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Identification, Determination and Quantification of Indole-3-Acetic Acid Produced by *Pseudomonas aeruginosa* UPMP3 and Its Effect on The Growth of Oil Palm (*Elaeis guineensis* Jacq)

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Abstract *Pseudomonas* species have founded as greatest and potentially most promising group of plant growth promoting rhizobacteria (PGPR). *Pseudomonas aeruginosa* UPMP3 is an important PGPR isolated from oil palm rhizosphere. This rhizobacteria is likely to synthesize and release phytohormone indole-3 acetic acid (IAA). Production of IAA is one of the main reasons to promote plant growth and yield. The aim of this study was to detect, identify and quantify the IAA production by *P. aeruginosa* UPMP3 *in vitro* and its influence on oil palm seedling growth. Nutrient broth medium supplemented with 1-5 mg/ml L-tryptophan and without L- tryptophan were used for bacterial culture. The pH levels of culture media were optimized under shaken and static conditions and incubated at 28±2°C in different incubation periods. The production of IAA by *P. aeruginosa* UPMP3 was extracted, purified, detected and quantified by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) analyses. Production of IAA was quantified by HPLC in liquid culture and achieved 12.08µg/ml with a retention time of 13.711 min. On the other hand, the maximum 52 µg/ml IAA was recorded in the medium supplemented with 4 mg/ml L- tryptophan in compare to control. The optimum pH level of the culture medium was recorded as 7 under shaken conditions at 150 rpm with 5 days incubation. The influence of IAA produced by the UPMP3 on oil palm seedling growth was carried out in the pot experiment. The germinated oil palm seedlings were treated with the extract of bacterial strain and observed a positive effect on seedling growth in respect to average root and leaf number, root, shoot, and leaf length compare to the synthetic IAA and the control.

Keywords: *Indole-3-Acetic Acid, Pseudomonas aeruginosa UPMP3, Thin Layer Chromatography, High Performance Liquid Chromatography, Oil Palm*

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1. Introduction

Plant growth regulators are those of carbonic composites which are able to control physiological processes of plants in low densities. The auxins are a group of indole ring compounds which have the ability to improve plant growth by stimulating cell elongation, root initiation, seed germination and seedling growth [1]. The phytohormone auxins play a central role in plant growth and development as a regulator of numerous biological processes, from cell division, elongation and differentiation to tropic responses, fruit development and senescence.

IAA is the member of the group of phytohormones and is a product of L- tryptophan metabolism in microorganisms. It is generally considered the most important native Auxin [2]. It functions as an important signal molecule in the regulation of plant development. IAA is one of the most physiologically active auxins and common product of L-tryptophan metabolism by several microorganisms including PGPR. L-tryptophan is considered an efficient physiological precursor of auxins in higher plants as well as for microbial biosynthesis of auxins. Diverse bacterial species possess the ability to

produce the phytohormone IAA. The recognition of plant growth-promoting rhizobacteria (PGPR), a group of beneficial plant bacteria, as potentially useful for stimulating plant growth and increasing crop yields has evolved over the past several years. Today researchers are able to use them successfully in field experiments. Some bacterial strains directly regulate plant physiology by mimicking synthesis of plant hormones, whereas others increase mineral and nitrogen availability in the soil as a way to augment growth. PGPR have potential agricultural and phytoremediation applications. It can increase the growth of plants by reducing pathogens, other biotic stress, or abiotic stress [3]. It could be a great ecological and agricultural importance, if they are reliably used in place of chemical fertilizers and pesticides without being pathogenic to plants. Rhizobacteria belonging to the genera Azotobacter, *Azospirillum*, *Bacillus*, *Pseudomonas*, *Enterobacter*, *Xanthomonas*, and *Rhizobium* have been shown to produce auxins which help in stimulating plant growth [4,5]. Some microorganisms produce auxins in the presence of a suitable precursor such as L-tryptophan. *Pseudomonas* is the most abundant auxin producer micro-organism [6]. Approximately 80% of rhizosphere bacteria can secrete IAA [7]. It is very likely that plant growth promotion by rhizobacteria is the result of combined action of several ways, but production of phyto-hormones (especially IAA) is considered as a direct mechanism used by bacteria to increase the growth and yield of plants [8,9]. There is also evidence that growth regulators such as IAA produced by bacteria can some instances increases and improve yields of the host plants. Bacterial production of IAA has been studied not only regarding its physiological effects on plants but also regarding its possible role as a phyto-hormone in plant- microbe interaction [10].

The use of PGPR producing IAA is a new concept to solve the replant problem to some extent. More specifically, the soil-borne *Pseudomonas* sp. has received particular attention because of their capacity to produce a wide range of phyto-hormones. It has been established and is now well accepted that normal plant growth and development throughout ontogeny is controlled by these compounds produced by the plant itself. However, plants may not have the capacity to synthesize sufficient endogenous plant hormones for optimal growth and development under sub optimal growth and environmental conditions. It was assumed that plant growth regulator IAA produced by *Pseudomonas* species could also influence plant growth. The aim of this study was to identify, optimization and quantify of IAA production by *P. aeruginosa* UPMP3 isolated from oil palm rhizosphere and its effect on oil palm seedling growth.

2. Materials and Methods

2.1. Bacterial Strains and Cultivation

Plant growth promoting rhizobacterial strain *P. aeruginosa* UPMP3 was previously isolated from roots of healthy oil palm [11]. The bacterium was collected from Plant Protection Department, Universiti Putra Malaysia and was used in this study.

2.2. Confirmation of Bacterial Strain on Biolog® System

Bacteria was grown on nutrient agar (NA) for routine use, and maintained in Nutrient Broth (NB) with “15% (w/v)” glycerol at -80°C for long-term storage. The bacterial isolate was confirmed with the Biolog® system (version 4.2). Fresh bacteria (24 h) on NA were streaked on Biolog® Universal Growth (BUG) medium. The bacterial suspension was inoculated in a GN Biolog 96-well microliter plate with 150 µl per well. The microliter plate was incubated at 28-30°C for 24 h, and the resulting pattern of coloured wells analysed using the MicrostationTM system and Biolog MicroLogTM software for bacterial identification.

