Characterization and application of nitrogen-fixing and indole-3-acetic acid producing bacteria A13 in Oil Palm (*Elaeis guineensis Jacq.*) seedling

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Istianah I, Mubarak NR, Tjahjoleksono A. 2021. Characterization and Application of Bacterial Isolate A13 as a Nitrogen-Fixing and *Indole-3-Acetic Acid* Producing Bacteria in Oil Palm (*Elaeis guineensis Jacq.*) Seedling. Journal of Microbial Systematics and Biotechnology 3(1), 32-40.

Abstract

Oil palm plantations have a good prospect in Indonesia. One of the efforts to improve the productivity of oil palm plantation is the application of bacteria as biological fertilizer. The research was conducted to characterize and apply the nitrogen-fixing and indole-3-acetic acid producing bacteria in oil palm seedlings. The bacteria was isolated from soil samples which taken from Taman Nasional Bukit Dua Belas (TNBD) Jambi. Nitrogen free bromthymol blue (NFB) is used as media for nitrogen-fixing bacterial isolation. Selected isolate named A13 had an ability to form white pellicle on the surface of the semisolid medium, increased the pH, and changed the color of medium from green to blue Isolate A13 was identified as Gram-negative bacteria and had a rods shape. Analysis of 16S rRNA gene sequence showed that isolate A13 had a similarity with *Pseudochrobactrum assacharolyticum*. Hypersensitivity assay on tobacco leaves showed that isolate A13 was not a pathogen. During 48 hours of incubation, isolate A13 produced a maximum of IAA at the 24th hour of incubation. Isolate A13 produced 0.675 ppm of ethylene/hour in Acetylene Reduction Assay and 69,839 ppm of IAA in HPLC methods. This was the first report on nitrogen fixation and IAA production by *Pseudochrobactrum assacharolyticum* and its application in the soil of oil palm seedlings. Application of isolate A13 in oil palm seedling increased significantly the number of lateral roots, stem diameter, and height of plants.

Keywords: indole-3-acetic acid, nitrogen-fixing bacteria, Taman Nasional Bukit Dua Belas Jambi, 16S rRNA gene.

Introduction

Microbes in the soil have great benefits for crop productivity. One of the example is nitrogen-fixing microbes that can help in the process of nitrogen fixation in nature and the synthesis of plant growth substances such as indole-3-acetic acid (IAA) (Tilak et al.2005). Nitrogen is a critical element for all organisms because nitrogen is required to synthesis of protein, growth, and reproduction of organisms. Nitrogen is one of the primary macronutrients that plants use large amount for their growth and survival. Therefore, when these elements decline, the plants can not grow normally (Sanchez 1993). While IAA is
known as the auxin hormone. IAA serves to control several physiological mechanisms of plants, such as the process of cell division and differentiation of plant tissue. Endogenous IAA is produced by plants while exogenous IAA is produced by microorganisms (Haq and Dahot 2007). Exogenous IAA is one of the factors that can improve plant growth (Alexander 1977). Soil microbes such as Azotobacter, Azospirillum, Pseudomonas, and Ochrobactrum are known as bacteria having ability to fix nitrogen and produce IAA. The production of IAA between species is dynamic and fluctuating and it is influenced by environmental conditions, growth rate, and availability of substrates such as amino acids (Arshad & Frankenberger 1993).

Excessive use of chemical fertilizers can cause environmental problems such as degradation in the quality of agricultural land (Las et al. 2006). One of the solutions for these problems is application of biofertilizer to reduce the use of chemical fertilizing. Biofertilizer contains one more kind of living microorganisms which are given into the soil as an inoculant to help or provide specific nutrients for plants. Interactions between biofertilizers and plants are mutually beneficial for both, i.e. plants get extra nutrients and microorganisms get organic material which is needed for growth. Biofertilizer play a role in influencing the availability of macro and micro nutrients, improve the efficiency of nutrient absorption, increase performance of enzyme system, and metabolism. Moreover, biofertilizers also have better prospects because of the real effect in improving outcomes (Cattelan et al. 1999).

