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Full Length Research Paper

Chromatographic detection of phytohormones from the bacterial strain UPMP3 of *Pseudomonas aeruginosa* and UPMB3 of *Burkholderia cepacia* and their role in oil palm seedling growth

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Different rhizospheric microorganisms of various plants are likely to synthesize and release phytohormones. Phytohormones act as signalling molecule in the regulation of plant growth. It influences the plant physiological process and plays a role in plant microbe interaction. The purpose of this study was to detect phytohormones from the two Plant Growth Promoting Rhizobacterial (PGPR) strain *Pseudomonas aeruginosa* UPMP3 and *Burkholderia cepacia* UPMB3 isolated from oil palm rhizosphere and their effect growth on plant growth responses. Three phytohormones namely Indole-3-acetic acid (IAA), Salicylic acid (SA) and Zeatin (Z) were detected by thin layer chromatography (TLC) from the both bacterial strains. The influence of phytohormones produced by the PGPR on oil palm seedling growth was carried out in the pot experiment. The germinated oil palm seedlings were treated with the extract of both bacterial strains and observed a positive effect on seedling growth in respect to average root number, root length, shoot length, leaf number and leaf length. Plant Growth Promoting Rhizobacteria producing phytohormones, play an important role in rhizobacteria-plant interactions. Moreover, bacterial IAA increases root surface area and length, and thereby provides the plant greater access to soil nutrients. Thus, rhizobacterial phytohormones are identified as an effector molecule in plant-microbe interactions, both in phytostimulation and pathogenesis.

Key words: Phytohormone, *Pseudomonas aeruginosa* UPMP3*, Burkholderia cepacia* UPMB3, oil palm, thin layer chromatography.

INTRODUCTION

Phytohormones are those of carbonic composites which are able to control physiological processes of plants in low densities. These composites are found in natural and artificial forms and are categorized into five main groups based on their chemical structure and physiological effects. These groups are auxins, cytokinins, gibberellins, abscisic acid and ethylene (Weyers and Paterson, 2001). Indole-3-acetic acid is the member of auxins and the product of L-tryptophan metabolism of microorganisms. It is generally considered as the most important native auxin (Ashrafuzzaman et al., 2009). It functions as an important signal molecule in the processes of plant development including organogenesis, cell expansion, division, differentiation and gene regulation (Ryu and Patten, 2008a). Different *Pseudomonas* and *Burkholderia* species have emerged and potentially promising group of plant growth promoting rhizobacteria. The phytohormone

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salicylic acid (SA) plays a key role to regulate the defences against pathogens and insects (Victoria et al., 2012). It influences the seed germination, seedling establishment, cell growth, respiration, senescence associated gene expression, responses to, thermotolerance, abiotic stresses, nodulation, and fruit yield (Rajou et al., 2006). Several genera of rhizo bacteria including Pseudomonads are known to synthesize SA and are an intermediate in the bio synthesis of pyochelin siderophores (Press et al., 1997). In this context, detection and subsequent characterization of SA derivatives is highly desirable. Phytohormone cytokinins such as zeatin, zeatin riboside, trans zeatin stimulate developmental processes in plants. Cytokinins were detected in several bacteria including *Halomonas desiderata*, *Proteus mirabilis*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Bacillus megaterium*, *Bacillus cereus*, *Bacillus subtilis* and *Escherichia coli* (Pooja et al., 2012).

Plant hormones regulate multiple physiological processes including root initiation, elongation, architecture and root hair formation. Similarly, many PGPR have the potential to affect multiple plant hormone groups, and alter the plant hormone concentration *in vivo*. Although PGPR can directly affect rhizosphere hormone concentrations there is increasing evidence that it affect root hormone concentrations, and can also alter root-to-shoot long-distance signalling to mediate shoot hormone status (Belimov et al., 2009). Therefore, an attempt was made to investigate phytohormones from the selected bacterial strains of Pseudomonas aeruginosa and Burkholderia cepacia and evaluate their potential growth enhancement of oil palm seedling.

