INFLUENCE OF INDOLE-3-ACETIC ACID (IAA) PRODUCED BY DIAZOTROPHIC BACTERIA ON ROOT DEVELOPMENT AND GROWTH OF in vitro OIL PALM SHOOTS (Elaeis guineensis Jacq.)

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INTRODUCTION

Diazotrophic plant growth-promoting rhizobacteria (PGPR) have the capabilities to stimulate plant growth by fixation of N₂, solubilisation of minerals and production of phytohormones (Bashan and de Bashan, 2005). The diazotrophs were reported to produce phytohormones such as indole-3-acetic acid (IAA) which is important in promoting plant growth (Arshad and Frenkenberger, 1991; Bashan et al., 2004). IAA is one of the common physiologically active auxins and it is a common product of L-tryptophan metabolism produced by PGPR which may result in pronounced effects on plant growth. Among the PGPR species, Azospirillum...
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is well-known for its ability to excrete auxins. The phytohormone causes morphological and physiological changes to the inoculated plant roots, which leads to plant-bacteria interaction (Costacurta and Vanderleyden, 1995; Tien et al., 1979; Harari et al., 1988; Pedraza, 2008; Malhotra and Srivastava, 2009). The modification, in turn, can affect root growth, leading to better nutrient and water absorption by the plant hosts (Bashan et al., 2008). It may result in bigger and more branched roots, thus providing a larger root surface area by which the host plant can access more nutrients (Vessey, 2003). The diazotrophs offer interesting perspectives for alternative fertilisation approaches which significantly influence plant growth (Baldani et al., 1983; Ladha and Reddy, 2000). It has been successfully applied and tested on rice, sugar-cane and oil palm seedling (Elbeltagy et al., 2001; Amir et al., 2001; 2003; Muthukumarasamy et al., 2006; Azlin et al., 2007; 2009; Noor Ai’ishah et al., 2010; Keyeo et al., 2011). Thus, the objectives of this study were to estimate IAA productivity of free-living diazotrophs and to observe the effects of IAA produced on root development and shoot growth of in vitro oil palm plantlets.

MATERIALS AND METHODS

Bacterial Isolates

Three locally-isolated diazotrophic rhizobacteria were tested in this study. These bacteria [Microbacterium sp. (E7), Acetobacter sp. (E9) and Microbacterium sp. (E14)] were isolated from root tissues of oil palm planted under field conditions (Azlin et al., 2005). Identification was done via 16S rDNA sequence analyses. Herbaspirillum seropedicae (Z78) (ATCC 35893) was used as a positive control, whereas killed Z78 was the negative control.

The Production of Indole-3-Acetic Acid and Viability of Free-living Diazotrophs

The IAA production by free-living diazotrophs (Z78, E7, E9 and E14) was assayed using high performance liquid chromatography (HPLC) system. The bacteria were cultured in a 250 ml conical flask containing 100 ml N-free liquid medium supplemented with 0.5 g litre⁻¹ L-tryptophan as a physiological precursor for biosynthesis of IAA in plants and microbes (Glickmann and Dessaux, 1995). The bacterial cultures were shaken at 160 rpm in room temperature and were harvested at every 12 hr intervals for the production of IAA and the viable cell count (cfu ml⁻¹) until 96 hr of growth. The experiment was laid out in a randomised complete block design (RCBD) with triplicates for each isolate at each harvesting time.

