Superior Nitrogen Fixing Bacteria Screening from Various Rhizobiome in Palm Oil Plantation, North Sangatta, East Kalimantan

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Abstract. Nitrogen fixing bacteria (NFB) plays an important role in increasing N availability for plants. Research to examine the ability of nitrogen fixing bacteria isolates to produce nitrogenase, phytohormone and the ability of nitrogen fixing bacteria isolates in the biological test process using the corn plant indicator as an indicator has been carried out from September 2018 to February 2019 in laboratories and greenhouses. The ability of nitrogen fixing bacteria was tested by the ARA method, while the phytohormone testing of nitrogen fixing bacteria was tested using the HPLC method. Bioassays using Murphy media and corn plants as indicators were performed using a randomized block design consisting of six treatments (one control and five selected NFB isolates from the selection results) and given five replications. Measurement of root length, plant height, and dry weight of plants were carried out every 2 days for 14 days. The results showed that the nitrogen fixing bacteria isolates used from North Sangatta rhizobiome, East Kalimantan had different nitrogenase and different phytohormone test results, and obtained five selected isolates based on the selection results. The results of the bioassay did not show any significant differences based on the Duncan test at the level of 5%. However, it can be seen visually the significant difference in which plants in the biological test using nitrogen fixing bacterial isolates have relatively higher plant growth and dry weight of plants than plants that are not given treatment or control

Key words: Nitrogen fixing bacteria, superior isolates, bioassay

1. Introduction

Oil palm (\textit{Elaeis guineensis} Jacq.) is a plantation crop that derivate many products such as cooking oil, cosmetic products, soaps, processed foods, and fuel. Oil palm plantations area in Indonesia has increased from 300 thousand hectares in 1980 to 11.6 million ha in 2016. Crude palm oil has increased too from 700 thousand tons in 1980 to 33.5 million tons in 2016 [1]. The significant increase in plantation area and production has mostly affected the soil health level to become worse due to the dependence of the high degree of synthetic fertilizer. For example, if the nitrogen requirement per plant can be supplied with 1 kg of urea, it will make microbial activity on the oil palm rhizosphere could be disrupted accumulatively. It is evidenced by the opinion of Rakhmawati et al. [2] which states that the continuous administration of inorganic fertilizers can cause a decrease in soil fertility due to the accumulation of soil residue causing soil to become saturated. In addition to the
consequences of residue, years of intensive land use for cultivation of plants can decrease chemical fertility and soil biology [3].

In the context of soil microbiology ecosystem, forests are the benchmark in measuring of the biological level of soil health through abundance and microbial activity of the rhizosphere versus oil palm plantation. Primary forest is a healthy and independent categorized ecosystem because it has a sustainable nutrient cycle. Forests can achieve ecosystem health and nutrient cycle sustainability because all components in the ecosystem are primarily the rhizomicrobiome or community of microbes found in the rhizosphere [4][5] performs its function in the recycling of nutrients and has functions as an external digestive system in both the forest and oil palm planting land [6]. However, due to continuous use of synthetic fertilizer, it can lead to degradation in oil palm planting land.

Efforts to overcome the negative impact of the use of inorganic fertilizer can be performed by utilizing the environmentally friendly fertilizer, namely biological fertilizer. Biological fertilizer is a material containing living microorganisms that are given to the soil as an inoculant to help providing certain nutrients for plants [7]. Biofertilizer is an active biological product consisting of microbes that can improve the efficiency of fertilization, fertility, and Soil Health [8]. Biological fertilizer aims to increase the number of microorganisms and accelerate the microbiological process to increase nutrient availability, so it can be utilized by plants [9].

Nitrogen fixing bacteria (NFB) is an important component in the soil against the availability of nitrogen for plants because the bacteria have the ability to convert free nitrogen in the atmosphere into ammonia needed by plants and have the ability to increase the efficiency of the use of N available in soil. NFB use free nitrogen to synthesize protein cells where the proteins will undergo a process of mineralization in the soil.

Research result of Gharib et al. [10] that inoculation with nitrogen-blocking bacteria can increase the population of N2 fixing rhizobacteria which is a sensitive indicator of soil health, able to decrease the use of urea, improve soil health and improve cultivation yield. According to Dixon and Kahn [11], NFB occupies an indispensable ecological niche because it acts as a supplier of nitrogen and transforms it in a form that can be absorbed by plants.

According to Zuberer [12], the conversion of N2 from the air becomes ammonia assisted by the enzyme nitrogenase. The number of N2 converted to ammonia depends heavily on condition, physical, chemical, and biological soil. The ARA method was chosen to measure the nitrogenase of bacteria because it has been widely used in previous studies and is a relatively easier and cheaper testing procedure than the analysis with spectrophotometry. The research results of Sukmadi [13] about indicating that from 34 isolates of bacteria tested, 20 isolates of bacteria can produce an IAA type of auxin hormone with vary concentrations. Phytohormones are essential to help accelerate growth and development of crops.

Based on the explanation above, it is necessary to improve the quality of biological fertilizer by conducting a superior isolate of NFB that can produce nitrogenase and phytohormones, as well as able to improve plant growth through biological test (bioassay).

