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Selection of bacteria producing indole-3-Acetic acid and its application on oil palm seedlings (*Elaeis guineensis* Jacq.)

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ABSTRACT

Aims: Oil palm (*Elaeis guineensis* Jacq.) is the largest plantation crop in Indonesia and has a high economic value. The use of synthetic fertilizers and pesticides is the largest plantation expenditure and causes many environmental problems. The objectives of the research were to select indole-3-acetic acid (IAA) producing bacteria and test its application in oil palm seedlings.
Methodology and results: The IAA producing bacteria selected in this research consisted of 9 chitinolytic and 16 cellulolytic bacteria. Screening results based on colorimetric method showed that bacterial isolates, KAHN 15.12 and *Bacillus thuringiensis* SAHA 12.08, has the ability to produce IAA. Both isolates were negative on hypersensitivity tests to the plant. Quantitative measurement using HPLC showed IAA was produced by peaks at a retention time of 21 to 22 min was the same as IAA standard. KAHN 15.12 had 99% of identify with *Serratia marcescens* when identified based on 16S rRNA gene sequences. The application of *S. marcescens* KAHN 15.12 and *B. thuringiensis* SAHA 12.08 on oil palm seedlings showed greater improvement in oil palm seedling growth than application of chemical fertilizer. The isolates increased the number of seedling lateral roots by up to 60.7-65%.
Conclusion, significance and impact of study: Bacterial isolates KAHN 15.12 and SAHA 12.08 can promote the growth of oil palm seedling roots. The isolates have potential as a biological fertilizer and to reduce chemical fertilizer usage on oil palm seedlings.

Keywords: biological fertilizer, oil palm seedlings, *Serratia marcescens*

INTRODUCTION

Oil Palm (*Elaeis guineensis*) is a crop which has high economic value in the production of vegetable oil in Indonesia. The eminent seed quality and seedling preparation of oil palm are some factors that affect plant productivity and become a basic step which determined the planting process in the field. Expensiveness in fertilizer expenses become basic matter for in preparation of plant maintenance. Long term uses of chemical fertilizers and pesticides cause environmental problems due the residue of this synthetic compound on the soil. One of solutions in those problems is the use of a natural compound or biological agent which can promote plant growth.

Indole-3-acetic acid (IAA) is the most active phytohormone in nature (Tsavkelova *et al*., 2005). IAA endogenously produced by plants and is also exogenously produced by bacteria. The exogenous IAA produced by bacteria has a greater effect in plant growth in increasing root hair formation and root differentiation. In low concentrations, exogenous IAA stimulates elongation of primary roots while in high concentrations it stimulates formation of lateral and adventitious roots (Patten and Glick, 2002).

Formation of root hairs is stimulated by presence of rhizosphere bacteria that produce IAA. The presence of the rhizosphere bacteria depends on root exudates. Root exudates determine the diversity and abundance of microorganisms in the rhizosphere. The root exudates contain sugars, amino acids, organic acids, phenolic compounds, vitamins, and various secondary metabolites (Castro *et al*., 2009). The IAA biosynthesis pathway relies on the production of tryptophan as the precursor of IAA (Spaepen *et al*., 2007).

Azospirillum and *Bacillus* spp. have been reported to fix N_2 in the rhizosphere of non-leguminous crops. Rhizobacteria have been pointed out as having potential to stimulate root growth and water absorption in oil palm seedlings (Amir *et al.*, 2005). Diazotrophic bacteria *Herbaspirillum sorepedicae* Z78 and *Microbacterium* sp. E7 isolated from oil palm root tissue were shown to have positive effects on oil palm shoot growth (Ai'shah *et al.*, 2013). Cellulolytic and chitinolytic rhizosphere bacteria have been isolated from soil of oil palm plantations in Jambi (Haryanto, 2013). One isolate, *Bacillus*

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thuringiensis SAHA 12.08, produced chitinase and had antagonistic activity on *Culvularia affinis* and *Colletotrichum gleosporioides* (Asril *et al.*, 2014). The cellulolytic bacteria could degrade oil palm wastes such as empty bunches (Zainudin *et al.*, 2013), while the chitinolytic bacteria have been widely reported as biological control agents (Saleem and Khan, 2011). The study of bacteria producing IAA and stimulating oil palm seedlings is thus promising. The objectives of the study were to obtained bacteria producing IAA from plantations soil and to apply the selected bacteria to oil palm seedlings.

