillum *(Sp7 and CCM 3863) is a potential biofertilizer and bioenhancer for sustainable oil palm plantlet cultivation and saves cost on nitrogen fertilizer.*

Keywords:

Elaeis guineensis, Azospirillum, biofertilizer, N_2 fixation, bioenhancer, photosynthesis, ^{15}N isotope dilution.

INTRODUCTION

Nitrogen fertilizer is the most expensive nutrient input in oil palm production. At a recommended rate of 0.5 to 1.0 kg nitrogen palm^{-1} yr^{-1} (148 palms ha^{-1}) and with the average price of urea at RM 587 t^{-1}, total nitrogen fertilizer cost to the industry is estimated to be RM 470 million yr^{-1}. The use of nitrogen fixing bacteria, such as *Azospirillum* spp., as biofertilizer can reduce the need for inorganic nitrogen fertilizer and consequently lower the production cost of this crop. According to Macalintal and Urgel (1992), *Azospirillum* inoculation contributed up to 60% of the nitrogen requirement for sugar cane through the N_2 fixation process.

Azospirillum is not a plant specific bacterium but a general root colonizer (Kapulnik, 1991). It is a prokaryotic diazotroph, capable of fixing atmospheric nitrogen in association with a non-leguminous crop (Bashan and Holguin, 1997). *Azospirillum* has been found within the roots and even in the aerial parts of various graminous plants such as sugar cane, wheat, maize, and in other monocots like oil palm (Dobereiner and

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Baldani, 1998), pupunha palm (*Bactris gasipaes*) (De Carvalho *et al.*,1996) and coconut palm (*Cocos nucifera)* (George, 1990). Its presence suggested a probable biological nitrogen fixation ability within the rhizosphere which benefited the host plants.

It has been shown by ^{15}N isotope dilution method that inoculation of *Azospirillum* spp. to oil palm plantlets grown in a liquid medium contributed 82%-89% of the total nitrogen requirement of the host plant (Shamsuddin, 1994). In addition, *Azospirillum* has also been shown to directly stimulate growth and yield of the host plant, *viz.* wheat, corn, sorghum and banana (Okon *et al.*, 1988; Mia *et al.*, 1999), and become a beneficial bioenhancer and hence is grouped as a plant growth promoting rhizobacterium (PGPR) (Bashan and Holguin, 1997).

The N_2 fixation rate by *Azospirillum* will depend on the efficiency of the photosynthetic process of the host plant in supplying energy (reducing sugar) and carbon skeleton for the ammonium assimilation process to the diazotrophic microorganism (Pausch *et al.,* 1996). However, the photosynthetic capacity of the host plant is positively correlated to leaf nitrogen concentration since it is fundamental in regulating chlorophyll development for better photosynthetically active radiation interception (Field and Mooney, 1986). Furthermore, the nitrogen requirement is also important for the synthesis of ribulose-1, 5-biphosphate carboxylase (Rubisco) and pigment protein complexes in the chloroplast for better CO_2 fixation (Pate, 1986).

Thus, this experiment was conducted to estimate the amount of nitrogen fixed by *Azospirillum* spp. in association with oil palm plantlets, and to observe the effects of *Azospirillum* inoculation on the growth and development, and photosynthetic rates of the host plants.

MATERIALS AND METHODS

The experiment was conducted in undrained pots with Selangor series soil (*Typic Sulfic Tropaquept*, pH 4.2 in 1:5 with 0.01 M $CaCl_2$) at 8 kg pot^{-1}. The nitrogen-free basal fertilizer solution (29.5 mM KH_2PO_4 and 13.8 mM $MgSO_4$. 7 H_2O) was applied weekly at 20 ml pot^{-1} for the first two months of growth. The liquid fertilizer formulation was prepared in 4.5l stock solutions based on the basal fertilizer requirements for oil palm seedlings using NPK Yellow (15:15:6:4) at the rate of 7 g 100^{-1} palms. In the following months, straight fertilizer was applied in the form of P_2O_5 (TSP), K_2O (MOP) and MgO (Kieserite) based on the rate of NPK Yellow (15:15:6:4) used, 7 and 14 g $palm^{-1}$ at months five and six, respectively (*Table 1*) (Foo and Mat, 1995). The soil was labelled with ^{15}N by adding 100 ml $(^{15}NH_4)_2SO_4$ 10% ^{15}N atom excess (a.e.)(20 kg N ha^{-1}) to each pot. The soil moisture level was maintained at field capacity (28% moisture as determined by pressure plate) daily, throughout the experiment to prevent any possible stagnant water effects and leaching out of the applied ^{15}N fertilizer. Two-month-old oil palm plantlets (clone P149, from MPOB) were planted at one plantlet/pot with three treatments and four replicates arranged in a randomized complete block design. The treatments were: 1) control [+ killed inoculum (Sp7)], autoclaved at 121°C for 20 min - a non-fixing system reference (Malik *et al.,* 1987), 2) *Azospirillum brasilense* (Sp7) (fixing system) inoculation, and 3) *A. lipoferum* (CCM 3863) (fixing system) inoculation. At planting, roots of the plantlets were soaked and washed clean of the nursery soil before planting them immediately in pots with the Selangor series soil. The plantlets were harvested four months after planting.