MATERIALS AND METHODS

Bacterial strain and cultivation

UPMP3 strain of *Pseudomonas aeruginosa* and UPMB3 of *Burkholderia cepacia* were isolated from oil palm rhizosphere at United Plantations, Perak in Malaysia and its NCBI accession no. GQ183951 - *P. aeruginosa* strain UPMP3 and GQ183952 - *B. cepacia* strain UPMB3 (Zaiton et al., 2006; Azadeh and Sariah, 2009). These two strains were collected from Plant Protection Department, Universiti Putra Malaysia. Both strains were cultured on nutrient agar medium for 24 h at 30 ± 2°C and incubated for the extraction and detection of phytohormones IAA, SA and Z.

Preparation of culture supernatant of IAA, SA, Z production

The two bacterial strains *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 were grown in nutrient agar medium for 24 h at 30°C. Then the bacteria were cultured in nutrient broth medium for 2 days and the concentration was found as 10^8 colony forming unit (CFU) mL^{-1} (cfu/mL). The bacteria were grown in NB medium supplemented with 4 mg/mL L- tryptophan or without L-tryptophan to prepare bacterial supernatant for IAA production. For SA and zeatin, the bacteria were grown in casamino acids and nutrient broth medium respectively. Each experiment was carried out with 250 mL of medium in 500 mL Erlenmeyer flasks. The pre-cultured (24 h) *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 were inoculated on three media. The initial pH of the media has been adjusted to 7.0. The cultures were incubated at 28 ± 2°C, 34 ± 2°C and 30 ± 2°C respectively at 150 rpm, 200 rpm and 150 rpm on an incubator shaker for 5 days in the dark condition. Thereafter, the bacterial cells were harvested by centrifugation at 10,000 rpm for 15 min at 4°C and the supernatant was used for extraction of phytohormones IAA, SA and Z.

Screening of IAA and SA production

To screen IAA, 2 mL of the supernatant was mixed with 2 drops of ortho-phosphoric acid and 4 mL of Salkowski's reagent (0.01 g of $FeCl_3$ dissolved in 35% $HClO_4$). Mixtures were incubated at 28°C for 30 min and observed for pink colour production. The indole compounds react with Salkowski's reagent and form a pink chromophore in absorbance at 530 nm. For salicylic acid (SA) production, the supernatant was acidified with 1 N HCl at pH 2.0 and extracted with chloroform (2 × 2 mL). 1 mL of extract was added with 2 mL of $FeCl_3$ (2M) and 1 mL of distilled water. The salicylic acid reacts with $FeCl_3$ and forms purple iron SA complex with an absorbance at 527 nm. The production of purple iron colour indicates the ability to produce SA of the both bacterial strains. The colour change for both was recorded in the spectrophotometer.

Extraction and purification of IAA, SA and Z

IAA was extracted and purified following the method described by Tien et al. (1979) with some modifications. The 250 mL bacterial supernatant was reduced to 50 mL by evaporation and acidified to pH 2.8 with 1 N HCl and extracted with double volume of ethyl acetate. Besides, SA was extracted and purified following the method described by Shanmugam and Narayanasamy (2009). For extraction and purification of SA production, 50 mL of cell free supernatant was reduced to 20 mL by evaporation and acidified at pH 2 and extracted with double volume of chloroform. The extraction and purification of zeatin was done following the method described by Tien et al. (1979) with some modifications. The bacterial supernatant (cell-free liquid culture medium) was harvested by centrifugation at 8000 rpm for 15 min at 4°C and reduced to 30 mL by evaporation under vacuum. The pH was adjusted to 2.8. This solution was then transferred to an evaporator flask and the extraction proceeded by the use of 30 mL of ethyl acetate, thrice. The ethyl acetate fraction was discarded

and aqueous fraction was taken to extract zeatin. The pH of the fraction was adjusted to 7.0 with 1 N NaOH. The aqueous fraction was again extracted with water saturated n- butanol. Ethyl acetate, chloroform, and n-butanol fraction were evaporated in a rotatory evaporator at 40°C and dissolved in 1 mL of methanol for each. After filtering through 0.45 µm membrane filter, the three phytohormone extracts were kept at -20°C for further analysis by thin-layer chromatography (TLC). The entire harvesting procedures were carried out under a semi dark condition and samples were maintained in ice bath.