Application of biofertilizer in oil palm (Elaeis guineensis Jacq.) seedlings growth is one of the bioprospection in establishing the interaction between microorganisms and plants. Oil palm seedlings are a type of plant that has many advantages. Efforts to improve the quality of oil palm seedling be realized because the quality of seedling determines the production of prospective oil palm plantation in Indonesia. One of the efforts in increasing productivity can be done by means of efficient and effective fertilization (Mardiana & Napitupulu 2009). Therefore, the exploration and selection of nitrogen-fixing bacteria which produce indole-3-acetic acid (IAA) need to be done in order to get the best strain of bacteria for biofertilizer.

Materials and methods

Materials

The material used in this experiment was isolate A13 which had been isolated from soil samples from oil palm rhizosphere in Taman Nasional Bukit Dua Belas (TNBD) in Jambi, Indonesia and Palm sprouts were obtained from Oil Palm Research Center Medan, Indonesia.

Growth curve

Total plate count (TPC) and turbidimetry methods (Cappuccino & Sherman 2008) were used to determine the bacterial growth curve. Total Plate Count method was done by serial dilution at 10⁻¹ until 10⁻⁷ in physiological salt solution (NaCl 0.85%). Serial dilution results then was taken 0.1 mL and spread into nutrient agar (NA) medium for 24 hour incubation time at 27°C. Colony of bacteria which had grown in plate then calculated to be compared with the absorbance values obtained from the method of turbidimetry. Then the steps for turbidimetry method were inoculating cultures in NB medium and then incubated for 24 hours. Culture was grown in NB medium then shaken in incubator in 120 rpm at 30°C. Measurement of growth curve carried out every three hours for 48 hour incubation time using Genesys spectrophotometer at 620 nm wave length.
Quantification of IAA

Analysis concentration of IAA produced by isolate A13 was measured using two methods, the colorimetric method and HPLC method. One loop of isolate A13 was inoculated to 100 ml of NB medium with the addition of 1.0 mM of amino acid tryptophan as a precursor for IAA and incubated for 48 hours in a shaking incubator at a speed of 120 rpm. Every 3 hours during incubation, 1.5 mL of culture was poured into a microtube and centrifuged for 10 minutes at 8000 rpm. Supernatant was then reacted with a Salkowski reagent (Gordon & Weber 1950) and incubated for 15 minutes in the dark. The density of red color produced by this reaction was measured using a spectrophotometer Genesysat a wavelength of 520 nm. The IAA concentration is obtained based on the results of calculations on the synthetic IAA standard curve that has been made previously.

The IAA production calculations were also analyzed by the HPLC method. The first step was concentrating the bacterial supernatant using ethyl acetate. Concentration was done by dissolving 100 mL of the supernatant using ethyl acetate with a volume ratio of 1: 1. The extraction was carried out using a rotary evaporator in order to obtain the necessary crude extracts in HPLC analysis, the crude extract was obtained then dissolved in 1 mL of methanol. Then about 20 μL solution was injected using a syringe into the HPLC instrument. The mobile phase used in this analysis were water: acetic acid: methanol (70: 1: 30) (Mehnaz&Lazarovits 2006). HPLC analysis was performed using chromatography Shimadzu Prominence 20 A column of type C18. The peak which was formed from the standard form of synthetic IAA and the crude extract of isolate were detected in a UV-VIS detector at a wavelength of 254 nm.

Acetylene reduction assay (ARA)

Nitrogenase activity was measured by the method of ARA according to Gibson and Turner (1980). Isolate A13 was grown in semisolid NFB media and incubated for 5 days. Once pellicle formed, tube was closed using a rubber stopper. The air inside the tube was removed using sterile injecting equipment as much as 1 mL. Then the acetylene gas (C2H2) was injected into the tube with the same volume as the volume of air which was removed. After 2 hours of incubation, ethylene gas was measured using gas chromatography HITACHI 263-70.

