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Effect of Plant Growth-Promoting Rhizobacteria on Root Formation and Growth of Tissue Cultured Oil Palm (*Elaeis guineensis* Jacq.)

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Abstract: An experiment was conducted to enhance rooting initiation for oil palm shootlets by using diazotrophic rhizobacteria (locally isolated *Acetobacter diazotrophicus* (R12) and *Azospirillum brasilense*, Sp7 (ATCC 29729)) and to observe the effects of inoculation on growth of oil palm plantlets under *in vitro* conditions. The experiment was laid out in a completely randomized design with twenty-five replicates and harvested after 60 days of growth (D_{60}). The observations involved N_2 fixation activities by acetylene reduction assay, root fresh weight (g), primary and secondary root numbers, leaf chlorophyll content and bacteria-root colonization at D_{60}. Results of the experiment showed that locally isolated *A. diazotrophicus* (R12) and *A. brasilense* (Sp7), were found to have the potential of inducing root formation of tissue cultured oil palm shoots (*Elaeis guineensis* Jacq. clone L295-1/177-1/16) and better shoot growth. *A. brasilense* (Sp7) could induce better rooting on the *in vitro* oil palm shoots compared to *A. diazotrophicus* (R12). Positive interactions between the bacterial inocula and roots of tissue cultured oil palm plantlets were recorded, which resulted in enhancing growth of the host plants. The plantlets inoculated with Sp7 showed the highest fresh weight (g) followed by control 1 (MS + NAA), R12 and Control 2 (Sp7k) at D_{60}. The root fresh weight (g) and number of primary and secondary roots of the inoculated plantlets (Sp7 and R12) were also high compared to the controls. The Acetylene Reduction Assay (ARA) indicated the N_2 fixation ability of the diazotrophs (Sp7 and R12 could fix up to 0.965 and 1.181 μmol C_2H_4 g^{-1} fresh weight h^{-1}, respectively). The inocula could hence be used as microbial fertilizer for the clonal propagated plantlets of oil palms.

Key words: Acetylene reduction assay, *Acetobacter diazotrophicus*, *Azospirillum brasilense*, microbial fertilizer, N_2 fixation, root induction, bacterial-root colonization

INTRODUCTION

Higher nutrient demand and high cost of mineral fertilizers for oil palm industry has encouraged the growers to find cheaper alternatives that may contribute to efficient nutrient use and better profits. Application of plant growth enhancer or microbial fertilizer (diazotrophic rhizobacteria) to oil palm can be considered as one of the potential alternatives to the mineral fertilizers that required by the host plants (Vestberg *et al*., 2004). It could successfully benefits the host plants through the ability to fix N_2 biologically and to produce phytohormones (Kefalogianni and Aggelis, 2002; Park *et al*., 2005). Potentials of the beneficial diazotrophs were found to be important for the establishment and growth of the host plants (Tsimilli-Michael *et al*., 2000). It has been successfully applied and tested to rice, sugarcane and oil palm plantlets (Elbeltagy *et al*., 2001; Biswas *et al*., 2000; Sevilla *et al*., 2001; Amir *et al*., 2001). Maximal potentials of the diazotrophs in promoting growth and development of the host plants could be achieved *via* fostering early bacterial colonization within the root systems of the host plants (James *et al*., 2001). Establishment of the inocula within the tissues of the host plants could be achieved through inoculating the tissues aseptically with diazotrophs as early as the callus stage (Varga *et al*., 1994). After inoculation, the bacteria will colonize on the surface and the inner tissues of the callus and will continue to remain in the tissues until regeneration of the plantlets.

The nitrogenase enzyme-complex is responsible for biological nitrogen fixation process for both symbiotic and associative diazotrophic rhizobacteria. In the process, the atmospheric nitrogen gaseous will be reduced to ammonia (NH_3) and ammonium (NH_4^+) involving nitrogenase enzyme and ATP (Azam and Farooq, 2003).

