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BIOFERTILIZER AND BIOENHANCER CONCEPTS FOR SUSTAINABLE OIL PALM SEEDLING PRODUCTION

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Introduction

In oil palm production, nitrogen fertilizer is the most expensive nutrient input required. At an average recommended rate of 0.5 to 1.0 kg N/matured palm/year (148 palms/ha) and with an average price of urea at RM 587/tonne, total nitrogen fertilizer cost to the industry is estimated to be RM 470 million/year. These phenomena had synergistically increased cost on oil palm production and make it less profitable especially during the current low price of the commodity. The concept of Biological Nitrogen Fixation (BNF) and plant growth enhancement by diazotrophic microorganisms (*Acetobacter diazotrophicus*, *Herbaspirillum* spp., *Azoarcus* spp., *Azospirillum* spp. and *Bacillus* spp.) in association with non-leguminous crops is becoming increasingly important in attempts to develop a sustainable agricultural system. The process could prevent ground water pollution, save nitrogen fertilizer and reduce the cost in crop production (Hardarson, 1993). Cocking (2000) has highlighted a new technique which could increase the biological nitrogen fixation capacity with cereals and other non-legumes by establishing N_2-fixing bacteria within roots of the host plants. This new inoculation technology is aimed at significantly reducing the use of synthetic nitrogenous fertilizer in the agricultural sector. Inoculation of the diazotrophic microorganisms could also benefit the host plant by improving root development, biomass, yield and nitrogen content (Okon and Kapulnik, 1986). The inocula (*Azospirillum*) have been shown to save at least 67% of inorganic-N requirement in sweet potato cultivation (Saad *et al.*, 1999). Exploitation of BNF concept through application of selected inoculum on oil palm seedlings can potentially save cost on nitrogen fertilizer and make the palm oil industry more profitable (Dobereiner and Baldani, 1998; Shamsuddin *et al.*, 2000b). Thus, this study was conducted to develop a sustainable oil palm fertilization system (especially N) in Malaysia beginning with oil palm seedlings at the nursery stage.

Materials and Methods

Two glasshouse experiments (**Expt. 1 & 2**) were conducted in UPM glasshouse (undrained pots) with Selangor series soil at 8 kg/pot. The soil was maintained at field capacity (28% moisture) and labeled with ^{15}N by adding 100 ml/pot of $(^{15}NH_4)_2SO_4$. **In Expt. 1**, each pot was planted with a two months old oil palm plantlets and applied with three treatments; 1) Control (+ killed inoculum (Sp7), 2) *Azospirillum brasilense* (Sp7), 3) *A. lipoferum* (CCM 3863) and harvested at 120 days after planting (DAP). **In Expt. 2**, newly germinated oil palm seeds were planted at one seed/pot with seven treatments: 1) Control 1 (+ killed Sp7, non-sterile soil), 2) Control 2 (+ killed Sp7, sterile soil), 3) Control 3 ($+N_i$), 4) *A.brasilense* (Sp7), 5) *A. lipoferum* (CCM 3863), 6) Locally isolated rhizobacteria (UPMB 10) and 7) UPMB 13, with three different harvests (130, 260 and 390 DAP). A duplicate of Expt. 2 was conducted under *in vitro* conditions using sterilized tissue cultured oil palm plantlets in large test-tubes (**Expt. 3**). In Expt. 1, effect of the inocula on photosynthetic rates was also determined. The samples

were recorded for dry weight and analyzed for N, P, K, Ca and Mg. The ^{15}N dilution was later analyzed by emission spectrometer at Malaysian Institute of Nuclear Technology (MINT) to estimate the amount of N_2 fixed.