Hypersensitivity assay

Hypersensitivity test was done by injecting 1 ml of isolate A13 using the result of a serial dilution at 10^-8 on the lower surface of on tobacco leaf (Nicotiana tabacum L.) (Vanneste et al. 1990). Pseudomonas syringae was used as a positive control and distilled water as a negative control. Hypersensitivity symptoms was observed after 48 hours of injection. The hypersensitive leaf showed discoloration (leaf color changed from green to yellow) around the injection area.

Application of isolate A13

Preparation of seedling

Planting medium was consisted of soil, compost, and rice husk with the ratio of 5: 3: 2. The planting medium was sterilized by autoclaving at 121 °C 1 atm for 2 hours and then put into a polybag. Before planted in the polybag, oil palm sprouts were rinsed with distilled water and Dithane-45 to reduce the risk of contamination. Sprouts were planted in polybags that already contained planting medium at a depth of ± 2 cm.
Experimental designs

Application of isolate A13 on oil palm seedling was conducted for 90 days. The design used completely randomized design (CRD) with 5 treatments, namely: negative control (without NPK fertilizer and bacterial culture), positive control (100% dose of NPK fertilizer), inoculation with culture isolate A13, combination 100% dose of NPK fertilizer and isolate A13, then a combination of 50% dose of NPK fertilizer and isolate A13.

Applied of NPK fertilizer and bacterial cultures

A total of 5 g of NPK fertilizer was given to the treatment of 100% of fertilizers and 2.5 g into the treatment 50% of fertilizers. NPK fertilizer was given at the beginning of planting by way of sown evenly around the area planting of palm seedlings in each polybag. While the bacterial cultures were given every two weeks up to 45 days after planting. Inoculant used was the culture of isolate A13 that had a density of $10^8$ cells/ml with the addition of tryptophan. A total of 10 mL cultures were inoculated using a syringe around the area planting palm sprouts.

Measurement of growth

The roots of oil palm aged 90 days after treatment were cleaned from planting medium. After that the oil palms in all treatments were measured growth. The growth parameters measured while the oil palm seedlings were maintained for 90 days of planting were: plant height, main root length, stem length, root diameter, stem diameter, number of lateral roots, number of leaves, and wet weight. Data analysis was performed using SPSS V.21 software and further tests using Duncan's test at a 95% confidence level.

Molecular identification of selected bacteria

Selected bacterial of isolate A13 was bred in Nutrient Agar media for 24 hours. DNA genome of bacteria was extracted using Presto™ Mini gDNA Bacteria Kit (Geneaid). The extracted DNA was then used as a template in Polymerase Chain Reaction (PCR). PCR was conducted to amplify 16S rRNA gene using 67F primer (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387R primer (5′-GGG CGG WGT GTA CAA GGC-3′) (Marchesi et al. 1998). The amplicon was sequenced. The sequence of isolate A13 was then aligned with the sequences available in the GenBank using BLAST-N program. Phylogenetic construction was performed by using MEGA 6.0 program.

Results

Growth curve

![Figure 1. Growth Curve (➜) and IAA production (✦) in isolate A13](image-url)
Isolate A13 produced optimum exogenous IAA at 24 hours of incubation time. IAA production increased until the end of the log phase and had an optimum production when bacteria entered the stationary growth phase (Figure 1).

**Quantification of IAA**

Results of the analysis of synthetic IAA as a standard solution showed that the peak was formed consistently on the concentration of 40-100 ppm in minutes 21.75 (Figure 2a). Results of standard solution were compared with the results obtained in the treatment using the crude extract isolate A13. Concentration of IAA that was produced by isolate A13 was 69.839 ppm with the retention time in minutes 19.605 (Figure 2b).