2.3. Molecular Confirmation of *P. aeruginosa* UPMP3 Using Polymerase Chain Reaction (PCR) Method

Genomic DNA of the strain UPMP3 of *P. aeruginosa* was prepared according to a modified of the cetytrimethyl ammonium bromide (CTAB) method [12,13]. PCR amplification of recA gene was performed in the PCR reaction mixture containing DNA (100 ng) as template, 1X taq buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 µM primers and 0.1 u Taq DNA polymerase in a total volume of 25 µl. The primers used in this study were designed by primer3 program (http://frodo.wi.mit.edu/primer3/) according to the sequences of recA gene obtained from the GeneBank database(http://www.ncbi.nlm.nib.gov/) (Table 1). The amplification was performed in a thermal cycler (T3 Thermocycler, Biometra, Syngene, UK) programed for pre-denaturing of 3 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min of appropriate annealing temperature (Table 1) and 2 min at 72 °C. After a final extension of 10 min at 72 °C, the samples were cooled at 4 °C. The PCR products were analysed and 1kb DNA ladder was used as marker to compare the bands with different molecular weights generated.

Table 1. Primer used for the PCR amplification of recA gene from *P. aeruginosa* UPMP3

Rhizobacteria	Accession	Sequence (5’ to 3’)	Annealing temperature (°C)	Product size (bp)
P. aeruginosa UPMP3	X05691.1	Forward: ATGGACGAGAACAAGAAGCG Reverse: TCAATCGGCTTCGGCGTCA	57	1087

2.4. Detection and Confirmation of IAA Production

2.4.1. Preparation of Culture Supernatant for IAA

The bacterial strain *P. aeruginosa* UPMP3 was grown in nutrient agar medium for 24 hours at 28 ± 2 °C. Then the bacteria was cultured in nutrient broth medium for 2 days and the concentration was found as 10^8 colony forming unit (CFU) ml^{-1} (cfu/ml). To prepare bacterial supernatant for IAA production the bacteria were grown in NB medium supplemented with 4 mg/ml L- tryptophan or without L-tryptophan. The initial pH of the media was adjusted to 7.0. The inoculated flasks were incubated at 28 ± 2°C on an incubator shaker at 150 rpm for 5 days in the dark condition. Thereafter, the bacterial cells were harvested by centrifugation at 10,000 rpm for 15 min at 4 ºC and the supernatant (cell-free liquid culture medium) was used for extraction of phytohormone IAA.

2.4.2. Screening of IAA Production

To screen indole-3-acetic acid production, 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. Indole compounds react with Salkowski's reagent to form a pink chromophore in absorbance at 530 nm. The IAA concentration in culture filtrate was determined using a calibration curve of authentic IAA (Sigma Chemicals Co. USA) as a standard.

2.4.3. Extraction and Purification of IAA

Indole-3-acetic acid was extracted and purified following the method described by Tien *et al*. [14] with some modifications. The bacterial supernatant was reduced from 200 ml to 50 ml by rotatory evaporation under vacuum and acidified to pH 2.8 with 1 N HCl. The supernatant was extracted thrice with double volume of ethyl acetate. Extracted ethyl acetate fraction was evaporated to dry in a rotatory evaporator at 40°C and dissolved in 1 ml of methanol. After filtering through 0.45 µm membrane filter the extract was kept at -20°C for further analysis by thin-layer chromatography (TLC). The entire harvesting procedures were carried out in dim light with samples maintained in a covered ice bath.

2.4.4. Confirmation of IAA by Using Thin Layer Chromatography

Ethyl acetate fractions (30 µl) were plated on TLC plates (Silica gel Gf 254, thickness 0.25 mm, Merck, Germany)) and was run by using three solvent systems such as benzene: acetone: acetic acid "65:25:10 (v/v/v)"; isopropanol: ammonia: water "80:10:10 (v/v/v)"; chloroform: ethyl acetate: formic acid "50:30:20 (v/v/v)" and sprayed with Ehrlich's reagent [0.2% p di- methyl aminobenzaldehyde was dissolved in the mixture of 95% ethanol and conc. HCl (1:1)] immediately after removal from the developing chamber. Spots with R_f values identical to authentic IAA were identified under UV light (254 nm & 365 nm). The IAA was detected as a blue band with an R_f value corresponding to that of standard IAA. The R_f value was calculated using the following formula according to Fried and Sherma [15].

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

Where,
A= distance spot travels
B= distance solvent travels.

2.4.5. Confirmation of IAA by Using High Performance Liquid Chromatography

The ethyl acetate extract of the culture supernatant was analyzed by HPLC with some modifications [14]. HPLC chromatograms were produced by injecting 20 µl of the filtered extract onto a 5µm reverse phase column (Waters Associates µBondapak C18, 250mm x 4mm) in a Waters Associates liquid chromatograph equipped with an ultraviolet detector absorbing at 208 nm. The solvent system used to separate IAA was water: acetonitrile [76:24 (v/v)], flow rate was 1 ml/min for 20 minutes. Retention times for peaks were compared to those of authentic standards (IAA) added to the medium and extracted by the same procedures used with bacterial culture. Quantification was done by comparison of peak heights.

2.5. Optimization and Quantification of IAA Production

2.5.1. Effect of L-tryptophan Concentration on IAA Production

To check the effect of L- tryptophan on IAA production, NB medium was amended with 0-5 mg/ml L-tryptophan, and inoculated with the selected strains. 1% inoculum of optical density $(O.D)_{600}$ 1.0 was incubated at 28±2°C for 3, 5, 7 and 10 days respectively. After incubation the broth was centrifuged at 7000 rpm for 10 min. Supernatant was collected. Two ml of Salkowski reagent was added in 1ml supernatant and extent of red colour. IAA production was measured spectrophotometrically at 530 nm. [16].