MATERIALS AND METHODS

Materials

The bacterial isolates consisted of 25 rhizosphere bacteria of which 16 were cellulolytic bacteria and 9 were chitinolytic bacteria originally collected from oil palm and rubber plantations soil around Taman Nasional Bukit Duabelas (TNBD), Jambi. All isolates are maintained in the IPB Culture Collection (IPBCC), Bogor Agricultural University. Oil palm seedlings were obtained from the Indonesia Oil Palm Research Institute (IOPRI) in Medan.

Cultivation of isolates

The twenty five bacterial isolates were cultivated on slant nutrient agar as stock cultures.

Hypersensitivity test

All of the 25 bacteria isolates used in hypersensitivity reaction (HR) were tested on 2-3 months old tobacco leaves (*Nicotiana tabacum* L.). Bacterial suspension (10^8 CFU/mL) was injected into mesophyll between the leaf veins of the tobacco. HR observations were carried out at 24 and 48 h after injection. Distilled water was injected as a negative control while the positive control was *Pseudomonas syringae* (Klement and Godman, 1967). Positive reaction was indicated by the formation of necrotic symptoms on the tobacco leaves. The isolates which showed no necrotic symptoms (negative reaction) in the tobacco leaves were then used in the IAA quantification using colorimetric method.

Measurement of IAA production

Colorimetric method

IAA content was measured using colorimetric method (Patten and Glick, 2002). All of isolates were cultured in 50 mL Nutrient Broth (NB) medium supplemented with and without L-tryptophan 1 mM and then incubated in a rotary incubator at 30 °C for 24 h. One milliliter of cultured bacteria was then centrifuged at 4600×*g* for 15 min. A total of 1 mL supernatant was homogenated with 4 mL Salkowski reagent which was made from 150 mL concentrated H_2SO_4, 7.5 mL $FeCl_3 \cdot 6H_2O$ 0.5 M and 250 mL sterile distilled water. This test was done in duplicates. The suspension was incubated for 15 min in the dark (without light). The light absorbance was measured using spectrophotometer at 520 nm wavelengths to estimate IAA concentration (Patil, 2011). Colorimetric method was used as an early indication of the ability of bacteria to produce IAA indirectly, so that the isolates which produced the highest concentrations of IAA were then used in the more precise quantitative analysis using HPLC.

HPLC method

Quantitative analysis of IAA was performed using a Shimadzu High Performance Liquid Chromatography (HPLC) LC 20A reversed phase with C 18 column. A total of 5 mL each of the liquid cultures of the isolates KAHN 15.12 and SAHA 12.08 were taken and then centrifuged at 8400× *g* for 15 min. The pH of supernatant was adjusted to 2.8 by adding hydrochloric acid and then extracted using ethyl acetate 3 times. The extracted product was evaporated (dried) and suspended into 1 mL methanol. Samples were analyzed by HPLC using methanol: acetic acid: double distillated water (30:1:70 v/v/v) as the mobile phase, with 1.2 mL/min flow rate, with the detector at 254 nm wavelength. IAA sample concentration was calculated based on the concentration of pure IAA measured in the same circumstances and conditions (Mehnaz and Lazarovits, 2006).

Bacterial growth and IAA production

Bacterial cultures were made from isolates (24 h after incubation) in 50 mL Nutrient Broth (NB) medium then incubated in a rotary incubator at 30 °C till the cell density reached 10^8 cell/mL. A total of 1 mL culture was inoculated in 100 mL NB medium supplement with L-tryptophan 1 mM. The bacterial growth curve and IAA production were measured every 6 h until the declining phase was obtained. Optical density of bacterial cell was determined using a spectrophotometer at 620 nm and the concentration of IAA supernatant homogenated with Salkowski's reagent was measured with a spectrophotometer at 520 nm wavelength.