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Though molecular nitrogen is the natural substrate for nitrogenase, other triple bonded molecules like acetylene (C_2H_2) can also undergo the reduction process mediated by nitrogenase. This process is known as the Acetylene Reduction Assay (ARA) (Somasegaran and Hoben, 1985; Azam and Farooq, 2003). Hence, the ARA was used to quantify nitrogenase enzyme activities of the diazotrophic rhizobacteria in association with the oil palm plantlets. Thus, the objectives of this study, 1) observe effects of nitrogen fixing rhizobacteria (diazotrophs) on rooting induction and root colonization of tissue cultured oil palm shoots, 2) to estimate the N_2 fixation potentials and to observe growth enhancement effects of the inocula to the associated oil palm plantlets and 3) to determine the possibility of using the diazotrophs as microbial fertilizer for tissue cultured oil palm plants.

MATERIALS AND METHODS

The experiment was conducted in Plant Tissue and Cell Culture Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang. The oil palm (*Elaeis guineensis* Jacq.) shootlets clone L295-1/177-1/16 were obtained through *in vitro* clonal propagation by somatic embryogenesis origin bred at the Biotechnology and Breeding Station, Malaysian Palm Oil Board (MPOB), Malaysia. Each vigorous shoots 5-7 cm sized (with 2-3 leaves) were selected and manually isolated from bunches and cultured in a test tube (Pyrex™ 150 mm length × 25 mm diameter) containing modified N-free (MS) liquid medium (pH 5.7) (Murashige and Skoog, 1962; Rohani *et al*., 2003). The shoots were cultured on i) Murashige and Skoog (MS medium supplemented with 16.7 mg L^{-1} NAA (Control 1), ii) N-free MS, + *Azospirillum brasilense*, Sp7 (ATCC 29729); 4.3×10^7 cfu mL^{-1}, iii) N-free MS, + *Acetobacter diazotrophicus*, R12 (locally isolated from oil palm roots); 5.5×10^9 cfu mL^{-1} and iv) N-free MS, + killed *Azospirillum brasilense* (Sp7k; Control 2). After 60 days of growth (D_{60}), ten of the plantlets were randomly sampled for analyzing the root formation (primary and secondary roots), shoot fresh weight, root fresh weight and leaf chlorophyll content. The nitrogenase enzyme activities were estimated by using the Acetylene Reduction Assay (ARA). The roots were then fixed with McDowell fixative (McDowell and Trump, 1976) for bacterial colonization study using Field Emission Scanning Electron Microscope (FESEM) (Leo Supra 50 VP). The remaining plantlets were transferred into polybags containing 30 g of soil mixture (soil, sand and coconut husk; 1:1:1) for acclimatization in the glasshouse. After 4 weeks of growth, the plants were transferred into bigger polybags (60×90) containing 1 kg of the same soil mixture. Then the survival rates of the tissue-cultured plants were determined (Aslin *et al*., 2005).

Bacterial culture: *Azospirillum brasilense*, Sp7 (ATCC 29729) and locally isolated *Acetobacter diazotrophicus,* R12 were used as the plant growth enhancers. The inocula were subcultured into nitrogen free broth medium (100 mL $flask^{-1}$) which contained in each liter of distilled water KH_2PO_4 0.4 g, K_2HPO_4 0.1 g, $MgSO_4$ 0.2 g, NaCl 0.1 g, $CaCl_2$ 0.02 g, $FeCl_3$ 0.01 g, Na_2MoO_4 0.002 g, malic acid 5.0 g and yeast extract 0.5 g (Eskew *et al*., 1977). The flasks containing the bacterial inoculum were shaken on a rotary shaker at 160 rpm for 48 h at room temperature (30-32°C). Prior to every inoculation process, each inoculum was determined for optical density (OD_{600nm}) and colony-forming unit, cfu mL^{-1}.