2.5.2. Effect of pH on IAA Production

To optimize the different pH level on IAA production by the strain UPMP3, NB medium was adjusted to pH as 3, 5, 6, 7, 8 and 9. Medium was inoculated with 1% inoculum incubated at 28±2°C for 3, 5, 7 and 10 days respectively. IAA production was studied by using Salkowski reagent after 24 hours [17] and measured spectrophotometrically at 530 nm.

2.5.3. Effect of Culture Conditions and Incubation Periods on IAA Production

The bacterial strain was grown in the NB media at a range of 3, 5, 7 and 10 days in static and shaken (150 rpm) conditions at 28±2°C on incubator shaker. IAA production was measured spectrophotometrically at 530 nm.

2.6. Effect of IAA on Plant Responses

The effect of IAA on plant growth was studied on germinated oil palm seeds. Before this bioassay, the

bacterial strain UPMP3 was cultured and extracted bacterial culture filtrate. The germinated oil palm seeds were surface 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 surface-sterilized germinated seeds were separately soaked in 250 ml of bacterial culture filtrate based on the production of IAA by the strain UPMP3 (Figure 1) and synthetic IAA.

Figure 1. *P. aeruginosa* UPMP3 treated germinated oil palm seeds

Sterile distilled water was used as non-treated control treatment to compare with the bacterial and synthetic treatments. Plastic Pot (9 cm x 9 cm x 6 cm) experiment was conducted to evaluate the effect of IAA on oil palm seedling growth. The seeds were soaked in each treatment for 48 hours and then planted in pots filled with sterile 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.

2.7. 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.

3. Results and Discussion

3.1. Confirmation of Bacterial Strain Using Biolog Reader System

P. aeruginosa UPMP3 is non-enteric bacterium that was confirmed with the Biolog® identification system and identified as *P. aeruginosa* based on 100% probability and similarity index of 0.57 [11].

3.2. Molecular Confirmation

RecA was amplified in *P. aeruginosa* UPMP3 through PCR. The size of *recA* amplicons for UPMP3 was 1087 bp. The nucleotide analysis of the amplified *recA* using BLAST showed 99% similarity between *P. aeruginosa* UPMP3 and clinical strain *P. aeruginosa* PAO1 (GeneBank accession number: AE004091.2) (Figure 2).

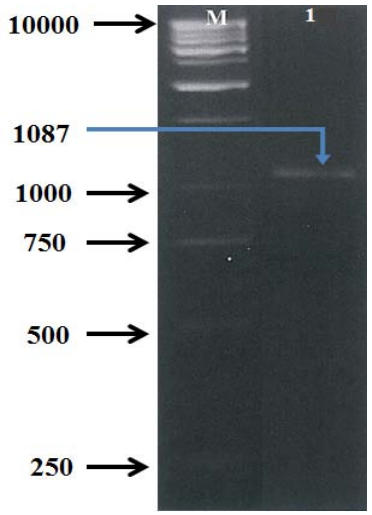

Figure 2. Amplification of recA for *P. aeruginosa* UPMP3(1). Size of DNA ladder used is 1 KB (M).

3.3. Screening and Confirmation of IAA Production

P. aeruginosa UPMP3 was screened for IAA production. Colour reaction with Salkowski reagent resulted in the appearance of pink colour. The strain showed pink colour reaction with Salkowski reagent which indicated the production of IAA (Figure 3A).

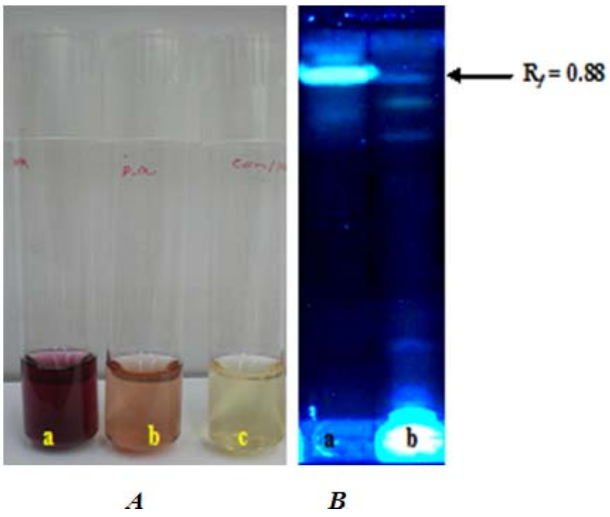

Figure 3. A. Screening of IAA production. a: Standard IAA, b: *P. aeruginosa* UPMP3 (pink colour), c: Control UPMP3; B. Thin layer chromatographic pattern on silica gel-G of partially purified auxin IAA of *P. aeruginosa* UPMP3, a: Standard IAA, b: *P. aeruginosa* UPMP3.

Indole acetic acid was detected by means of paper chromatography methods. Silica gel thin layer chromatography (TLC) was found to be a powerful technique in purification, separation and possible identification of natural and synthetic indole derivatives. The IAA produced by the bacterial strain was extracted with ethyl acetate and confirmed by the appearance of blue bands with that of authentic IAA bands on pre-coated silica gel plates under UV illumination. Three solvent systems were used to detect the IAA production individually for the bacterial strain. The result revealed that all solvent systems can be used to

detect the IAA compound. Among the three solvent systems in Benzene: Acetone: Acetic acid, the R_f value of standard IAA was found 0.88 for *P. aeruginosa* UPMP3 (Figure 3B). In the case of other two solvent systems, Isopropanol: Ammonia: Water and Chloroform: Ethyl acetate: Formic acid showed R_f value 0.64 and 0.59 for *P. aeruginosa* UPMP3 that was similar to the standard IAA R_f value. From the experimental results different R_f values were obtained for the different solvent systems (Table 2).