Confirmation of IAA, SA and Z by using thin layer chromatography

The extracted and purified IAA (ethyl acetate fractions), SA (chloroform fractions) and zeatin (n- butanol fraction) (20 µL) were plated on TLC plates (Silica gel G f254, thickness 0.25 mm, Merck, Germany) and were run by using the solvent systems benzene: acetone: acetic acid (65:25:10), chloroform: acetic acid: ethanol (95:2.5:2.5) and n- butanol: acetic acid: water (12:3:5) respectively. The plates were viewed under UV light (254 and 365 nm) sprayed with Ehrlich's immediately after removal from the developing chamber. The IAA, SA and Z were detected by observing a UV reflected band with an R_f value corresponding to that of individual standards (Sigma Aldrich).
The R_f value was calculated by the following formula according to Fried and Sherma
(1982):

$$R_f = \frac{A}{B}$$

where,

A = distance spot travels;
B = distance solvent travels.

Effect of bacterial phytohormones on plant responses

The effect of bacterial phytohormones was studied on germinated oil palm seeds. Before this bioassay, both bacterial strains were cultured and extracted from each bacterial culture filtrate. The germinated oil palm seeds were sterilized with 95% ethanol for 2 min and then with 10% Chlorox (Sodium hypochloride) for 15 min. After this, successive washing was done with sterile distilled water to remove the chemicals completely. The sterilized seeds were separately soaked in 250 mL of each bacterial culture filtrate based on the production of phytohormones (IAA, Z and SA) by the two strains (Figure 1). Sterile distilled water was used as non-treated control treatment to compare with the bacterial treatments. Plastic Pot (9 cm × 9 cm × 6 cm) experiment was performed to study the effect of phytohormones produced by *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 strains on oil palm seedling growth.

The seeds were soaked in each treatment for 48 hours and then planted in pots filled with sterile mixed soil. The pots were kept in partial sunlight and observed daily. Watering was made as required to maintain the soil moisture. After 4 weeks, the plants were carefully uprooted and recorded data subsequently.

Experimental design

The experiment was designed as Completely Randomized Design (CRD). Three treatments were used under this experiment namely: T1= *P. aeruginosa* UPMP3 + GOPS, T2 = *B. cepacia* UPMB3 + GOPS, T3 = non treated oil palm germinated seed. Data were collected on average shoot length, root number, root length, leaf number and leaf length with three replications.

Statistical analysis

Completely Randomized Design (CRD) was performed for all experiments. Data were analysed using statistical analysis system (SAS v9.3) and means were statistically compared using LSD test. The significance level was set up at $p < 0.05$. Three replications were considered for each treatment and repeated twice.

RESULTS AND DISCUSSION

Screening and confirmation of IAA production

The bacterial strains were screened for IAA production. Both the strains *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 showed pink colour reaction with Salkowski reagent which indicated the production of IAA (Figure 2A).

For purification separation and possible identification of natural and synthetic indole derivatives, the silica gel thin layer chromatography (TLC) was found to be a powerful technique. The production of IAA by the two bacterial strains were detected and confirmed with the appearance of blue bands on pre-coated silica gel plates under UV illumination at 365 nm with that of authentic IAA bands. The R_f value of standard IAA was found to be 0.88 similar to the R_f value of bacterial strains in solvent systems benzene: acetone: acetic acid (Figure 2B).