![Chromatogram of synthetic IAA as a standard solution (a) and isolate A13 (b)](image)

**Figure 2.** Chromatogram of synthetic IAA as a standard solution (a) and isolate A13 (b)

**Acetylene reduction assay (ARA)**

Nitrogenase assay using ARA test showed that isolate A13 could tie up the nitrogen 0.675 ppm per hour.

**Hipersensitivity assay**

Hipersensitivity test results showed that the tobacco leaves were inoculated with *Pseudomonas syringae* in the control treatment positive (+) showed necrosis symptoms after an incubation period of 48 hours. While the leaves were inoculated by using distilled water in the treatment of negative control (-) showed no symptoms of necrosis. Injection using the isolate A13 also did not show necrosis symptoms until the incubation period of 48 hours (Figure 3).
Figure 3. Hypersensitivity test on the positive control on a 48-hour incubation period (a), the negative control on a 48-hour incubation period (b), the treatment of isolate A13 at 0 hour incubation period (c), the treatment of isolate A13 at the 48-hour incubation period (d).

Application of nitrogen-fixing and IAA producing bacteria

Comparison of the main root length parameter indicated that the treatment of the culture isolate A13 (P4) affected the main root length compared to the negative control (P0) although when Duncan test was done there was not the differences (Table 1).

Table 1. Application isolate A13 in oil palm seedling for 90 days.

| Treatments        | Primary Roots Length (cm) | Root Diameter (cm) | Number of Lateral Roots | Stem Length (cm) | Stem Diameter (cm) | Plant Height (cm) | Number of Leaves | Wet Weight (g) |
|-------------------|---------------------------|--------------------|-------------------------|------------------|-------------------|-------------------|-----------------|---------------|
| P0 (Negative control) | 16.02<sup>a</sup> | 0.18<sup>a</sup> | 141.00<sup>b</sup> | 2.82<sup>a</sup> | 0.40<sup>b</sup> | 18.17<sup>ab</sup> | 3.25<sup>a</sup> | 4.20<sup>a</sup> |
| P1 (Positive control) | 16.50<sup>a</sup> | 0.19<sup>a</sup> | 150.75<sup>b</sup> | 2.77<sup>a</sup> | 0.47<sup>ab</sup> | 13.05<sup>b</sup> | 3.75<sup>a</sup> | 3.83<sup>a</sup> |
| P2 (NPK + A13) | 20.37<sup>a</sup> | 0.18<sup>a</sup> | 367.75<sup>a</sup> | 3.02<sup>a</sup> | 0.49<sup>ab</sup> | 15.75<sup>ab</sup> | 2.75<sup>a</sup> | 4.08<sup>a</sup> |
| P3 (½ NPK + A13) | 18.27<sup>a</sup> | 0.16<sup>a</sup> | 366.00<sup>a</sup> | 2.70<sup>a</sup> | 0.49<sup>ab</sup> | 18.10<sup>ab</sup> | 3.25<sup>a</sup> | 4.17<sup>a</sup> |
| P4 (A13) | 22.27<sup>a</sup> | 0.17<sup>a</sup> | 417.75<sup>a</sup> | 3.20<sup>a</sup> | 0.55<sup>a</sup> | 22.40<sup>a</sup> | 4.00<sup>a</sup> | 4.37<sup>a</sup> |

Note: Test using the Duncan test at 95% confidence level. Means that do not share a letter are significantly different.

Identification of nitrogen-fixing and IAA producing bacteria

NFB media did not contain nitrogen unsure in its composition. Isolate grown in the media was capable of fixing free nitrogen. Moreover, the selected isolate could form the white pellicle on the surface of the media showed successfully of the isolation. The media color change to bluish green (Figure 4). The selected isolate, namely isolate A13, had these following characteristics: rod shapes, the color of colony was red, and classified into Gram-negative bacteria according to Gram staining (Figure 5).