Two bacterial cultures of *Azospirillum* spp. were used for inoculation: *A.brasilense* (Sp7) and *A. lipoferum* (CCM 3863), which were obtained from EMBRAPA, Brazil and the Czechoslovakian Collection of Microorganisms, Republic of Czech, respectively. The strains were subcultured in 100 ml Okon medium (Okon *et al.,* 1977) and shaken continuously for 48 hr (150 rpm, 28°C). Each pot was inoculated monthly with the respective inoculum at 20 ml inoculum/pot/application.

The measurements taken in this experiment were: 1) percentage of N_2 fixed, 2) photosynthetic efficiency (light and CO_2 response characteristics), 3) leaf chlorophyll content, 4) total dry weight (tops and roots), 5) root volume (cm^3), and 6) root length (cm). Two weeks before the harvest, the first fully expanded leaf (FEL) from each plantlet was analysed for gas exchange measurements (light and CO_2 response characteristics) using a closed system of Portable Infra-red Gas Analyzer LI-6200®(Licor Inc. Nebraska, USA) which comprised a leaf chamber, infra-red gas analyser and data logger. The light response curve of the inoculated plantlet

was characterized by measuring the photosynthetic rate at eight different intensities of photosynthetically active radiation (PAR) (0-1000 µmole quanta $m^{-2} s^{-1}$, attenuated as necessary using wire mesh filter) at air temperatures between 30°C-37°C with a constant CO_2 concentration of 360 µmole CO_2 $mole^{-1}$. The quantum yield (efficiency of light trapping) was determined by regression analysis of the initial phase of the light response curve.

The CO_2 response characteristics (C assimilation rate) was also based on the photosynthetic rate of oil palm plantlets at eight different concentrations of CO_2 (0-1000 µmole CO_2 $mole^{-1}$) in air passing over the leaf. The CO_2 concentration was varied from saturated level to zero by passing varying proportions of the incoming air through a soda lime column. All responses to CO_2 concentration were measured with PAR of at least 800 µmole quanta $m^{-2} s^{-1}$. The efficiency of carboxylation was calculated by the regression line in the initial phase of the CO_2 response curve. Each plantlet was analysed for leaf greenness using a SPAD meter (Minolta® SPAD-502). The actual leaf chlorophyll content was determined based on the proposed standard curve of SPAD value and leaf chlorophyll content (*Figure 1*).

At harvest, the plantlets were separated into tops and roots, and the roots further soaked and washed clean of the nursery soil. The root volumes and length were determined using the

TABLE 1. N-FREE BASAL FERTILIZER RATES BASED ON RECOMMENDED FERTILIZER APPLICATIONS FOR OIL PALM SEEDLINGS AT THE NURSERY STAGE

Recommended fertilizer rate		N-free fertilizer rate (g plantlet^{-1})		
Months	**Fertilizer application**	**P_2O_5 (TSP)**	**K_2O (MOP)**	**MgO (Kieserite)**
5	NPK Yellow (15:15:6:4) 7 g $palm^{-1}$	1.05	0.42	0.28
6	NPK Yellow (15:15:6:4) 14 g $palm^{-1}$	2.10	0.84	0.56