Bacterial cells were separated by filtration through a membrane filter (Whatman TM, 0.20 µm). The filtrate was acidified to pH 2.8 and reconstituted in 1.0 ml acetonitrile (Asghar et al., 2002; Patten and Glick, 2002). A total of 20 µl of filtrates were subjected to the HPLC system equipped with UV-VIS variable-wavelength detector (SPD-10 AVP Shimadzu) and two Shimadzu LC-10 ATVP reciprocating pumps. The analysis was carried out in a low-gradient condition with C-18 reverse phase HPLC column, Supelco (LC 18-DB, 3.3 cm x 4.6 mm ID x 3 µm particle size) at ambient temperature. The analysis was conducted using 1% (v/v) acetic acid adjusted to pH 2.8 as solvent A and 100% acetonitrile as solvent B. The gradient programme for solvent B was adjusted from 13% to 20% and from 20% to 35% within 1.25 min and 5 min, respectively. The run time was 5 min at a flow rate of 1.0 ml min⁻¹ with a wavelength of 280 nm. The peak retention time and areas were compared with the IAA standard curve. The standard curve was prepared from serially diluted 98 µg ml⁻¹ (ppm) of 98% purity (Merck) IAA in the range of 0 to 16 µg ml⁻¹. A total of 0.01 g IAA was dissolved in 95% ethanol and the volume was made up to 100 ml with deionised water. The standard solution (20 µl) was injected into the HPLC column. The retention time and peak area for each concentration were recorded (Asghar et al., 2002; Patten and Glick, 2002).

Growth of in vitro Oil Palm Shoots Inoculated with Diazotrophs

Tissue-cultured oil palm shoots (Elaeis guineensis Jacq.) of the clone S69 1.436-1/26 T-1/10 were obtained from the Tissue Culture Laboratory of the Malaysian Palm Oil Board (MPOB), Bangi, Selangor. The shoots were individually transferred aseptically into the shoot development medium. Shoots of more than 5 cm in height and at two to three leaf-stages were considered ready for root initiation in the rooting medium supplemented with plant hormone. Selected in vitro oil palm shoots with vigorous growth were transferred into N-free MS liquid medium for root induction stage. The inoculation treatments were: 1) + Herbaspirillum seropedicae (Z78), 2) + Microbacterium sp. (E7), 3) + Acetobacter sp. (E9), 4) + Microbacterium sp. (E14), 5) + Z78 killed (Z78K) and 6) N-enriched MS medium [+ 1.90 mg litre⁻¹ KNO₃ + 1.65 mg litre⁻¹ NH₄NO₃ + 16.7 mg litre⁻¹ NAA (naphthalene acetic acid)]. The inoculated shoots were monitored for growth and root initiation after 90 days of growth (D₉₀). Shoot height, number of new shoot formation, number of secondary roots initiated, shoot fresh weight and shoot protein content were determined and recorded. The experiment was laid out in a RCBD.
RESULTS AND DISCUSSION

IAA Production and Viability of Free-living Diazotrophs

In this study, the results revealed that all isolates tested were capable of producing IAA (Figure 1a). This finding is in line with other studies by Vessey (2003), Bashan et al. (2004) and Gravel et al. (2007), which reported that diazotrophic bacteria were not only able to fix atmospheric nitrogen but were also capable of producing plant growth regulators such as auxins. IAA is one of the most studied natural auxins, which was reported to be able to stimulate root growth and thus, consequently increases access to more nutrient uptake from the soil.

As shown in Figure 1a, isolate Z78, E7, E9 and E14 produced IAA in concentrations ranging from 0.2 to 11.5 µg ml⁻¹ within 96 hr of growth incubation. The productivity started to increase rapidly during the log phase growth stages of the isolates tested (Figures 1a and 1b). During this phase, the cells were very well adapted to their environment and were rapidly multiplying. Furthermore, this is a period of balanced bacterial cell growth, in which all components of a cell grow at the same rate (Shuler and Kargi, 2002). This is in agreement with El-Khawas and Adachi (1999), who reported that Azospirillum brasilense produced more IAA during the log (exponential) growth stages from 48 hr until 96 hr of incubation. Results have recorded the highest concentration of IAA produced at 11.542 µg ml⁻¹ (0.318 µg ml⁻¹ of IAA production rates) after 60 hr of bacterial growth (Figure 1a, Table 1). In addition, optimal IAA productivity of isolate E7 also propelled the highest maximum overall IAA productivity of 0.185 µg ml⁻¹ hr⁻¹ (Table 1). Similarly, maximum IAA productivity of Z78 was recorded at 0.261 µg ml⁻¹ hr⁻¹ (10.058 µg ml⁻¹ of IAA produced) after 60 hr of incubation (Table 1). Subsequently, the IAA was consistently produced at 72 hr, 84 hr and 96 hr in concentrations ranging from 10.746 to 11.057 µg ml⁻¹. The overall IAA productivity of Z78 was 0.165 µg ml⁻¹ hr⁻¹, while the viable cell count of Z78 increased gradually and was stable until the end of the incubation period (96 hr) at 1.05 x 10¹³ cfu ml⁻¹ (Figure 1b). Whereas, IAA production of other isolates declined, while reflecting the decline in viable cell numbers. The results exhibited that higher viable cell numbers of Z78, E7 and E14 (which was up to 10¹⁰ cfu ml⁻¹) influenced the production of optimum IAA at 84 hr, 60 hr and 48 hr of incubation, respectively (Figure 1b).