Acetylene reduction assay: The assay (ARA) was performed on oil palm plantlets immediately after 60 days of culture (D_{60}). The plantlets were transferred to sterile airtight bottles (64 mL) aseptically. By using an airtight syringe, 10% of the air from the head-phase of each bottle was removed and replaced by purified acetylene gas (99.8%). The plantlets were incubated for 30 min for the reduction of acetylene to ethylene. At the end of the incubation period, 5 mL of the gas mixture was withdrawn and transferred into a sterile vacuum tube (Vacutainer™ 7 mL) to assay for ethylene by using G-300 Hitachi gas chromatography (fitted with carboxen 1004 micro packed, 2 m × 1/16 in stainless steel column and Flame Ionization Detector (FID)). Nitrogen was used as the carrier gas with a flow rate of 3.5 kgf cm^{-2} and the temperatures were maintained at 165°C (column) and 230°C (injector and detector), respectively.

Experimental design: The experiment was laid out in a completely randomized design with four inoculation treatments and twenty-five replicates. The data were statistically analyzed by the statistical procedures as in SPSS v11.0. Following the analyses of variance procedure, differences among treatment means were determined using the Least Significant Difference (LSD) comparison methods, $p = 0.05$.

RESULTS AND DISCUSSION

The results of the study indicated that the fresh weight of oil palm shoots was influenced by the inoculation treatments. The *in vitro* oil palm shoots cultured on N-free MS medium and inoculated with *A. brasilense*, Sp7 produced high shoot biomass although

it was not significantly different compared to the biomass of shoots cultured on nitrogen enriched MS medium supplemented with naphthalencacetic acid (NAA). However, the biomass of oil palm shoots cultured on N-free MS medium, inoculated with *A. diazotrophicus*, R12 and Control 2 (N-free MS medium + killed *A. brasilense*, Sp7k) were much lower. The *in vitro* oil palm plantlets inoculated with Sp7 also produced higher root biomass and more secondary roots compared to the plantlets supplemented with 16.7 mg L^{-1} NAA (Control 1), R12 and Sp7k (Control 2). This indicated that Sp7 could induce better rooting on the *in vitro* oil palm shoots compared to R12 (Table 1). Well developed root system of the plantlets inoculated with Sp7 after 60 days of growth could have contributed to higher shoot fresh weight due to efficient nutrient and water uptake of the plantlets. Developments of the root systems were due to effective colonization of bacterial inoculums which had changed the root morphology and induced additional root hairs and lateral roots formations (Torres-Rubio *et al*., 2000). Another suggested mechanism which could directly influence root developments was phytohormone production by the bacteria which colonizing the root systems of the host plants (Steenhoudt and Vanderleyden, 2000; Mantelin and Touraine, 2004). Similar beneficial effects of microbial inoculants to *in vitro* plantlets were noted in potato with increased plant's stem length, shoot and root biomass (Bensalim *et al*., 1998). Presence of endophytic *Flavobacterium* sp. had also promoted growth and rooting of statice (*Limonium sinuatum*) (Van Zaayen *et al*., 1992). Similarly, Sevilla *et al*. (2001) have shown enhanced growth of sugar cane due to early inoculation of wild type *Acetobacter diazotrophicus*.

The ARA results indicated that the plantlets inoculated with Sp7 and R12 had the capabilities of reducing acetylene to ethylene at 0.965 and 1.181 μmol C_2H_4 g^{-1} fresh weight h^{-1}, respectively. However, no ethylene gas was produced from the control treatments (Control 1 and 2). The ability to reduce acetylene to ethylene had indicated the N_2 fixation potentials of the inocula tested in association with oil palm plantlets. Several inoculation studies had proven that diazotrophs could contribute biologically fixed nitrogen and later enhanced growth of the host plants such as oil palm plantlets, rice and sugarcane (Amir *et al*., 2001, 2003; Elbeltagy *et al*., 2001; Sevilla *et al*., 2001). Elbeltagy *et al*. (2001) had detected the nitrogenase activities in rice seedlings associated with *Herbaspirillum* sp. Higher nitrogenase activity was also reported for *in vitro* sugar beet plants inoculated with *Azotobacter chroococcum* (Mrkovaèki *et al*., 1997). In the associative relationship between the bacterium and the host plant, the former will provide fixed N_2 in the form that can be utilized by host plant.