Results and Discussion

Results from the preliminary study have shown the ability of *Azospirillum* spp. to fix N_2 in association with oil palm plantlets grown under glasshouse conditions after 120 days of growth (D_{120}). Inoculation of *A. lipoferum* CCM 3863 and *A. brasilense* Sp 7 showed higher contributions of N from N_2 fixation process (42.1 and 40.7% Ndfa (175.38 and 125.71 mg N shoot^{-1}), respectively), which clearly demonstrated the efficiency of *Azospirillum* to fix N_2 in association with oil palm plantlets. This result leads to the following experiment which involved association of *Azospirillum* and locally isolated *Bacillus sphaericus* UPMB 10 and *B. subtilis* UPMB 13 with oil palm seedlings at D_{130}, D_{260} and D_{390} under glasshouse conditions. In this experiment, the inocula tested also successfully contributed fixed N_2 to the host plant in the range of 25-50% Ndfa (28-800 mg N plant^{-1}) and 30-40% Ndfa (30-500 mgN plant^{-1}), respectively, relative to reference plants inoculated with killed inocula. The highest %Ndfa was recorded at D_{130} with a reduction in the fixation rates at D_{260} for all the inocula tested. Total amount of N_2 fixed was also increased and the highest amount was recorded at D_{390}, which corresponded to a greater plant nitrogen requirement with increased plants growth. Distribution of the fixed N_2 also varied, with more being present in the leaf at D_{130} and in the stem at D_{260}. However, at D_{390}, homogenous percentages of fixed N_2 were found in leaves, stems and roots of the host plants. Estimation of N_2 fixation rates relies on the use of proper non-fixing control plants against which the dilution of ^{15}N enrichment is assessed (Chalk and Ladha, 1999). Contribution of fixed N_2 by the indigenous microorganisms in the non-sterile soils had lowered the $\%^{15}Na.e.$ of the reference plants and reduced the actual estimated rates of N_2 fixed. Besides that, competition with the indigenous microorganisms to fully colonize the host plants had also reduced the N_2 fixation efficiency of the inocula (Giller and Cardish, 1995). Important role of the rhizobacteria (*Azospirillum* and locally isolated *Bacillus* spp.) as a potential N_2 fixer with oil palm seedlings was improved through axenically *in vitro* N_2 fixation trial, which showed a higher contribution of fixed N_2 to the host plants, compared to the glasshouse experiment with non-sterile soil. Inoculation with Sp 7 resulted in higher accumulation of fixed N_2 (66.07% Ndfa; 4.38 mg N_2 fixed) followed by CCM 3863 with a lower fixing capacity (41.67% Ndfa; 2.37 mg N_2 fixed). Meanwhile, inoculation with UPMB 10 and 13 accumulated up to 46.57 and 54.56% Ndfa (2.79 and 3.52 mg N_2 fixed), respectively.

The study had also shown a progressive increment in essential nutrient uptake due to inoculation with the N_2 fixing bacteria. N and K concentration was higher in shoot of the inoculated plantlets compared to the control. In the subsequent glasshouse experiment, response of the inoculation on N, P and K accumulation and concentration were similar or better than the control plants fertilized with inorganic-N_i fertilizer (N_i). However, the N accumulation declined especially at D_{390} although the N_2 fixation rates was the highest, since there was insufficient supply of N from the fixation to compensate the higher N demand by the host plants with time (Rojas *et al.*, 2001). The *in vitro* inoculation trial (sterile conditions) also highlighted a significant increment in total N content of the inoculated host plants compared to the control (Sp 7 k). However, the response was comparable to the control supplied with complete fertilizer-N.

The inocula tested (*Azospirillum* and locally isolated *Bacillus* spp.) are potential plant growth promoting rhizobacteria (PGPR) through enhancement in top and root growth of the host plants. Inoculation with CCM 3863 has shown up to 37% increment in root dry weight while Sp 7 has increased root volume (82%) and length, indicative of stimulatory effects of

the inocula in promoting higher root hair formation of the host plants. Besides that the inoculation process also enhanced the plant growth through higher accumulation of dry matter, top dry weight and root dry weight which could be related to the potential of the inoculum to fix N_2. Inoculation of CCM 3863 and locally isolated *Bacillus* spp. (UPMB 10 and UPMB 13) had stimulated root dry weight, volume and primary root numbers of the host plants, from the initial phase (D_{130}) until the end of the experiment (D_{390}). The trends were similar to the control plants supplied with inorganic fertilizer-N_i (Sp 7 k +N_i). One of the alternative explanations for the observed plant growth stimulation by *Azospirillum* inoculation involves the production of plant growth regulatory substances by the bacterium. Three types of plant growth promoting substances could be detected in the supernatant of *Azospirillum* cultures (auxins, cytokinins and gibberellins). The quantitatively most important phytohormones produced by *Azospirillum* is the auxin indole-3-acetic acid (IAA). Bacterial phytohormones production is assumed to cause the detected changes in root morphology after *Azospirillum* inoculation, which in turn may be related to enhanced mineral uptake (Kapulnik *et al.*, 1985b; Jain and Patriquin, 1985). Similar stimulatory effects of inoculation treatments on growth of the host plants were also shown in the *in vitro* trial. The inoculation process had affected root growth and development including primary and secondary root formation and primary root length of the host plants. Enhancement in root formation was significantly better compared to the control plants with fertilizer-N_i. In the earlier growth stage (D_{130}) the inoculation process significantly stimulated top dry weight, total leaf area and leaf chlorophyll content of the host plants. The inoculation process also enhanced top dry weight of the host plants at D_{260} but not until D_{390}. Response of the inoculation process on leaf chlorophyll content was only significant at D_{130} but not at D_{260} and D_{390}, especially in the glasshouse experiment which could be related to insufficient supply of N for the host plant requirement (Rojas *et al.*, 2001). Enhancement in plant growth (tops) was related to the N_2 fixation capacity of the inocula tested and lower contribution of fixed N_2 for plant N requirement was reflected in lower top dry weight and leaf area of the host plants. The phenomenon was further strengthened by a reduction in total leaf chlorophyll content of the host plants. However, the dry matter accumulation for the respective plants was significantly influenced by different inoculation treatments especially at D_{130} and D_{260}. Response of the inoculation treatments was higher or similar compared to the control with complete fertilizer-N, but not at D_{390}. Similar response of inoculation was also shown in enhanced dry matter production of the plantlets under *in vitro* conditions although the response was less effective compared to the control fertilized with full N_i (Sp 7 k +N_i). Similar trend of results has also been shown by Rojas *et al.* (2001) that the average number of true leaf formation of mangrove seedlings had increased due to *Phyllobacterium* sp. and *Bacillus licheniformis* inoculation. This phenomenon indicated a direct transfer of nitrogen from the N_2 fixing bacterium to the host plant. However, the total nitrogen content of the plant decreased and might be speculated that the supply of nitrogen by the N_2 fixing bacterium was insufficient to keep pace with the increased growth of the seedlings and the N requirement.