Ritika *et al.* [18] reported that different isolates of *Pseudomonas* sp. produced auxins like substances in the stationary phase of growth at 72 hours 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, (v/v)". Pink spots corresponding to auxins or auxins like substances were visible when sprayed with Salper reagent. María *et al.* [19] used the solvent system, chloroform: ethyl acetate: formic acid "5:3:2 (v/v/v)" 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 band with that of authentic IAA bands on TLC plates with R_f value 0.66 in solvent system isopropanol : ammonia : water "80:10:10(v/v/v)" and sprayed with Ehrlich's reagent [20]. Thin layer chromatography analysis of auxin compounds obtained from the two fluorescent Pseudomonas isolates An-1-kul and An-13-kul confirmed the presence of IAA with R_f value 0.80 and 0.81 respectively identical to the R_f of the standard 0.85 [21].

3.4. Confirmation of IAA by HPLC

IAA was detected from *P. aeruginosa* UPMP3 and confirmed for production through High Performance Liquid Chromatography. The HPLC responses (peak areas) of IAA was observed to have highly positive correlation with the standard concentration. The retention time of IAA from the sample was 13.711 min which matched the retention time of authentic IAA of 13.810 min at 208 nm (Figure 4A & 4B). IAA was quantified from the respective standard curve. The maximum amount of IAA was calculated as 12.08 µg/ml which was the same as results done by Sharma *et al.* [21]. They reported the range of IAA production from fluorescent Pseudomonas isolates An-1-kul and An-13-kul was 11.59µg/ml and 13.76µg/ml culture respectively. Crozier *et al.* [22] measured the IAA amount of twenty *Azospirillum lipoferum* and *A. brasilense* strains through both HPLC and Salkowski methods. It was reported the IAA amount in *A. lipoferum* through Salkowski method from 0 to 14.9µg/ml; and in *A. brasilense* from 0 to 26.0µg/ml. They also reported the IAA amount in *A. lipoferum* through HPLC method from 0.05 to 14.9µg/ml; and in *A. brasilense* from 0 to 4.5µg/ml. Khalifah *et al.* [23] reported that the maximum excitation wavelength for auxins (IAA) was 290 nm. IAA extracted from the culture of fluorescent Pseudomonas isolates showed a similar peak to that of the standard IAA (Hi-media) in HPLC analysis. Crozier and Reeve [24] reported that HPLC is a powerful method for simplifying the auxins identification in comparison with mass spectrophotometer method. In HPLC method the goal is to promote the measurable IAA and reduce the amount of unexpected substances in samples.

Table 2. Thin layer chromatographic analysis of partially purified bacterial plant growth regulators viz. auxins (IAA) from *P. aeruginosa* UPMP3

Plant Growth Regulator	Bacterial Strains	Solvent Systems	Spraying Reagent	Colour of Band	*R_f Value
Indole-3 Acetic Acid (IAA)	*P. aeruginosa* UPMP3	Benzene: Acetone: Acetic acid "65:25:10 (v/v/v)"	Ehrlich's reagent	Blue	0.88
		Isopropanol: Ammonia: Water "80:10:10 (v/v/v)"	Ehrlich's reagent	Blue	0.64
		Chloroform: Ethyl acetate: Formic acid "50:30:20 (v/v/v)"	Ehrlich's reagent	Blue	0.59

*R*f* (Retention factor) values of three different solvent systems, used for IAA detection.

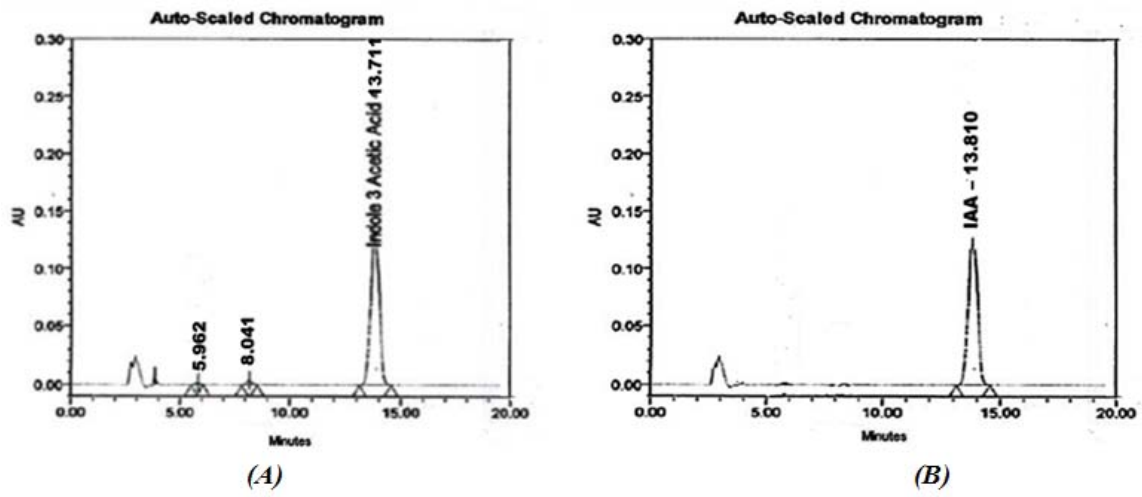

Figure 4. Chromatograph of separation of indole acetic acid (IAA) from sample *P. aeruginosa* UPMP3 (A) and from standard (B) with retention time at 208 nm wave length