Molecular identification using 16S rRNA gene

Bacterial isolate KAHN 15.12 was grown in NB medium for 24 h. Genomic DNA was extracted using a PrestoTM gDNA Bacteria Mini Kit (Geneaid, Japan). DNA was extracted and then used as a template in PCR (Polymerase Chain Reaction). The primers 63F (5' - CAG GCC CAC ATG TAA CAA GTC - 3') and 1387R (5' - GGG CGG WGT CAA GGC GTA - 3') were used to amplify the sequences of 16S rRNA gene (Marchesi *et al*., 1998). The PCR conditions followed pre-denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and elongation at 72 °C for 1.5 min. Post PCR products were extended at 72 °C for 10 min. PCR products were sequenced and aligned with

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data sequences from GenBank were using the BlastN program. The phylogenetic trees were constructed using MEGA 6.0 software.

Application of bacteria oil palm seedlings

Growth medium consisted of soil from Cikabayan Farm at the Bogor Agricultural University with compost and rice husk mixed at a ratio of 5: 3: 2, autoclaved at 121 °C and 1 atm, and placed in 30 cm × 30 cm polybags. Oil palm seedlings (3 months old) were treated with Dithane M-45 fungicide for 2 min, were rinsed with sterile distilled water, and then planted in the polybags and acclimatized for 2 weeks.

Experimental design

The experimental design was compiled using one factor in complete randomized design (CRD). The treatments included: (P1) without bacteria as negative control, (P2) 5 grams (100% standard dose) of Rock phosphate (RP) fertilizer as positive control, (P3) culture isolate KAHN 15.12, (P4) culture isolate SAHA 12.08, (P5) ½ dose of RP fertilizer + isolate KAHN 15.12, (P6) ½ dose of RP fertilizer + isolate SAHA 12.08, (P7) 100 % dose of RP fertilizer + isolate KAHN 15.12, (P8) 100 % dose of RP fertilizer + isolate SAHA 12.08. Each treatment had 4 replicates.

The observed parameters in this study included: the number of lateral roots, plant height, main root diameter, stem length, stem diameter, main root length, number of leaves, and fresh weight.

Data analysis

The data were analyzed using ANOVA and Duncan's Multiple Range Test (DMRT) at the significance level of α = 5%.

RESULTS AND DISCUSSION

Hypersensitivity Test

A total of 25 bacterial isolates derived from TNBD Jambi were tested on tobacco leaves, 13 isolates did not cause necrotic symptoms (negative hypersensitivity) in the tobacco leaves. This indicated that the isolates were not pathogenic to the plants. When pathogens invade the host, the plant tissue reacts with the formation of necrosis to prevent the further spread of pathogens (Klement and Godman, 1967). The isolates that did not cause necrotic symptoms on the leaves were then tested for their ability to produce IAA in media supplemented with and without tryptophan by colorimetric method (Table 1).

Table 1: Screening of bacteria for plant pathogenicity and IAA production.

Isolates Code	Hipersensitivity Test	IAA Concentration(ppm)	
		With tryptophan	Without tryptophan
SAHA 2.1	+	*)	*)
SAHA 2.2	-	2.43	0.00
SAHA 3.02	+	*)	*)
SAHA 3.4	-	2.88	1.16
SAHA 3.5	+	*)	*)
SAHA 3.6	-	0.00	0.00
SAHA 32.6	-	0.01	0.00
SAHA 5.8	+	*)	*)
SAHA 12.04	+	*)	*)
SAHA 12.07	-	1.11	0.00
SAHA 12.08	-	3.99	0.35
SAHA 12.17	+	*)	*)
SAHN 13.28	+	*)	*)
KAHA 7.01	+	*)	*)
KAHN 10.03	+	*)	*)
KAHN 10.06	+	*)	*)
KAHN 10.10	-	2.20	0.00
KAHN 10.11	-	3.23	0.00
KAHN 10.12	+	*)	*)
KAHN 10.13	-	1.76	0.00
KAHN 10.14	-	2.81	0.00
KAHN 10.15	-	1.34	0.00
KAHN 10.16	+	*)	*)
KAHN 15.38	-	2.91	0.00
KAHN15.12	-	3.75	0.038

−, Negative Hypersensitivity; +, Necrotic (Positive hypersensitivity)
*) Isolates causing necrotic symptoms were not selected for IAA production testing

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Selection of IAA producing bacteria and IAA analysis using HPLC

Isolate SAHA 12.08 and KAHN 15.12 produced high of IAA concentration in the presence of 1 mM tryptophan (Figure 2). Concentrations of IAA produced by *Azotobacter* strains were between 1.47-11.88 ppm after induction with 1 mg/mL tryptophan. Some microorganisms are able to produce auxin in the presence of L-tryptophan as IAA precursor (Ahmad *et al*., 2005).