The inoculated host plants also showed higher photosynthetic activities compared to the control (Sp 7 k), which is important to maintain the N_2 fixation ability of the inocula tested. This experiment has shown that inoculation with Sp 7 significantly enhanced the photochemical efficiency (50% increment in efficiency of ATP formation) and light saturated photosynthesis of the host plants. Higher ATP formation of the inoculated plantlets is important in maintaining efficiency of the N_2 fixation process by the diazotophs, since the process (A_{max}) require high amounts of energy and consequently benefit the host plants through the N_2 fixed. James (2000) has proposed that endophytic diazotrophs actually fix N_2 *in planta* and transfer the fixed-N products to their hosts in return for the photosynthate provided by the host plants. Symbioses between legumes/rhizobia, actinorhizal plants/*Frankia* and

Azolla/Anabaena, all involve a significant net transfer of N from the microsymbionts to the host in return for photosynthetically derived carbon substrates from the host plants.

Another factor that will ensure success of the inoculation process, which would benefit the host plant, is through ability of the inoculum to establish with the host. Burdman *et al.* (2000) have pointed out that the attachment of *Azospirillum* to the roots of the host plants is essential for the establishment of an efficient association. *Azospirillum* and locally isolated rhizobacteria have been shown to successfully colonize roots of oil palm plantlets, which is vital to ensure successful establishment of the inocula with the host plant. Shishido *et al.* (1999) have highlighted the ability of *Bacillus* and *Pseudomonas* to colonize spruce seedlings endophytically under controlled environment. It also raises the possibility of planting 'pre-inoculated seedlings' with well-established endophyte population at reforestation sites. Similar findings have also been highlighted by Struz and Nowak (2000) for early establishment of selected communities of endophytic micoorganisms within the root systems of any planting materials (tissue culture technique) before transplanting to the soils.

Conclusion

The rhizobacterial strains tested; Sp 7, CCM 3863, UPMB 10 and UPMB 13 are potential biofertilizer for oil palm seedlings with a N_2 fixing capacity of 25-50% Ndfa (28-800 mg N plant^{-1}). The response was more promising (40-60% Ndfa) in the *in vitro* experiment under sterile conditions with no competition from indigenous microbes to colonize the roots and provide beneficial effects to the host plant. The inoculation process had also stimulated uptake of essential plant nutrients especially N, P and K; thus the PGPR could be considered a biofertilizer and bioenhancer for oil palm seedling. As a PGPR the inocula had successfully colonized the root-surface of the host plants, enhanced growth and development of roots (root dry weight, volume and primary root numbers), tops (total dry matter, top dry weight and chlorophyll content) and promoted photosynthetic activities of the host plant. The inoculation process could also shorten the one-year nursery period of the seedlings to only 8 months before being transplanted to the field. These strains are suitable for sustainable oil palm seedling production and environmental friendly oil palm cultivation. In addition to that, more in-depth studies on the effects of phytohormones on growth of oil palm seedling need to be done to strengthen the biofertilizer and bioenhancer concepts.

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