3.5. Effect of L-tryptophan Concentration on IAA Production

Most of the micro-organisms produce IAA in presence of L- tryptophan. In the present study it was observed that *P. aeruginosa* UPMP3 was found to be the efficient producer of IAA. The effect of different concentrations of L- tryptophan in the medium increases the amount of IAA production. Different concentrations of L-tryptophan (0-5 mg/ml) were supplemented in the culture media. The pH of the media was adjusted 7.0 and the culture incubation periods were 3, 5, 7 and 10 days respectively. From the result it was observed that the bacterial strain was able to produce IAA at a lower amount, when the medium was supplemented without L- tryptophan. But the IAA production was increased with the increase amount of L- tryptophan concentrations. The range of IAA production of *P. aeruginosa* UPMP3 was 9.71 µg/ml to 52.41 µg/ml. A significant increase in the production of IAA was recorded in the absence and presence of L-tryptophan. The maximum IAA production in *P. aeruginosa* UPMP3 was found 52.41 µg/ml after 5 days incubation at 4 mg/ml L- tryptophan concentration (Figure 5). IAA production was found to be decreased when the media were supplemented with 5 mg/ml L- tryptophan in 10 days incubation. This decrease might be due to the release of IAA degrading enzymes such as IAA oxidase and peroxidase as was reported earlier in *Rhizobium* sp. from *Cajanus cajan* [25]. Various researchers reported variable IAA production ability of bacteria. Ahmad *et al.* [16] reported that rhizosphere *Azotobacter* spp. and *Pseudomonas* spp. produced a high level of IAA when theses bacteria were cultured in a nutrient broth amended with 1, 2 and 5 mg/ml of L-tryptophan. The concentration of IAA in *P. fluorescens* AK1 and *P. aeruginosa* AK 2 isolates without L-tryptophan was 3.1 pmol /ml and 3.3 pmol /ml. A further increase in IAA production was observed in the presence of different concentrations of L-tryptophan (100, 200 and 500 µg/ml). A significant increase in the production of IAA was recorded in *P. fluorescens* AK1 and *P. aeruginosa* AK2 in the presence of 100, 200 and 500 µg/ml of L-tryptophan, i.e. 3.8, 5.2 and 6.9 pmol/ml, 3.9 pmol/ml, 4.0 pmol/ml and 4.2 pmol/ml respectively **[26]**. Karnwal [26] tested *Fluorescent Pseudomonas* isolates for their ability to produce indole acetic acid in pure culture in the absence and presence of L-tryptophan and found that indole production by the strain, increased with increases in tryptophan concentration. The rhizobacterial strain *Burkholderia cepacia* UPMB3 produced maximum 50.88 µg/ml IAA at 4 mg/ml L-tryptophan concentration in culture medium reported by Parvin *et al.* [27].

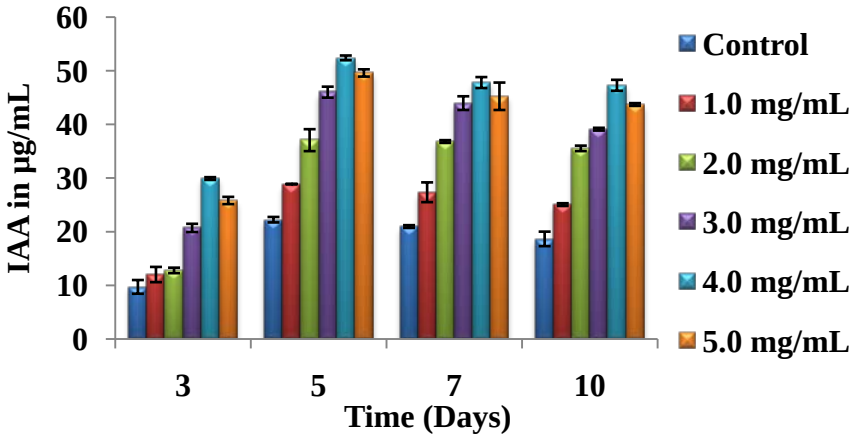

Figure 5. Production of IAA by *P. aeruginosa* UPMP3 at various concentrations of L - tryptophan. Each value is the mean of 3 replications. Vertical bars represent standard error.

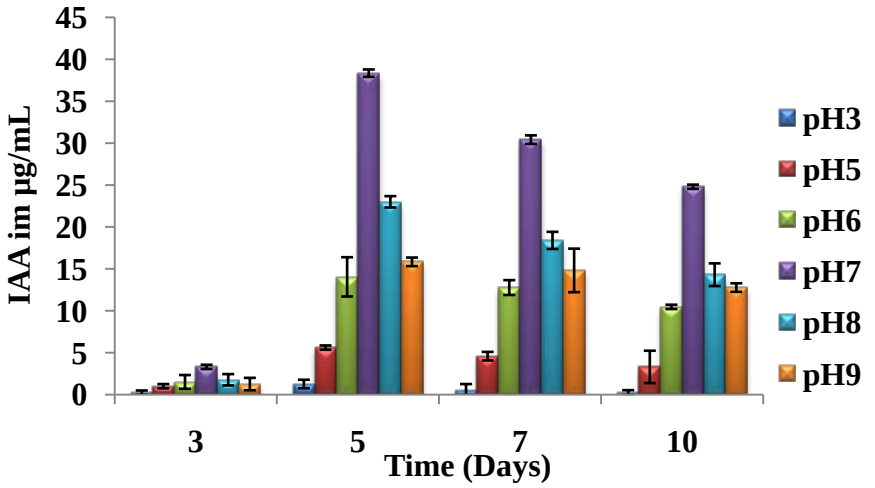

Figure 6. Production of IAA by *P. aeruginosa* UPMP3 at different pH. Each value is the mean of 3 replications. Vertical bars represent standard error.

3.6. Effect of pH on IAA Production

To optimize the different levels of pH on IAA production, the bacterial strain was inoculated in NB medium amended with 4 mg/ml L-tryptophan. Different pH levels such as 3, 5, 6, 7, 8, and 9 were maintained at 3, 5, 7 and 10 days incubation period. No IAA was produced by *P. aeruginosa* UPMP3 at pH 3. Maximum IAA production was achieved for *P. aeruginosa* UPMP3 38.35 µg/ml at pH 7 on 5 days incubation period (Figure 6).