Figure 4. Color of Nitrogen Free Bromthymol Blue (NFB) semisolid media before the isolation (a) and color of NFB semisolid media after isolation on day 7, the white pellicle formed on the surface of the media (b).
Amplification of the 16S rRNA gene in isolate A13 generated 1300 bp amplicon size. Then amplicons were sequenced and the results of sequencing data analysis were performed using Blast-N program at NCBI. Results showed that isolates A13 had the closest genetic relationship with *Pseudochrobactrum assacharolyticum* species until 99%. Phylogenetic tree showed that isolate A13 similar with *Pseudochrobactrum assacharolyticum*, which amounted to 99% (Figure 6).

Isolate A13 aligned on ChromasPro software and the Phylogenetic tree was constructed along some sequence comparison using MEGA 6.0 software. In this Phylogenetic tree, *Beijerinckia fluminensis*, one of Nitrogen-Fixing and IAA producing bacteria, also used as one of the sequence comparison and *Bacillus thuringiensis* was used as an outgroup. The result is *Pseudochrobactrum assacharolyticum* was a nitrogen-fixing bacteria on isolate A13.

**Discussion**

The selected isolate could form the white pellicle on the surface of the media showed successfully of the isolation. It showed that the isolate had the ability to be able to fix free nitrogen. Bacteria fix free nitrogen and can change the pH to a more alkaline one (Kumar & Pannerselvam 2013).

Isolate A13 produced optimum exogenous IAA at 24 hours of incubation time. IAA production increased until the end of the log phase and had an optimum production when bacteria entered the stationary growth phase. According to Spaepen *et al.* (2007), IAA was produced and increased when the bacterial growth slowed down and entered the stationary phase.IAA concentration resulted using HPLC method was a bit different from colorimetric method used previously. This is due to the colorimetric method using supernatant from bacterial cultures while the HPLC analysis uses a crude extract sample from the supernatant. Thus analysis used HPLC had a higher accuracy (Presits&Molndr-Per 2003).
Hypersensitivity test results showed that isolate A13 did not cause hypersensitivity symptoms on leaves tobacco showed that the isolate was not pathogenic on plants (Vanneste et al. 1990). Isolate A13 could be applied in Oil Palm (Elaeisguineensis Jacq.) seedling.

Isolate A13 produced high amounts of IAA, which was about 60-90 ppm (derived from the results of quantitative analysis IAA using colorimetric method and HPLC) or approximately 3.42 x 5.13 x 10^{-5} M to 10^{-5} M. The results obtained were consistent with the statement Patten & Glick (2002) which found a high concentration of IAA could initiate the growth of lateral roots. While low concentrations of IAA, which is about 10^{-9} M to 10^{-12} M only trigger the main root elongation. Observations of stem diameter parameter indicated that most large diameter was owned by P4 (A13 bacterial culture), while P0 had the smallest diameter, which amounted to 0.40 cm. It showed that variation result of plant height also gave varying results. P4 treatment (A13 bacterial culture) had an average plant height greater than 20 cm. Similar results were obtained from the parameters of the number of leaves, P4 had an average number of leaves highest if it was compared to other treatments. Results of calculation parameters indicated that P4 (A13 bacterial culture) wet weight had the greatest weight compared to four other treatments.

Amplification of the 16S rRNA gene in isolate A13 generated 1300 bp amplicon size. Amplicons were sequenced and the results of sequencing data analysis were performed using Blast-N program at NCBI. Results showed that isolates A13 had the closest genetic relationship with Pseudochrobactrum assacharolyticum species until 99%. Phylogenetic tree showed that isolate A13 similar with Pseudochrobactrum assacharolyticum, which amounted to 99%.

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Conflict of interest

The authors state no conflict of interest in this manuscript.

Acknowledgement

This research was funded by The Directorate General of Higher Education awarded to NRM in 2017.

Author contributions

All authors have reviewed the final version of the manuscript and approved it for publication. NRM collected samples. II performed research and collected the data. II, NRM, AT wrote and reviewed the paper. II is the main contributor of this manuscript.

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40