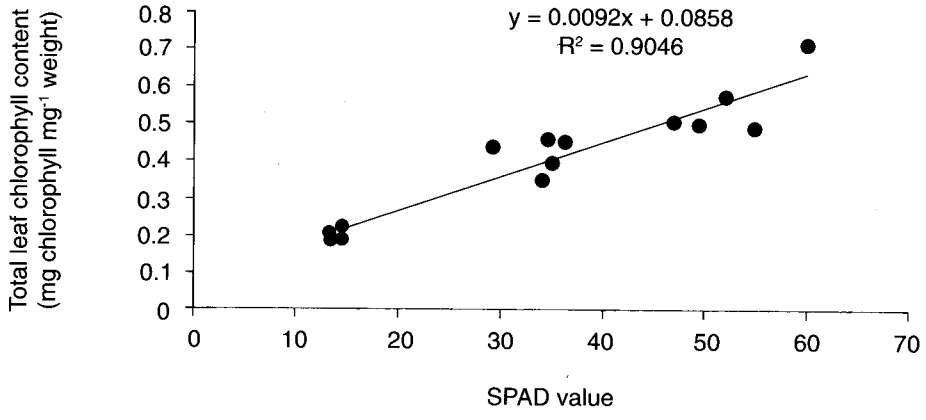

Figure 1. Standard curve for total leaf chlorophyll content (mg choloropyll mg^{-1} leaf fresh weight) for oil palm plantlets.

digital image analysis system, v. 1.12 (1993), Delta T-Device. The samples were dried at 70°C for 48 hr, weighed and ground for total nitrogen analysis by semi-micro Kjeldahl methods (Bremner, 1996). Distillates were collected via Buchi® K314 distillation unit and concentrated for N analysis. Dilution of ^{15}N was determined by mass spectrometer at the International Atomic Energy Agency (IAEA), Vienna. The proportion of biologically fixed nitrogen (%Ndfa) was calculated using the following equation:

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

RESULTS AND DISCUSSION

^{15}N Isotope Dilution Analysis

Azospirillum inoculation caused a significant increase (16%) in shoot nitrogen concentration of the oil palm plantlet when compared to the control (*Table 2*). The ^{15}N% a.e. of the inoculated plantlet shoots was less than that of the uninoculated plants, indicating dilution of ^{15}N due to uptake of biologically fixed nitrogen (Ndfa). The estimation of nitrogen derived from fixation by the plantlets inoculated with Sp7 and CCM 3863, based on ^{15}N isotope dilution analysed by mass spectrometer, amounted to 40.7% and 42.1%, respectively.

TABLE 2. EFFECTS OF *Azospirillum* INOCULATION ON SHOOT NITROGEN CONCENTRATION (%), ^{15}N% ATOM EXCESS (a.e.) AND ESTIMATED BIOLOGICALLY FIXED NITROGEN (% Ndfa) IN OIL PALM PLANTLETS AFTER FOUR MONTHS OF GROWTH IN ^{15}N LABELLED SELANGOR SERIES SOIL

Treatment	Shoot nitrogen conc. (%)	^{15}N % atom excess	% Ndfa
Sp7 killed (control)	3.60b	0.2328a	–
Sp7	3.58b	0.1380b	40.7a
CCM 3863	4.17a	0.1348b	42.1a

Note: means with the same letters are not significantly different at 5% level.

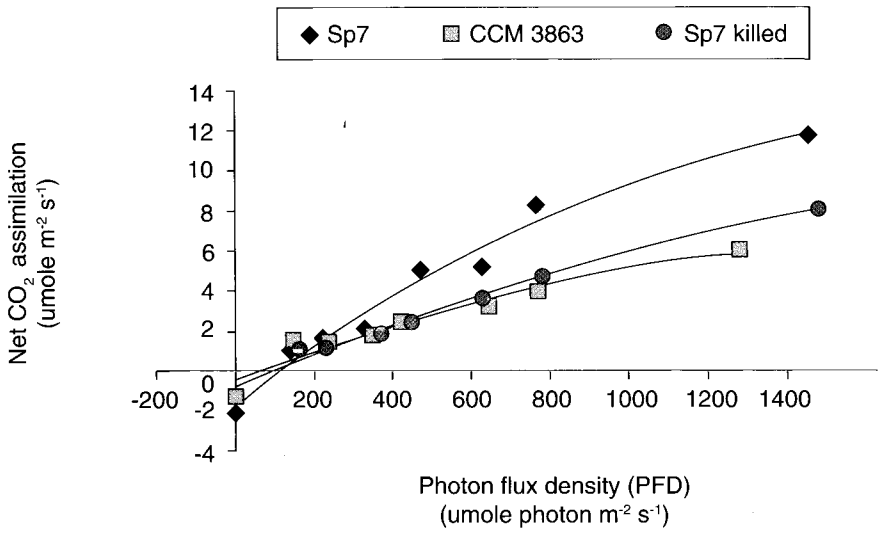

Figure 2. Effects of Azospirillum *inoculation (Sp7, CCM 3863 and Sp7 killed) on the efficiency of light trapping mechanism of oil palm plantlets after four months of growth in Selangor series soil.*