Similar studies by Baca and Elmerich (2007) and Bashan et al. (2008), found that the amount of IAA obtained varied based on the species and strain as well as on the conditions of their cultivation, including the presence of tryptophan, oxygenation level, pH and growth phase. Similarly, our results also exhibited that the addition of 0.5 g ml⁻¹ L-tryptophan into the culture medium stimulated the excretion of the IAA, which implicated the role of exogenous tryptophan as a precursor of the IAA, and supports the existence of a tryptophan dependent route for IAA biosynthesis. Such routes are known to induce multiple enzymatic pathways in both plants and bacteria (Pedraza et al., 2004; Baca and Elmerich, 2007).

Overall, amongst the isolates tested, E14 showed high IAA productivity at 0.432 µg ml⁻¹ hr⁻¹, during the mid-exponential growth phase (48-60 h). Concentration of IAA recorded at the maximum productivity rate was 9.066 µg ml⁻¹ (Table 1), whereas, the overall productivity was 0.180 µg ml⁻¹ hr⁻¹ which was higher compared to E9 and Z78 (Table 1). However, IAA productivity by isolate E9 was much lower even at the maximum cell density (2.55 x 10¹¹ cfu ml⁻¹). The IAA productivity for E9 peaked slightly at 36 hr and gradually declined thereafter until 96 hr of incubation, giving a low

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Maximum productivity (µg ml⁻¹ hr⁻¹)</th>
<th>Concentration at maximum productivity (µg ml⁻¹)</th>
<th>Overall productivity (µg ml⁻¹ hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z78</td>
<td>0.261c</td>
<td>10.058c</td>
<td>0.165c</td>
</tr>
<tr>
<td>E7 (Microbacterium sp.)</td>
<td>0.318c</td>
<td>11.542cd</td>
<td>0.185c</td>
</tr>
<tr>
<td>E9 (Acetobacter sp.)</td>
<td>0.117b</td>
<td>2.795b</td>
<td>0.059b</td>
</tr>
<tr>
<td>E14 (Microbacterium sp.)</td>
<td>0.432c</td>
<td>9.066c</td>
<td>0.180c</td>
</tr>
<tr>
<td>Z78K (Control)</td>
<td>0a</td>
<td>0.066a</td>
<td>0a</td>
</tr>
</tbody>
</table>

Note: Means in the same column followed by the same letter do not significantly different at P < 0.05.
of new leaves formation. This was probably because *Herbaspirillum seropedicae* is a facultative endophytic bacterium that is known to promote growth and yield of the host plants due to N$_2$ fixation and phytohormone production activities. However, no significant differences were recorded between controls (Control 1 and 2) and inoculated treatments (E7, E9 and E14) for two factors observed which was percentage increment of height and also number of new shoot formation (Table 2).