IAA production decreased at pH 8.0 successively. Acidic or high alkaline pH is unsuitable for IAA production because *P. aeruginosa* UPMP3 grows poorly in these conditions. Parvin *et al.* [27] reported that the rhizobacterial strain *Burkholderia cepacia* UPMB3 produced maximum 39.12 µg/ml IAA at pH 7 in the culture medium. Shirokikh *et al.* [28] reported the distribution of *Streptomyces* sp. from acidic soils is lower than neutral soils. A significant correlation was also observed between bacterial growth and IAA production. The pH affects the function of enzyme systems and also the solubility of many substances that are important for bacterial growth. Yurekli *et al.* [29] reported that the synthesis of the highest IAA level was determined in cultures cultivated in an alkaline media at a pH of 7.5. According to Madhuri [30], different *Rhizobium* strains produced maximum IAA at pH level 7.0.

3.7. Effect of Culture Conditions and Incubation Period on IAA Production

From the result it was observed that IAA production for the bacterial strain *P. aeruginosa* UPMP3 was influenced by the culture conditions and incubation periods. The culturing of *P. aeruginosa* UPMP3 under static and shaken conditions was tested for the production of IAA. The maximum production of IAA for *P. aeruginosa* UPMP3 (52 µg/ml) was achieved in shaken condition on 5 days incubation period supplemented with 4 mg/ml L- tryptophan which was statistically significant compared to the static condition (Figure 7).

The reason hypothesized that during shaken condition, the bacterium might be able to get maximum L-tryptophan supplied in the culture medium, which could result in more IAA production.

The bacterial strain *Burkholderia cepacia* UPMB3 was influenced by the culture conditions and incubation periods as well. Both the static and shaken conditions were tested for the production of IAA. The maximum production of IAA for *B. cepacia* UPMB3 was achieved in shaken condition on 7 days of incubation period [27]. Ahmad *et al.* [16] reported that production of IAA in fluorescent *Pseudomonas* isolates increased with an increase of L- tryptophan concentration from 1 to 5 mg/ml in the majority of isolates. In presence of 5mg/ ml of L- tryptophan, 5 isolates of *Pseudomonas* produced high levels (41.0 to 53.2 µg/ml) of IAA while 6 other isolates produced IAA in the range of 23.4 to 36.2 µg/ml at 7 days incubation period. Production of IAA in *Bacillus licheniformis* MML2501 with a maximum of 23 µg/ml under optimised conditions such as pH 7.0, temperature 35 °C, L- tryptophan at a concentration of 16 mM and at 200 rpm shaken conditions [20]. Ritika *et al.* [18] reported that the production of auxins like substances by the different strains of *Pseudomonas* sp. ranges from 1 to 30 µg/ml under shaken conditions.

3.8. Effect of Bacterial IAA on Oil Palm Seedling Growth

A pot experiment was conducted to study the influence of the bacterial extracts (UPMP3) on growth promotion of oil palm germinated seedlings. The results of the pot experiment revealed that among the treatments, T1 (germinated seeds inoculated with *P. aeruginosa* UPMP3) and T2 (germinated seeds inoculated with synthetic IAA) 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 produced average 14.66 roots per seedlings whereas the synthetic and control treatment produced average 9.55 and 9.00 roots per seedling after 4 weeks of planting. The average root length, shoot length, number of leaves, and leaf length per seedling also found higher than the control treatment. Results are presented in Table 3, Figure 8 and Figure 9.

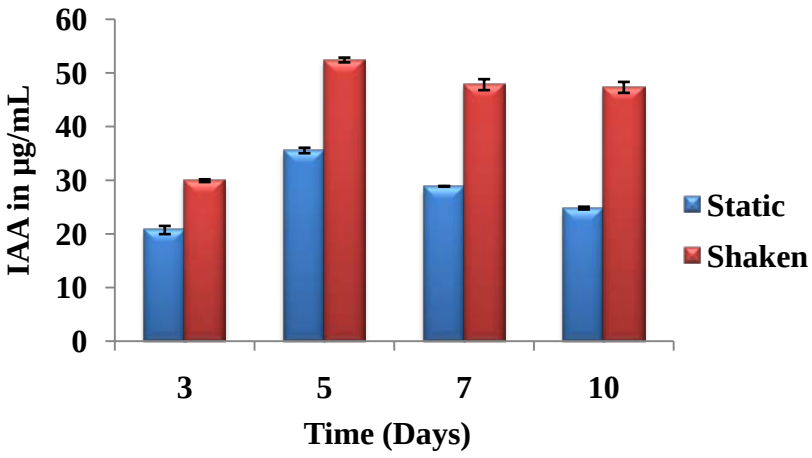

Figure 7. Effect of culture conditions and incubation period on IAA production by *P. aeruginosa* UPMP3. Each value is the mean of 3 replications. Vertical bars represent standard error.

Table 3. Morphogenic response of oil palm seedlings inoculated with bacterial supernatant after 4 weeks

Treatments		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	Synthetic IAA + GOPS	18.11± 0.92^{a}	1.90 ± 0.12^{cd}	9.55 ± 1.07^{a}	1.66 ± 0.16^{cd}	3.07 ± 0.32^{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.

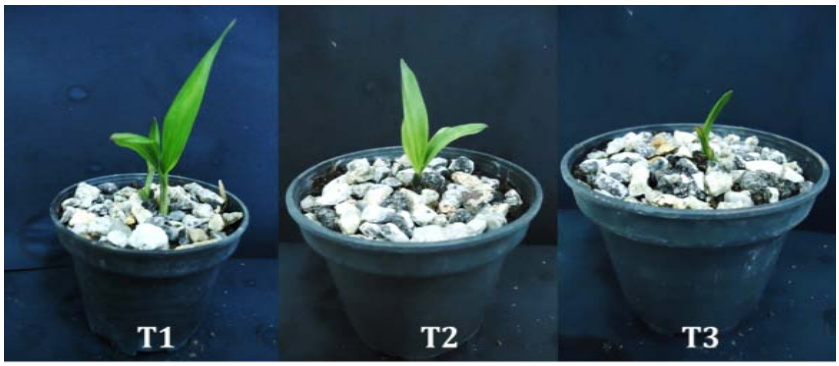

Figure 8. Growth performance of bacteria treated oil palm seedlings in pot experiment after 4 weeks. T1: *P. aeruginosa* UPMP3 extract + Oil palm seedling, T2: Synthetic IAA + Oil palm seedling and T3: Untreated oil palm seedling.