The concentrations of IAA from isolates of SAHA 12.08 and KAHN 15.12 were subsequently confirmed and measured by reversed phase high performance liquid chromatography (HPLC). The resulting IAA concentrations derived from extracts of SAHA 12.08 and KAHN 15.12 was 94.015 and 91.568 ppm (Table 2).

IAA measurement by the colorimetric method differed from the HPLC method. IAA measurement by the colorimetric method used the supernatant of culture which had been was incubated for 24 h (before the optimum time of IAA production was known). IAA measurement by HPLC method used samples extracted supernatant from cultures incubated for the optimum time of IAA production. The HPLC method detects IAA compounds from extracted IAA samples based on polarity level (Sweetser and Swartzfager, 1978). The colorimetric method measures the visible color of the solution using a spectrophotometer (Patten and Glick, 2002), but it possess disadvantages, lacking specificity, sensitivity, or stability of color complex formed (Gordon and Weber, 1951). HPLC has a high separation or resolution power as well as excellent selectivity to separate various solutes, which make HPLC a high quality method (Skoog *et al.*, 2010).

IAA chromatogram peaks were obtained by injecting IAA extracted sample. IAA extraction was obtained from condensation of SAHA 12.08 and KAHN 15.12 supernatant from the optimum time of IAA production. Active compounds of IAA in both samples had the same retention time with the IAA standards. Extract samples injected into the HPLC showed chromatogram peaks of IAA at 20.912 min (retention time) for SAHA 12.08 and at 20.167 min (retention time) for KAHN 15.12. The chromatogram peaks of the samples were then compared with IAA standards (synthetic IAA) with a range of retention times between 20 and 22 min (Figure 1). Mujahid *et al*. (2011) reported that there were various retention times for several compounds involved in biosynthesis pathways of *Rubrivivax benzoatilyticus* JA2. The retention time of indole 3- aldehyde was 17.5 min, IAA was 18.55 min, indole 3- carboxylic acid was 18.9 min, indole 3- acetonitrile was 23.1 min, and indole was 24.6 min.