Efficiencies of the nitrogen fixation activities were found to be correlated with the degree of bacterial root colonization on the root surfaces of the host plants. The scanning electron micrograph showed that both Sp7 and R12 had colonized and attached to the surface and the ruptured epidermal cells of the roots of the *in vitro* oil palm plantlets (Fig. 1a-d). It was reported earlier that associative diazotrophs such as *Azospirillum* sp. could colonize the root hairs, elongation zone, between the epidermal cells and the outer cortices of the host plants (Baldani and Baldani, 2005). As shown in Fig. 1a and b, Sp7 were attached to the root surfaces and between the epidermal cells of the roots. However, the colonization pattern of R12 were different, since it tend to group together only on the root surfaces (Fig. 1c and d). According to Dalla Santa *et al*. (2004), the bacterial cells tend to attach and aggregate on the root surfaces due to high chemo-attractant concentrations. The root could be a specific attachment site for the putative receptor (capsular glycoprotein, polysaccharides, fimbriae and flagella) of the bacteria, contributed to bacterial adhesion to the root surfaces and hence improved growth of the host plants (Karpati *et al*., 1999; Vande Broek *et al*., 1998). There were differences in the morphology of the root epidermal cells between the plantlets inoculated with Sp7 and R12. The plantlets inoculated with R12 showed more ruptured regions between the epidermal cells (Fig. 1c) suggesting that more R12 could be colonized within the intercellular spaces which could suggest for higher nitrogen fixation activities by R12. *Acetobacter diazotrophicus* is also known as endophytic diazotrophic

Table 1: Inoculation effects of *A. brasilense* (Sp7) and locally isolated rhizobacteria *Acetobacter diazothrophicus* (R12) on shoot fresh weight (g), root fresh weight (g), primary and secondary root formation, acetylene reduction (μmol C_2H_4 g^{-1} fresh weight h^{-1}), leaf chlorophyll content (mg chlorophyll mg^{-1} leaf fresh weight) of oil palm plantlets at D_{60} and survival rate of acclimatized bacterized oil palm plantlets

Treatments	Fresh weight (g)		Root (No.)		Acetylene reduction	Leaf chlorophyll	Survival rate
medium	Shoot	Root	Primary	Secondary	assay	content	(%)
N-free MS + Sp7	3.14^{a}	0.102^{a}	2^{a}	5^{a}	0.965^{a}	0.240^{a}	86.0
N-free MS + R12	2.51^{b}	0.076^{a}	1^{b}	3^{b}	1.181^{a}	0.231^{a}	100.0
MS + NAA 16.7 mg L^{-1} (Control 1)	2.67^{ab}	0.085^{a}	2^{a}	2^{b}	0.000^{b}	0.218^{a}	63.0
N-free MS +Sp7k (Control 2)	2.14^{b}	0.024^{b}	1^{b}	3^{b}	0.000^{b}	0.168^{b}	86.0

Mean values in each column with the same letter(s) are not statistically significant (Least Significant Different Test, (LSD)), p = 0.05)