Ritika et al. (2012) reported that different isolates of *Pseudomonas* sp produced auxins like substances in the stationary phase of growth at 72 h of incubation period at 28°C. The homogeneity of the partially purified auxins was checked by thin layer chromatography. Auxins gave the maximum R_f value of 0.81 in solvent system isopropanol: water (30:20). Pink spots corresponding to auxins or auxins-like substances were visible when

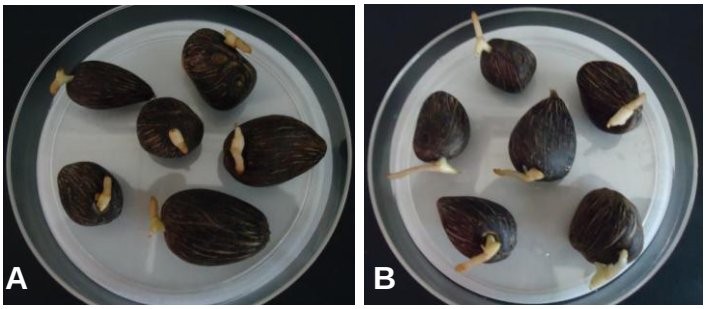

Figure 1. Bacteria treated germinated oil palm seeds. A: Seeds treated by *P. aeruginosa* UPMP3 and B: Seeds treated by *B. cepacia* UPMB3.

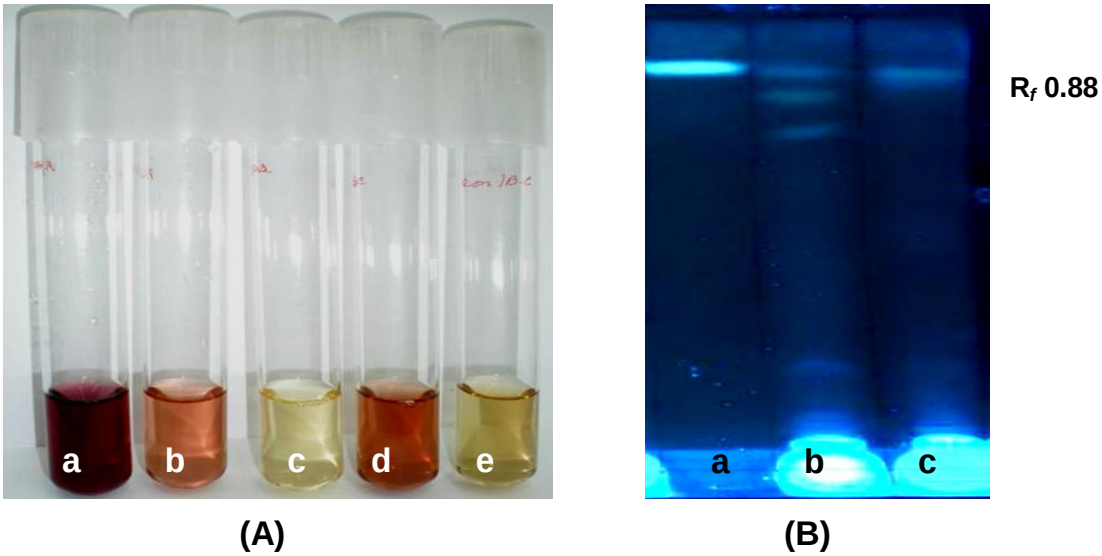

Figure 2. A: Screening of IAA production. (a): Standard IAA, (b): *P. aeruginosa* UPMP3 (pink colour), (c): Control UPMP3, (d): *B. cepacia* UPMB3 (pink colour), (e): Control UPMB3. **B:** Thin layer chromatographic pattern on silica gel-G of partially purified auxin IAA of *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3. (a): Standard IAA, (b): *P. aeruginosa* UPMP3, (c): *B. cepacia* UPMB3.

sprayed with Salper reagent. María et al. (2000) used the solvent system, chloroform: ethyl acetate: formic acid (5:3:2) to detect IAA in *Azotobacter sp.* and *Pseudomonas sp.* and developed with Salkowski reagent giving the correct R_f value (0.57). The IAA produced by the *B. licheniformis* MML2501 was confirmed by the appearance of blue bands with that of authentic IAA bands on TLC plates with R_f value of 0.66 in solvent system isopropanol: ammonia: water (8:1:1 v/v) and sprayed with Ehrlich's reagent (Prashanth and Mathivanan, 2010).