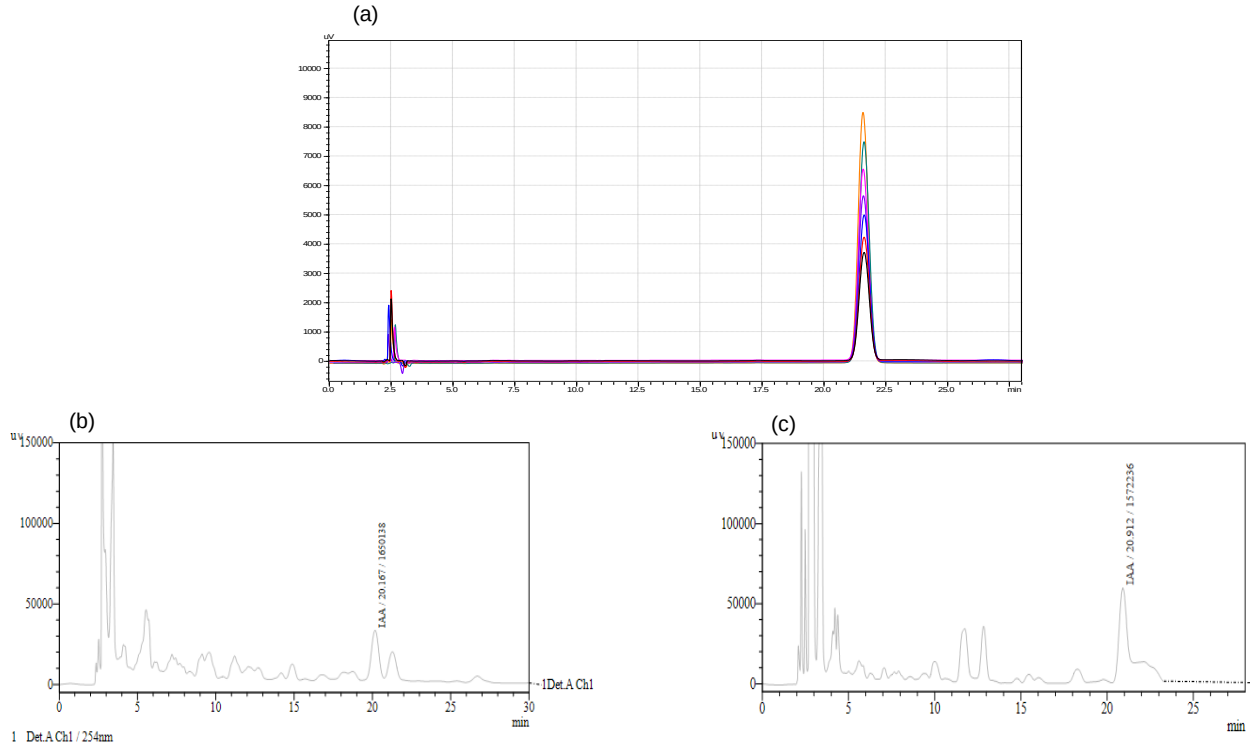

Figure 1: IAA analysis using HPLC. (a) standard IAA chromatogram; (b) KAHN 15.12 chromatogram; (c) SAHA 12.08 chromatogram.

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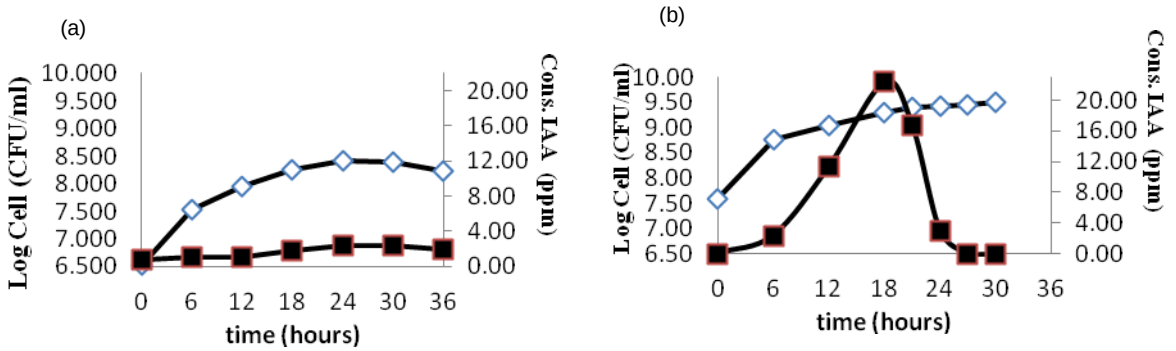

Figure 2: Bacterial growth and IAA production of SAHA 12.08 (a) KAHN 15.12; (b) cell log, IAA concentration (ppm).

Table 2: Measurement of IAA concentration using HPLC.

Isolate	Retention time	Area	Concentration (ppm)
KAHN 15.12	20.167	1650138	94.015
SAHA 12.08	20.912	1607267	91.568

Molecular identification

SAHA 12.08 was already previously identified molecularly based on 16S rRNA gene as *Bacillus thuringiensis* (Asril *et al*., 2014). *Bacillus thuringiensis*-KR1 is suitable for development as a commercial inoculant and potential as plant growth promoter (Mishrah *et al.*, 2009). The amplification of the 16S rRNA gene of the KAHN 15.12 isolate produced DNA fragments of 1300 bp length (Figure 3). The analysis of the 16S rRNA gene sequences via BLASTN program showed that isolate the KAHN 15.12 is 99% identify with *Serratia marcescens* (Figure 4). *Serratia marcescens* isolated from rhizosphere of coconut tree had chitinase activity as a biocontrol agent and was able to stimulate plant growth, produce siderophore, fix nitrogen (Tilak *et al*., 2005; George *et al*., 2013), and solubilize phosphate (Chakraborty *et al*., 2010).