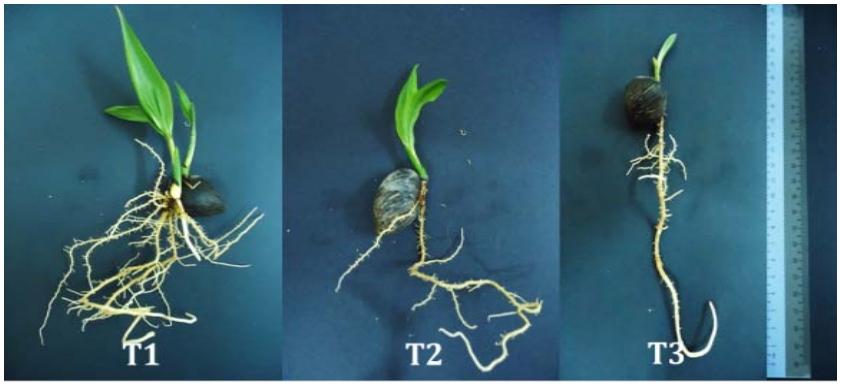

Figure 9. Influence of phytohormones in different treatments on morphogenic response of oil palm seedlings in pot experiment. T1: Oil palm seedling + *P. aeruginosa* UPMP3, T2: Oil palm seedling + Synthetic IAA, T3: Untreated Oil palm seedling

This result confirmed that the auxins especially (IAA) produced by the bacterial strain *P. aeruginosa* UPMP3 in the culture filtrate plays a role in plant growth promotion of oil palm seedlings. Parvin *et al.* [27] reported that the rhizobacterial strain *Burkholderia cepacia* UPMB3 produced IAA and it significantly promoted oil palm seedling growth compare to the synthetic IAA. Patten and Glick [10] explained the role of IAA produced by *Pseudomonas putida* in the development of the host plant root system. Seed treatment of *B. licheniformis* MML2501 in groundnut showed a significant increase in seed germination, plant growth and yield under potted plant experiments [20]. El-Tarabily [1] reported that *Streptomyces* spp. from a tomato rhizosphere had the ability to produce IAA and improved tomato growth by increasing root dry weight. The culture filtrate from the strain *Streptomyces* CMU- H009 stimulated a significant increase in the germination and root elongation of maize and cowpea seedlings [31]. 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 [32].

4. Conclusion

The findings of the present investigation highlighted that the bacterial strain *P. aeruginosa* UPMP3 are capable to produce IAA which may contribute in the promotion of plant growth as well as the contributor of other Plant Growth Promoter (PGP) traits. So the strain *P. aeruginosa* UPMP3 could be formulated as biofertilizer for commercial use instead of chemical fertilizer. Although rhizobacterial hormone production has received more attention, the role of rhizobacterial hormone degradation may be particularly important, and may partially account for unsuccessful attempts in applying hormone-producing PGPR to stimulate plant growth. However, further studies using IAA mutant strains of the isolate are needed to explore the exact contribution of IAA production.

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Conflict of Interest

The authors declare that they don't have any competing interests.

References

[1] El-Tarabily, K.A., "Promotion of tomato (*Lycopersiconesculentum* Mill.) plant growth by rhizosphere competent 1-aminocyclopropane-1-carboxylic acid deaminase-producing streptomycete actinomycetes", *Plant and Soil*, 308: 161-174, 2008.

[2] Ashrafuzzaman, M., Hossen, F.A., Ismail, M.R., Hoque, M.A., Islam, M.Z., Shahidullah, S.M. and Meon, S., "Efficiency of plant growth promoting Rhizobacteria (PGPR) for the enhancement of rice growth", *African Journal of Biotechnology*, 8:1247-1252, 2009.

[3] Compant, S. and Clement, C., "Plant growth-promoting bacteria in the rhizo- and endorhizosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization", *Soil Biology & Biochemistry*, 42: 669-678, 2010.

[4] Patten, L. and Glick, R.B., "Bacterial biosynthesis of indole-3-acetic acid", *Canadian Journal of Microbiology*, 42: 207–220, 1996.

[5] Mastretta, C. and Barac, J., "Endophytic bacteria and their potential application to improve the phytoremediation of contaminated environments" *Biotechnology & Genetic Engineering Reviews*, 23: 175-297, 2006.

[6] Khakipour, N., Khavazi, K., Mojallali, H., Pazira, E. and Asadirahmani, H., "Production of Auxin Hormone by Fluorescent Pseudomonads", *American-Eurasian Journal of Agricultural & Environmental Sciences*, 4: 687-692, 2008.

[7] Bhavdish, N., Johri, A., Sharma, J. and Virdi, S., "Rhizobacterial diversity in India and its influence on soil and plant health" *Advances in Biochemical Engineering / Biotechnology*, 84: 49-89, 2003.

[8] Arkhipova, T.N., Veselov, S.U., Melentiev, A.I., Martynenko, E.V. and Kudoyarova, G.R., "Ability of bacterium *Bacillus subtilis* to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants". *Plant Soil*, 272: 201-209, 2005.

[9] Idris, E.E., Iglesias, E.J., Talon, M. and Borriss, R., "Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42", *Molecular Plant-Microbe Interactions*, 20: 619-626, 2007.