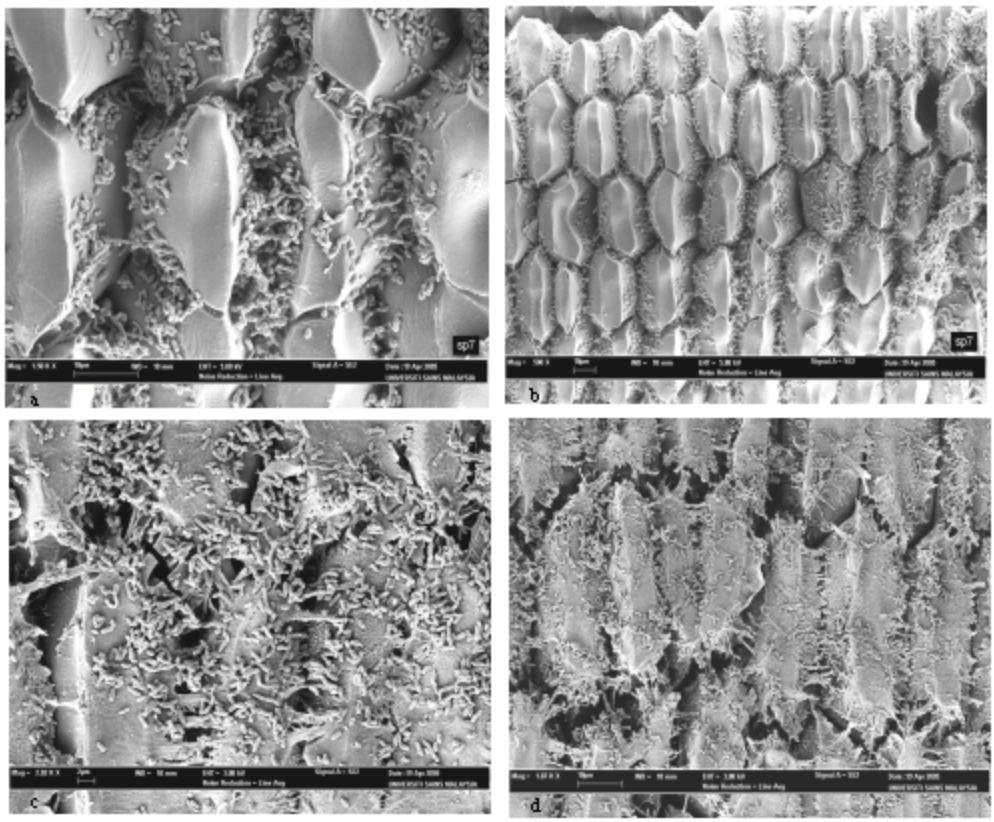

Fig. 1: Scanning electron micrographs showing (a, b) *Azospirillum brasilense*, Sp7 and (c, d) locally isolated *Acetobacter diazothrophicus*, R12 attached and colonizing the epidermal cells of oil palm roots 60 days after inoculation

rhizobacteria which were able to penetrate deeply into plant and root tissues (James *et al*., 2001) and effectively fixing N_2 in association with the host plants (Mehnaz and Lazarovits, 2006).

Development of improved root system by the rhizobacteria would later improved growth of the host plants due to enhance mineral and water uptake of the hosts. The response was shown successfully in the higher leaf chlorophyll content of the plantlets inoculated with Sp7 and R12 compared to Control 2 (plantlets cultured in N-free MS, + killed Sp7). Tilak *et al*. (2005) reported that *Azospirillum* sp. inoculation had improved root growth and later enhanced mineral and water uptake for both maize and rice host plants.

For the acclimatization process in the glasshouse, most of the inoculated oil palm plants could survive and grew well compared with uninoculated plants supplemented with inorganic nitrogen fertilizer (Control 1). The plants inoculated with R12 showed 100% survival rates, while only 86% of the plants inoculated with Sp7 survived after the acclimatization process. Sevilla *et al*. (2001) had reported earlier that the sugarcane plantlets inoculated with *Acetobacter diazotrophicus* could survive and grew well after transferred to the field. Vestberg *et al*. (2004) had also shown that effective transplanting and good growth promotion process were recorded for strawberry plantlets inoculated with *Bacillus subtilis* and *Pseudomonas fluorescens*. Frommel *et al*. (1991) also stated that survival of *in vitro* grown bacterized plants was 20 to 50% higher than the plants without bacterial inoculation after transplanted to the field. The results indicated or proved the beneficial effects upon bacterial inoculation into the host plants. In addition to N_2 fixation and phytohormone production, the bacteria could also suppress disease by inducing systematic resistance in the plants against root and foliar pathogens. Besides that, the bacteria could suppress deleterious microorganisms that are harmful to the host plants.

The experiment successfully showed that the rhizobacterial inoculation could promote root induction and growth of oil palm shootlets and later colonize the newly formed root surfaces. In the association, the rhizobacteria will benefits the host plants through N_2 fixation activities and phytohormone production.

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