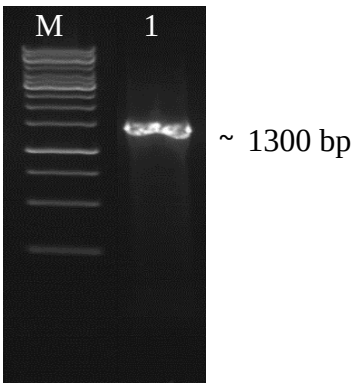

Figure 3: Electrophoresis result of 16S rRNA gene of KAHN 15.12. (M) 1 kb marker; (1) amplified 16S rRNA gene of KAHN 15.12.

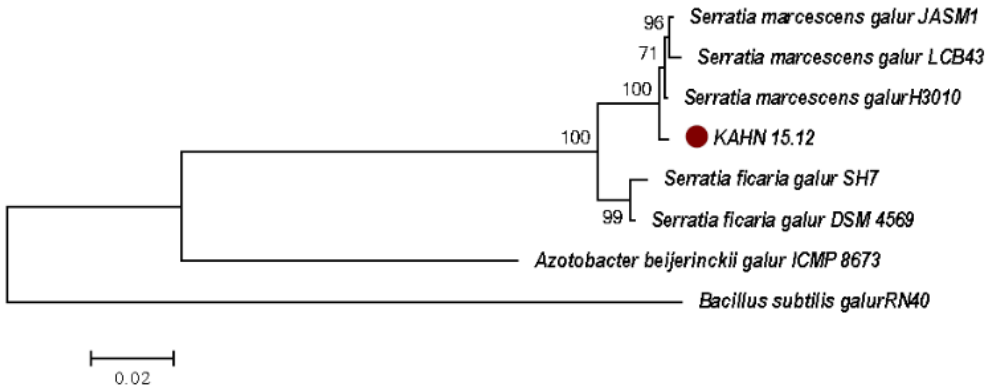

Figure 4: Construction of KAHN 15.12 isolate phylogeny based on 16S rRNA gene sequences.

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Application in oil palm seedlings

The cultures of SAHA 12.08 and KAHN 15.12 were applied to oil palm seedlings growing in polybags. The application of KAHN 15.12 and SAHA 12.08 isolates to oil palm seedlings showed greater improvement on oil palm seedling growth than chemical fertilizer treatment alone. These isolates increased the number of lateral roots up to 60.7-65% higher than negative control (Table 3).

The application of RP fertilizer and bacteria separately or together (in combination) increased the number of lateral roots significantly compared to the negative control. The bacterial culture treatments (P3 and P4) increased the number of lateral roots (Figure 5). This might be related to their ability to produce IAA. IAA synthesized by microbe primarily affect the root system, increasing the size and number of adventitious roots (Ribeiro and Cardoso, 2012). High concentrations of IAA will stimulate the formation of lateral and adventitious roots (Patten and Glick, 2002).

The 100% dose of RP fertilizer combined with the KAHN 15.12 isolate (P7) had the best effect on the lateral roots compared to KAHN 15.12 without fertilizer. This was perhaps because KAHN 15.12 could work synergistically in solubilizing P in the rhizosphere containing RP fertilizer. Addition of P fertilizer in the soil is often not absorbed by plant directly, because 75-90% of the P fertilizer will be bound to Fe, Al, and Ca compounds in the soil (Gyaneshwar *et al*., 2002). The isolate KAHN 15.12 may have the ability to solubilize phosphate and potassium, thus the availability of 100% dose of RP fertilizer in the soil can be optimized. The increased number of lateral roots will affect the density and length of root hairs.

The combination treatment of half dose of RP fertilizer with isolate SAHA 12.08 (P6) increased the number of lateral roots higher than the 100% dose RP fertilizer application (P2). Combination of 100% dose of RP fertilizer with SAHA 12.08 isolate did not show better effect than SAHA 12.08 without fertilizer.