[10] Patten, C.H. and Glick, B.R., "Role of *Pseudomonas putida* Indole acetic Acid in development of the host plant root system" *Applied and Environmental Microbiology*, 68: 3795-3801, 2002.

[11] Zaiton, S., Sariah, M. and Zainal Abidin, M.A., "Isolation and Characterization of Microbial Endophytes from Oil Palm Roots: Implication as Biocontrol Agents against *Ganoderma*", *The Planter*, 82: 587-597, 2006.

[12] Sambrook, J., Fritsch, E. F. and Maniatis, T., "Molecular cloning: a laboratory manual, 2nd ed." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989.

[13] Sapak, Z., Sariah, M. and ZainalAbidin, M.A., "Effect of endophytic bacteria on growth and suppression of *Ganoderma* infection in oil palm", *International Journal of Agriculture and Biology*, 10: 127-132, 2008.

[14] Tien, T.M., Gaskins, M.H. and Hubbell, D.H., "Plant growth substances produced by *Azospirillumbrasilense* and their effect on the growth of pearl millet (*Pennisetumamericanum* L.)", *Applied and Environmental Microbiology*, 37: 1016-1024, 1979.

[15] Fried, B. and Sherma, J., "Thin-Layer Chromatography: Techniques and Applications", Marcel Dekker, New York, 1982.

[16] Ahmad, F., Ahmad, L. and Khan, M.S., "Indole Acetic Acid Production by the Indigenous Isolates of Azotobacter and Fluorescent Pseudomonas in the Presence and Absence of Tryptophan", *Turkish Journal of Biology*, 29: 29-34, 2005.

[17] Yasmin, S., Bakar, M. A. R., Malik, K. A. and Hafeez, F. Y., "Isolation, characterization and beneficial effects of rice associated plant growth promoting bacteria from Zanzibar soils", *Journal of Basic Microbiology*, 44: 241-252, 2004.

[18] Ritika, K.R., Ajay, K., Amit, K., Sandip, P., Amit, P. and Mohinder, K., "Indole Acetic Acid production by fluorescent Pseudomonas isolated from the rhizospheric soils of Malus and Pyrus", *Recent Research Science Technology*, 4: 06-09, 2012.

[19] María, G.T.-R., Sandra, A.V.-P., Jaime, B.-C., and Patricia, M.-N., "Isolation of *Enterobacteria*, *Azotobacter* sp. and *Pseudomonas* sp., Producers of Indole-3-Acetic acid and siderophores, from Colombian rice rhizosphere", *RevistaLatinoamericana de Microbiología*, 42: 171-176, 2000.

[20] Prashanth, S. and Mathivanan, N., "Growth promotion of groundnut by IAA producing rhizobacteria *Bacillus licheniformis* MML2501", *Archives of Phytopathology and Plant Protection*, 43: 191-208, 2010.

[21] Sharma, S., Verma, P. P. and Kaur, M., "Isolation, Purification and Estimation of IAA from *Pseudomonas* sp. using High-performance liquid Chromatography", *Journal of Pure and Applied Microbiology*, 8(4): 1-6, 2014.

[22] Crozier, A., Arruda, P., Jasmin, J.M., Monterio, A.M. and Sandberg, G., "Analysis of IAA and related indoles in culture medium from *Azospirillumlipoferum* and *A. brasilense*", *Applied and Environmental Microbiology*, 54: 2833-2837, 1988.

[23] Khalifah, R.A., Lowell, N., Lewis and Coogins, C.W., "Differentiation between indole acetic acid the Citrus auxin by column chromatography", *Plant Physiology*, 41: 208-210, 1966.

[24] Crozier, A. and Reeve, D.R., "The application of high performance liquid chromatography to the analysis of plant hormones. In (ed.) Plant growth regulation", Proc. 4 Int. Conf. Plant Growth Substances, New York: P.E. Pilet, pp 67- 76, 1977.

[25] Datta, C. and Basu, P.S., "Indole acetic acid production by a *Rhizobium* species from root nodules of a leguminous shrub, *Cajanuscajan*", *Microbiological Research*, 155: 123-127, 2000.

[26] Karnwal, A., "Production of Indole Acetic Acid by Fluorescent Pseudomonas in the Presence of L-Tryptophan and Rice Root Exudates" *Journal of Plant Pathology*, 91: 61-63, 2009.

[27] Parvin, W., Jahan, Q. S. A., Rahman, M.M. and Wong, M.Y., "In vitro Screening and Optimization of IAA Production from Plant Growth Promoting Rhizobacteria *Burkholderiacepacia* UPMB3", *Plant Tissue Culture and Biotechnology*, 28(1): 25-34, 2018.

[28] Shirokikh, I.G., Zenova, G.M., Merzaeva, O.V., Lapygina, E.V., Batalova, G.A. and Lysak, L.V., "Actinomycetes in the prokaryotic complex of the rhizosphere of oats in a soddy-podzolic soil", *Eurasian Soil Science*, 40: 158-162, 2007.

[29] Yurekli, F., Geckil, H. and Topcuoglu, F., "The synthesis of indole-3-acetic acid by the industrially important white-rot fungus *Lentinussajor-caju* under different culture conditions", *Mycological Research*, 107: 305-309, 2003.

[30] Madhuri, M., "Screening of rhizobia for indole acetic acid production", *Annals of Biological Research*, 2 (4): 460-468, 2011.

[31] Sutthinan, K., Akira, Y., John, F.P. and Saisamorn, L., "Indole-3-acetic acid production by *Streptomyces* sp. isolated from some Thai medicinal plant rhizosphere soils", *EurAsian Journal of BioSciences*, 4: 23-32, 2010.

[32] Molina-Favero, C., Creus, C.M., Lanteri, M.L., Correa-Aragunde, N., Lombardo, M.C., Barassi, C.A. and Lamattina, L., "Nitric oxide and plant growth promoting rhizobacteria: Common features influencing root growth and development", *Advances in Botanical Research*, 46: 1-33, 2007.