Screening and confirmation of SA production by TLC

The bacterial strains were screened for SA production. *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 showed purple iron colour react with $FeCl_3$ which indicated their ability to produce SA (Figure 3A). Detection and confirmation of SA production was done by thin layer chromatography (TLC) with the appearance of blue bands matched with that of authentic SA bands under UV illumination at 365. The R_f value was found as 0.74 both for *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 that was similar to the R_f value (0.74) of the authentic SA nm in chloroform: acetic acid: ethanol solvent system (Figure 3 B).

SA in the culture filtrate of the *P. aeruginosa* IE-6S$^+$ strain was detected in TLC with blue spot at an R_f value of 0.91 after exposure to ammonia fumes(Imran et al., 2003). The SA produced by the *B. licheniformis*

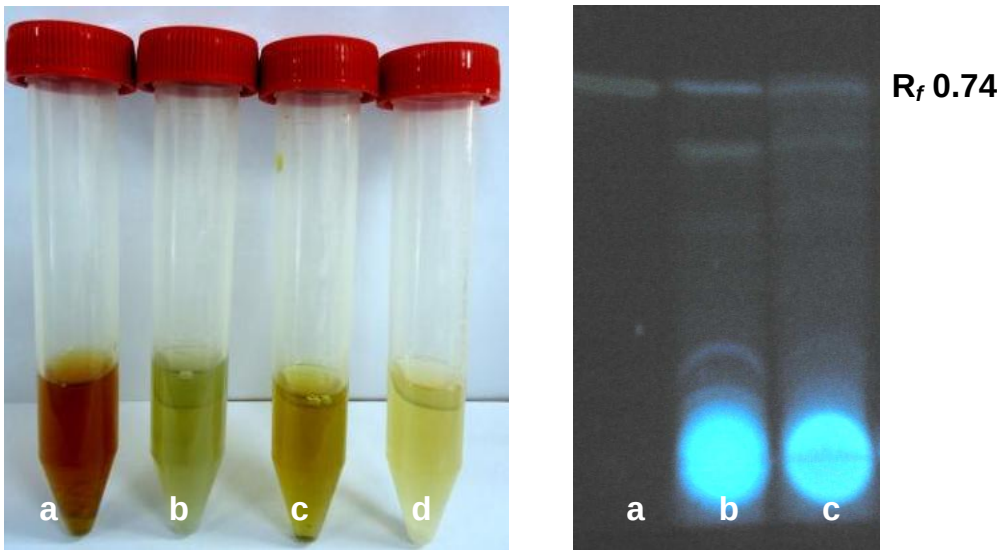

Figure 3. A: Screening of SA produced by *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3. a: UPMP3 (purple iron colour), b: UPMP3 (control), c: UPMB3 (purple iron colour), d: UPMB3 (control). **B:** Detection of SA by TLC; a: Standard SA, b: UPMP3, c: UPMB3.

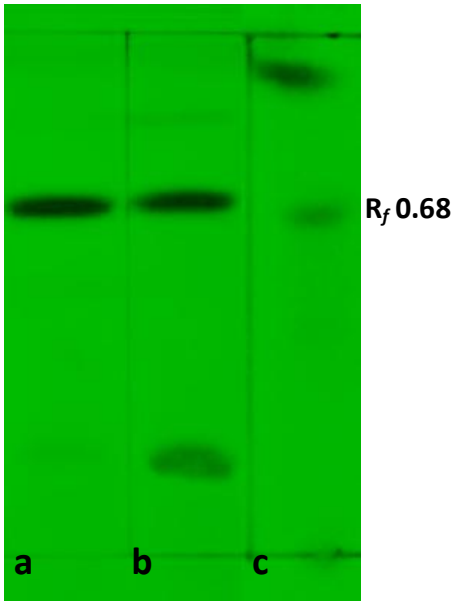

Figure 4. Detection of zeatin (Z) by TLC produced by *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3. a: Standard Z, b: *P. aeruginosa* UPMP3, c: *B. cepacia* UPMB3.