This result is in agreement with previous findings by Dobereiner and Baldani (1998) who suggested that high association of diazotrophic microorganisms (*e.g. Azospirillum*) with oil palm roots, stem and leaves (10^6 cfu g^{-1}) could consequently contribute fixed nitrogen from the atmosphere for growth and development of the host plant. Inoculation of *Azospirillum* spp. to oil palm plantlets grown in a liquid medium under *in vitro* conditions has been shown to contribute from 82%-89% of the total nitrogen requirement of the host plant (Shamsuddin, 1994). George (1990) showed the presence of *Azospirillum* in different root zones of the coconut palm and suggested a possible biological nitrogen fixation process due to this association. De Carvalho *et al.* (1996) has highlighted the occurrence of *Azospirillum* spp. and other groups of diazotrophic microorganisms in the rhizosphere of the pupunha palm in Brazil. These results have clearly demonstrated the efficiency of *Azospirillum* to fix N_2 in association with oil palm plantlets which could partly replace the expensive fertilizer - nitrogen requirement.

Growth of Oil Palm Plantlets

Within the *Azospirillum* inoculation treatments, significant increases in the root dry weight and volume of oil palm plantlets were observed when compared to the control (*Table 3*). Between the inocula used, CCM 3863 produced the highest significant increment in plant top dry weight (33%), root dry weight (37%) and volume (82%) compared to the control, but not with root length. However, the effects of inoculation on leaf chlorophyll content of the host plant compared to the control was not statistically significant. *Azospirillum* was reported to induce and promote plant growth through improvement in mineral and water uptake of the host plant (Kapulnik, 1991). Similar results have also been reported by Shamsuddin *et al.* (1999) and Mia *et al.* (1999) for banana; *Azospirillum* inoculation significantly enhanced growth (tops and roots) and nutrient uptake (N, P, K, Ca and Mg) of banana seedlings.

Photosynthetic Activities of Oil Palm Plantlets

There were significant differences in the detailed analyses of photosynthetic activity (light response) for inoculated (Sp7 and CCM 3863) oil palm plantlets compared to the control (Sp7 killed). The plantlets inoculated with Sp7 showed a significantly higher quantum yield (increase in efficiency of light trapping; mole CO_2 $mole^{-1}$ quanta) and net photosynthesis (increase in A_{max}; µmole CO_2 m^{-2} s^{-1}) (*Table 4* and *Figure 2*). The inoculated plantlets showed a positive trend but no significant response in carboxylation efficiency (Rubisco activity) and RuBp regeneration (J_{max}) capacity when compared to the control (Sp7 killed) (*Table 5* and *Figure 3*).

The higher net photosynthetic rates (A_{max}) at light saturation was related to the higher efficiency of RuBp (Ribulose Biphosphate) regeneration capacity of the host plant. This phenomenon is related to higher stomatal conductance [higher intercellular CO_2 concentration (C_i)] and maximal interception of light through increased leaf surface area and minimal carbon lost in the dark respiration process (Henson, 1991). Bondada and Oosterhius (1998) have pointed out that both the photosynthetic rates and the stomatal conductance of the host plant are related to the nitrogen content in the leaf. The same finding was also reported in cotton by Midgley *et al.* (1999) where, across the plant kingdom, the photosynthetic rates correlated with leaf nitrogen content in elevating higher net photosyn-

TABLE 3. EFFECTS OF *Azospirillum* INOCULATION ON THE GROWTH PARAMETERS OF OIL PALM PLANTLETS AFTER FOUR MONTHS OF GROWTH IN SELANGOR SERIES SOIL

Treatment	Chlorophyll content	Top dry wt. (g)	Root dry wt. (g)	Root vol. (cm^3)	Root length (cm)
Sp7 killed	0.57a	7.49c	3.57b	21.25b	332.35a
Sp7	0.59a	8.58b	3.83b	38.75a	381.41a
CCM 3863	0.61a	9.99a	4.90a	30.00a	361.96a

Note: means with the same letters are not significantly different at 10% level.