The inoculation effect on the development of the root system, such as on root length, and volume, have been frequently observed. The development of root systems may promote water absorption and mineral uptake (Dobereiner *et al*., 1993; Bastian *et al*., 1998). Among the proposed mechanisms for the increase in root development, is the bacterial production of phytohormonal substances such as cytokinins, auxins and gibberellins (GA). A quantitative analysis performed previously by capillary gas chromatography-mass spectrometry also reported that *H. seropedicae* produced 7.0 ng ml$^{-1}$ and 12.5 ng ml$^{-1}$ of IAA and GA$_3$, respectively (Bastian *et al*., 1998). This shows that both GA$_3$ and IAA are produced by *H. seropedicae* and further shows the importance of studying phytohormonal production, when inter-relationships between plants and endophytic microorganisms are analysed. These results may also explain, in part, the beneficial effects of endophytic bacteria on the host plant, as demonstrated in this present study.
The significance of inoculation (+Z78, +E7) response was also observed in the initiation of secondary roots. The results of the treatments (Z78 and E7) were significantly different compared to E9 and E14. More secondary root formation was recorded in the host plants inoculated with Z78 and E7, followed by E14 and Control 2 at D∞ (Table 2). However, lesser number of secondary roots were initiated in the host plants inoculated with E9 and Control 1 at D∞. As reported by Tsimilli-Michael et al. (2000), the diazotrophs were important for the establishment and growth of the host plants. Inoculation with Z78, E7 and E14 had promoted better growth performance of the host plants at D∞. In addition, shoots inoculated with Z78 and E7 showed significant influence on the development of secondary roots compared to E14 and Z78K (control 2) (Table 2). This indicated that the endophytic bacteria Z78 (H. seropedicae) and E7 (Microbacterium sp.), induced better rooting for in vitro oil palm shoots. This may be ascertained by the exogenous IAA production by the inocula which altered and improved root growth of host plants (Tables 1 and 2). As noted previously, the ability to produce phytohormone as a regulator of growth and development in plants is an important trait of diazotrophic bacteria. This regulatory ability includes modification of root morphology, such as an increase in length and in root branching and density of root hairs and root surface area (Tien et al., 1997; Baca and Elmerich, 2007). This is consistent with the results in the present study where the inoculated roots were bigger in size, while the surface areas were wider than the controls supplied with NH₄NO₃ and KNO₃ as N source and NAA synthetic auxin (Control 1). Consequently, a higher root surface area and a higher number of secondary roots led to enhanced water and mineral uptake efficiency.

Similar beneficial effects were observed in another study on Herbaspirillum sp. inoculation on rice and sugar-cane under axenic conditions. These beneficial effects on plants are attributed mainly to an improvement in root development and consequent increase in the rate of water and mineral uptake by roots (Njoloma et al., 2006; Zakria et al., 2007). Better plant growth was observed with increments in fresh weight, height and number of shoot formation of oil palm shoots inoculated with Z78, E7 and E14 at D∞. These effects may be attributed to IAA production by the bacteria. These diazotrophs would have affected plant growth through phytohormone synthesis and later through improved nutrient uptake. This result is in agreement with the previous study, which revealed that in vitro oil palm plantlets inoculated with A. brasilense produces high shoot and root biomass compared to the control treatment supplemented with NAA and inoculated with killed Sp7 (Azlin, 2007). Azospirillum sp. is also well-known for its ability to produce plant hormones in vitro, mainly IAA and possibly other plant hormones (Azlin et al., 2007).

Similar beneficial effects of PGPR inoculants on in vitro plantlets have been noted in potatoes, which have shown significant increases in biomass and the production of more top and root growth than those of non-inoculated plants, owing to sufficient auxin production by the inocula (Bensalim et al., 1998). IAA excretion has been similarly shown to improve plant growth in other micropropagated crops, such as sugar-cane inoculated by G. diazotrophicus and B. vietnamiensis (Govindarajan et al., 2006). This strong influence of bacterial colonisation on root development via excretion of IAA, gibberellins and other phytohormones, is well documented (Steenhoudt and Vanderleyden, 2000; Martinez-Morales et al., 2003; Mantelin and Tourine, 2004). In addition, better growth of inoculated shoots particularly Z78, E7 and E14 were observed apart from influencing the root development. This might be due to an effective