MML2501 was confirmed by the blue bands that appeared on pre-coated silica gel viewed under UV illumination. The R_f value of SA (0.61) produced by *B. licheniformis* MML2501 was matched with the R_f value (0.61) of the authentic SA (Shanmugam and Narayanasamy, 2009).

Confirmation of zeatin production by TLC

Zeatin production by P. *aeruginosa* UPMP3 and *B. cepacia* UPMB3 was detected by thin layer chromatography (TLC) and confirmed that the band matched with the authentic band on pre-coated silica gel plates under UV illumination at 254 nm. The R_f value was found as 0.68 both for *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 which was identical to the authentic R_f value (0.68) in n- butanol: acetic acid: water solvent system (Figure 4).

Different cytokinins were detected from several bacteria including *H. desiderata*, *P. mirabilis*, *P. vulgaris*, *K. pneumoniae*, *B. megaterium*, *B. cereus*, *B. subtilis* and *E. coli* (Pooja et al., 2012).

Pooja et al. (2012) reported that zeatin was extracted from the culture of *Corynebacterium aurimucosum* using ethyl acetate and the zeatin production was confirmed by TLC analysis with R_f value of 0.53 identical to the R_f of the standard zeatin. Methanol fractions (bacterial extract) were co-chromatogramed on Merck silica gel 60 Pf254 with authentic cytokinins (Trans-zeatin, zeatin riboside, kinetin and adenine) using n-butanol: acetic acid: water (12:3:5) (v/v) as mobile phase and TLC chromatograms were observed under 254 nm UV light to detect cytokinin. The R_f values were calculated for both standards and samples 0.50, 0.54, and 0.58 respectively (El-Tarabily, 2003; Anwar and Shahida, 2009).

Table 1. Morphogenic response of oil palm seedlings inoculated with bacterial supernatant after 4 weeks.

Treatment		Different parameters				
		Root length* (cm)	Shoot length* (cm)	No. of roots*	No. of leaves*	Leaf length* (cm)
T1	*P. aeruginosa* UPMP3 + GOPS	17.56 ± 1.68^{a}	2.10 ± 0.26^{cd}	14.66 ± 1.75^{a}	2.33 ± 0.28^{cd}	5.00 ± 0.75^{c}
T2	*B. cepacia* UPMB3 + GOPS	17.00 ± 0.83^{a}	1.86 ± 0.35^{cd}	15.66 ± 1.04^{a}	2.00 ± 0.00^{cd}	4.50 ± 1.39^{cd}
T3	Control	9.86 ± 1.75^{b}	1.73 ± 0.12^{cd}	9.00 ± 1.32^{b}	1.33 ± 0.28^{d}	1.33 ± 0.14^{d}

* Average;
GOPS = Germinated oil palm seeds.
Values followed by the same letter are not significantly different according to LSD test at P<0.05 level.