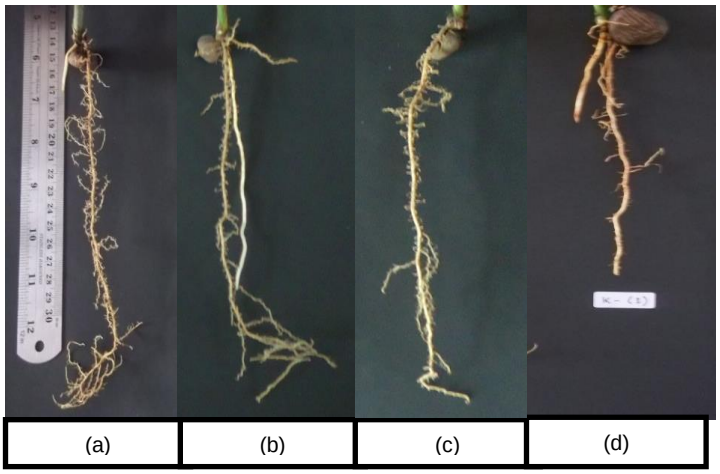

Figure 5: Effect of treatments on oil palm seedlings 90 days after planting (a) inoculation with KAHN 15.12 (P3); (b) SAHA 12.08 (P4); (c) RP (Rock Phosphate) fertilizer as positive control (P2); (d) without bacteria and fertilizer addition as negative control (P1).

Table 3: Effect of KAHN 15.12 and SAHA 12.08 bacterial treatment in oil palm seedlings at 90 DAP (days after planting).

P	Number of lateral roots	Plant height (cm)	Roots diameter (mm)	Stem length (cm)	Stem diameter (mm)	Primer roots length (cm)	Number of leaves	Wet weight (g)
P1	141^{d}	18.17^{b}	0.18^{a}	2.82^{c}	0.400^{c}	16.02^{b}	3.00^{b}	4.19^{ab}
P2	322^{c}	22.72^{ab}	0.16^{a}	3.45^{ab}	$0.44b^{c}$	20.70^{ab}	3.00^{b}	4.11^{ab}
P3	359^{bc}	20.97^{ab}	0.17^{a}	3.87^{a}	0.48^{abc}	22.92^{ab}	3.00^{b}	4.90^{ab}
P4	403^{ab}	20.22^{ab}	0.22^{a}	3.45^{ab}	0.51^{abc}	21.45^{ab}	3.00^{b}	5.57^{a}
P5	408^{ab}	20.92^{ab}	0.20^{a}	3.57^{a}	0.54^{ab}	21.10^{ab}	3.50^{a}	4.57^{ab}
P6	411^{ab}	23.82^{a}	0.16^{a}	3.30^{ab}	0.52^{abc}	21.32^{ab}	4.00^{a}	4.75^{ab}
P7	437^{a}	23.87^{a}	0.19^{a}	3.60^{a}	0.61^{a}	23.97^{a}	4.00^{a}	5.21^{ab}
P8	340^{c}	20.17^{ab}	0.15^{a}	2.90^{bc}	0.44^{bc}	20.25^{ab}	4.00^{a}	3.54^{b}

The number followed by different letters in the column showed significantly different results based on Duncan test at $\alpha = 0.05$. P : treatment; P1, without bacteria addition as negative control; P2, Rock phosphate fertilizer; RP, as positive control; P3, culture isolates KAHN 15.12; P4, culture isolates SAHA 12.08; P5, ½ dose of RP fertilizers + isolates KAHN 15.12; P6, 1/2 dose of RP fertilizers + isolates SAHA 12.08; P7, RP + culture isolates KAHN 15.12; P8, RP + culture isolates SAHA 12.08.

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CONCLUSION

The bacterial isolates *B. thuringiensis* SAHA 12.08 and *Serratia marcescens* KAHN 15.12 produced Indole acetic acid during their stationary phase in culture. Both isolates stimulated the amount of lateral roots growth in oil palm seedlings. Isolate SAHA 12.08 and KAHN 15.12 with a half dose of rock phosphate fertilizer showed a better effect oil palm seedlings such as number of lateral roots, stem diameter, number of leaves.

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