TABLE 4. EFFECTS OF *Azospirillum* INOCULATION ON THE LIGHT RESPONSE CHARACTERISTICS OF OIL PALM PLANTLETS AFTER FOUR MONTHS OF GROWTH IN SELANGOR SERIES SOIL

Treatment	Quantum yield (mole CO_2 $mole^{-1}$ quanta)	Net photosynthesis (A_{max}) (μmole CO_2 m^{-2} s^{-1})
Sp7 killed	0.007b	5.9c
Sp7	0.014a	11.6a
CCM 3863	0.007b	7.9b

Note: means with the same letters are not significantly different at 5% level.

TABLE 5. EFFECTS OF *Azospirillum* INOCULATION ON THE CO_2 RESPONSE CHARACTERISTICS OF OIL PALM PLANTLETS AFTER FOUR MONTHS OF GROWTH IN SELANGOR SERIES SOIL

Treatment	Carboxylation efficiency (μmole CO_2 m^{-2} s^{-1})	RuBp reg. capacity (J_{max})
Sp7 killed	0.03a	33.2a
Sp7	0.04a	35.4a
CCM 3863	0.03a	38.3a

Note: means with the same letters are not significantly different at 5% level.

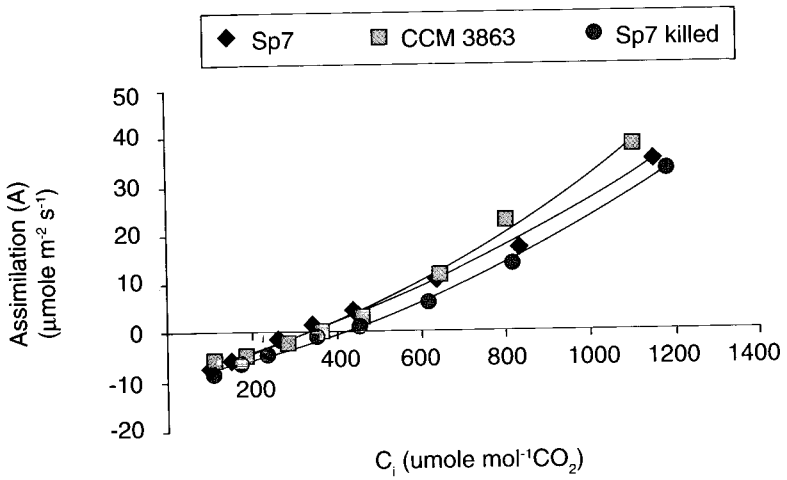

Figure 3. Effects of Azospirillum *inoculation (Sp7, CCM 3863 and Sp7 killed) on carboxylation efficiency of oil palm plantlets after four months of growth in Selangor series soil.*

thetic rates (A_{max}), carboxylation efficiency (effective Rubisco) and highly efficient RuBp regeneration capacity (J_{max}). Effective Rubisco and J_{max} were linearly co-correlated, probably due to nitrogen resource optimization between light harvesting and carboxylation capacity.

There is a need to investigate whether the higher photosynthetic rates are related to the N_2 fixation capacity of the host plant. Quilici and Medina (1998) have shown that the photosynthetic capacity of a N_2-fixer species was higher compared to a mineral nitrogen user, since the

former needed more energy (photosynthate) to cover the requirement of the microsymbiont (diazotrophic microorganism). Reduction in the nitrogen fixation capacity of a nodulated soyabean was related to inadequate translocation of photosynthate product to the rhizobia in root nodule, thereby limiting the carbon compound necessary for the rhizobial energy requirement and carbon skeleton for nitrogen assimilation (Pausch *et al.*, 1996).

CONCLUSION

Association of certain *Azospirillum* strains (Sp7 and CCM 3863) with oil palm plantlets contributed 40% of the nitrogen requirement of the host plant. This association also stimulated top and root growth by 30% and 60%, respectively and increased the photosynthetic rates of the host plant. The experiment indicated that *Azospirillum* spp. is a potential biofertilizer and bioenhancer for the sustainable cultivation and production of oil palm plantlets in an environmental friendly land management. The findings should now be investigated in mature palms.

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