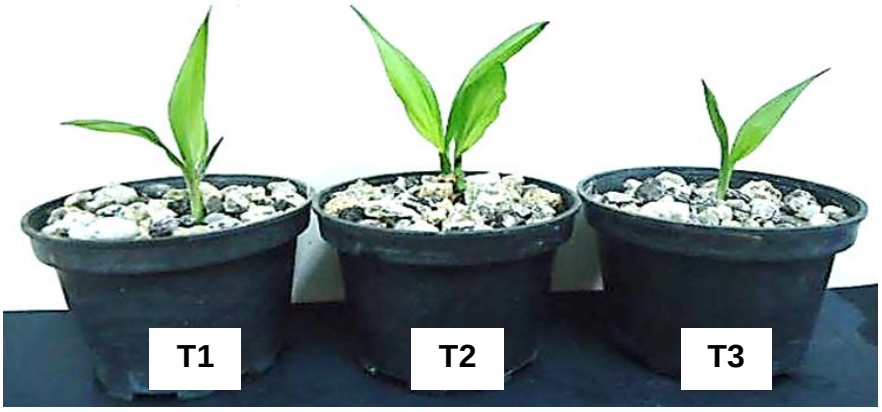

Figure 5. Growth performance of bacteria treated oil palm seedlings in pot experiment after 4 weeks. T1: *P. aeruginosa* UPMP3 extract + oil palm seed, T2: *B. cepacia* UPMB3 extract + oil palm seed, and T3: Untreated oil palm seed.

Effect of bacterial phytohormones on oil palm seedling growth

The influence of two bacterial extracts on growth promotion of oil palm germinated seedlings was done by the pot experiment. The results revealed that the treatments, T1 (germinated seeds inoculated with *P. aeruginosa* UPMP3) and T2 (germinated seeds inoculated with *B. cepacia* UPMB3) showed a positive effect on growth promotion in oil palm seedlings compared with control treatment T3 (non-inoculated germinated seeds). It was observed that the oil palm seedlings treated with *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 produced an average of 14.66 and 15.66 roots per seedlings respectively, whereas the control treatment produced an average of 9.00 roots per seedling after 4 weeks of planting. The average root length, shoot length, number of leaves, and leaf length per seedling were also found to be higher than the control treatment. Results are presented in Table 1, and Figures 5 and 6.

This result confirmed that the phytohormones especially auxins (IAA) and cytokinin (zeatin) produced by the two bacterial strains *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 play a vital role in oil palm seedlings growth. The role of IAA produced by *Pseudomonas putida* in the development of the host plant root stem was explained by Patten and Glick (2002). Seeds of groundnut treated with *B. licheniformis* MML2501 showed a significant increase in seed germination, plant growth and yield under potted plant experiments (Prashanth and Mathivanan, 2010). *Streptomyces* spp. from a tomato rhizosphere had the ability to produce IAA and improved tomato growth by increasing root dry weight (El-Tarabily, 2008). Sutthinan et al. (2010) reported that the culture filtrate of *Streptomyces* CMU- H009 significantly stimulated the germination and root elongation of maize and cowpea seedlings. Similarly, Markmann and Parniske (2009) reported that the impact of cytokinin producing bacteria on plant cell division was investigated in root nodule formation. A rhizobacterial strain *Azospirillum brasilense* inoculated with maize roots increased root surface area compared to the control.

Azospirillum brasilense has been also reported to enhance cell division in root tip of inoculated wheat

Figure 6. Influence of phytohormones in different treatments on morphogenic response of oil palm seedlings in pot experiment. T1: Oil palm seedling + *P. aeruginosa* UPMP3, T2: Oil palm seedling + *B. cepacia* UPMB3, T3: Untreated oil palm seedling.

(Molina-Favero et al., 2007).

CONCLUSION

Microbial synthesis of the phytohormones auxins, cytokinin and salicylic acid has been known for a long time. Directly, PGPR promote plant growth by either facilitating resource acquisition or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents. The two PGPR, *P. aeruginosa* UPMP3 and *B. cepacia* UPMB3 isolated from oil palm rhizosphere have been considered to evaluate their ability to produce different phytohormones and their subsequent effect on oil palm seedling. The influence of phytohormones produced by the PGPR on oil palm seedlings growth was carried out in the pot experiment and it was found that the seedlings growth of oil palm was significantly influenced by the inoculation of the bacterial strains in soil, compared to the control treatment. Thus, the application of microorganisms to improve plant growth as bio fertilizer and control diseases, as bio pesticide, could be an environment-friendly approach.

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