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of secondary roots</th>
<th>Shoot protein content</th>
<th>Percent of increment (%)</th>
<th>New shoot formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1 (+N₄+NAA)</td>
<td>2a</td>
<td>17.2c</td>
<td>35.5ab</td>
<td>28.5bc</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z78 (Herbaspirillum sp.)</td>
<td>4c</td>
<td>23.6de</td>
<td>52.8bcd</td>
<td>36.5d</td>
</tr>
<tr>
<td>E7 (Microbacterium sp.)</td>
<td>4c</td>
<td>19.6c</td>
<td>51.8bcd</td>
<td>29.0bc</td>
</tr>
<tr>
<td>E9 (Acetobacter sp.)</td>
<td>2a</td>
<td>13.1b</td>
<td>40.3abc</td>
<td>29.0bc</td>
</tr>
<tr>
<td>E14 (Microbacterium sp.)</td>
<td>3b</td>
<td>22.8d</td>
<td>46.3bc</td>
<td>30.3c</td>
</tr>
<tr>
<td>Control 2 (+Z78K)</td>
<td>3b</td>
<td>10.3a</td>
<td>34.2ab</td>
<td>23.5a</td>
</tr>
</tbody>
</table>

Note: Means in the same column followed by the same letter do not significantly different at P < 0.05.
colonisation of bacterial inocula which had changed root morphology and induced more secondary roots (Table 2). Phytohormone production has been cited as the main factor of growth improvement besides N₂-fixing capability of many diazotrophic PGPR strains such as Azospirillum, Herbaspirillum, Gluconoacetobacter and Burkholderia (Bashan and Holguin, 1998; Govindarajan et al., 2006; Pedraza, 2008). A positive response of inoculation on the growth of oil palm shoots could also be contributed through the biological nitrogen fixation (BNF) process. The oil palm shoots had increased root initiation (Table 2) and fresh weight, when the diazotrophs were used.

From the current study, it was observed that the well developed rooting system of in vitro oil palm induced by diazotrophs has influenced the growth of shoots. Stimulation by PGPR has been shown to improve root growth and functions, thus promoting crop yield which leads to an increased uptake of water and minerals (Matiru and Dakora, 2004; Kennedy et al., 2004; Bashan et al., 2008). This evidence can be used to explain the result of total shoot protein content obtained in this experiment (Table 2). Higher protein concentration was recorded in plant tissues of oil palm shoots inoculated with Z78 and E14. This may be related to the capacity of phytohormone production by the bacteria which might provoke changes in root morphology after the inoculation. This would later enhance the ability of host plants to absorb more nutrients and subsequently increase the protein content. In other studies, Azospirillum-inoculated plants exhibited an enhanced root branching and surface area due to phytohormone production, which in turn, can explain the enhancement of nutrient uptake and water status in both inoculated plants (maize and rice) (Okon and Labendera-Gonzalez, 1994; Tilak et al., 2005; Chi et al., 2005). Shoots inoculated with Z78 showed the highest shoot protein content (23.6 mg BSA ml⁻¹ protein) followed by E14, E7 and Control 1 (Table 2). In overall, the inoculation treatments, particularly Z78, E7 and E14, successfully improved certain plant growth parameters in this study.

CONCLUSION

The potential of selected diazotrophs (Z78, E7, E9 and E14) in producing phytohormones under free-living conditions was successfully observed. The diazotrophs, Z78 and local isolates E7 and E14 successfully influenced root development of in vitro oil palm shoots (clone S69 L436-1/26 T-1/10) at D₀. This may be partly due to the excretion of growth hormones by the tested inocula. Inoculation of E7 and Z78 also revealed positive influences on growth of oil palm shoots (height, fresh weight, new shoot formation and total shoot protein content) compared to the controls (+N, +NAA and +Z78K). The study concluded that phytohormone (IAA) produced by Herbaspirillum sp. (Z78) and locally-isolated E7 and E14 could in part enhance growth and development of in vitro oil palm shoots and may be further developed into a potential biofertiliser.

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REFERENCES


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