2. Material and Method

- Biological material

Eight isolates of nitrogen fixing bacteria (NFB) are isolated from eight different soil samples from the upper ground surface (0-10 cm) with a weight of ± 20 g respectively: (1) primary forest, (2) secondary forest, (3) fallow land, (4) palm oil plantation which has not produced yet, (5) planted palm oil plantation in 2008, (6) planted palm oil plantation in 2009, (7) planted palm oil plantation in 2010, and (8) planted palm oil plantation in 2011 in the PT. Kalimantan Agro Nusantara, the Thomas Square C-5 complex, North Sangatta Sub-district, East Kutai District, East Kalimantan Province in August 2018. The isolation of NFB was started by diluting pour plate method on dilution factor $10^3$ in
Jensen’s media and incubated for ± 9 days. The pure culture was kept in Jensen's media using a streak plate method.

- Acetylene reduction assay (ARA) test
  Gas chromatograph was conditioned earlier for the hours before injecting the sample in it. The chromatograph, injector, and detector initial temperatures were set to 100 °C, 150 °C, and 200 °C, respectively. Gas carrier used were N₂ (40 psi), H₂ (1.5 kgf/cm²), and O₂ (0.5 kgf/cm²). Ethylene standard curve was created into 0 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL, 175 µg/mL, 200 µg/mL, and 225 µg/mL. Then, the chromatogram result was plotted into ethylene standard curve that is a relation between nitrogen concentration from tested samples and standard area extent.

- Phytohormone test
  Tissue samples which have been dried-freeze (1g) and enveloped with the flour was inserted into liquid and homogeneous nitrogen and from 100% methanol. Samples were stirred in methanol 80% at 4 °C for one night, then filtered. The remaining results were extracted back with methanol 80% for 4 hours, filtered, and mixed with supernatant. Methanol was separated from the filtrate mixture by reducing it at 35 °C pressure and residual water was added until pH reached 2.5 (2 M HCl). This solution has been partitioned 3 times with the same volume with ethyl acetate and an organic combination phase that has been partitioned with 5% (M/V) sodium bicarbonate (3x1/5 volumes) and separated gibberellic acid and injected into the HPLC. For HPLC analysis Using the isocratic system. The extracts in the vial were injected into the HPLC system with the Hichrom Waters 6000 A pumps, ultraviolet detectors (Unicam Analytical Systems, Cambridge, UK) and Bondapak C18 column (Waters Hichrom) using acetonitrile (12.00%; pH 4.98) such as the automobile phase. Flow rate, pressure, and wavelengths were 2 cm³ min⁻¹, 13.8 MPa, and 265 nm, respectively.

- Bioassay test on maize plant
  This test was arranged by randomized complete block design (RCBD) with five replications. The treatments were used consisted of no NFB application (a₀), primary forest NFB (a₁), fallow land NFB (a₂), non-producing palm plantation NFB (a₃), 2010 planted palm oil plantation NFB (a₄), and 2009 planted palm oil plantation NFB (a₅).
  This test utilised Murphy selective medium. The sterilised maize seed has been germinated at petri dish which was covered by sterilised merang paper for about 5 days at 30 °C. Then, the maize sprout was grown on Murphy medium in test tube.
  Data were documented such as plant height, root length, dry root mass, and dry shoot mass after being incubated for 14 days in glass house. Those data were analysed by F test and it was continued by Duncan’s multiple range test.

- Treatments ranking
  The treatments were ranked based on the accumulation of the score from ARA, phytohormone, and bioassay test. The lesser the rank shows the better treatment.

3. Results

3.1. ARA Test

ARA analysis (Acetylene Reduction Assay) is an easy and sensitive test to determine whether the activity of the enzyme nitrogenase in a type of bacteria. The results of these tests are presented in Table 1.

Data in Table 1 shows that the eight isolates used in this study had nitrogenase activity, which is demonstrated by the ethylene (area) content that is readable by gas chromatography. The injected ethylene Gas in the tubes is converted into acetylene by the component nitrogenase bacteria [14]. Consequently, the eight isolates are capable of fixation of nitrogen from the atmosphere and transferring it to plants for use in metabolic processes.
Table 1. Isolate ability to produce nitrogenase

| Source of rhizosphere isolates | Result (mg/kg) |
|-------------------------------|---------------|
| Not producing plant (NPP)     | 1.654         |
| Producing plant (PP) 2009     | 1.280         |
| Fallow land                   | 1.143         |
| Primary Forest                | 1.083         |
| Secondary forest              | 0.961         |
| PP 2008                       | 0.793         |
| PP 2010                       | 1.001         |
| PP 2011                       | 0.825         |

3.2. Phytohormone test

Bacterial isolates have the ability to produce hormones growing IAA, zeatin, kinetin and gibberellin. Phytohormones (hormone growing plants) are essential to help accelerate the growth and development of plants, as presented in Table 2.

Table 2. Isolate ability to produce phytohormone

| Sources of rhizosphere isolates | Result (mg/kg) |
|---------------------------------|---------------|
|                                 | IAA | Zeatin | Kinetin | Gibberellin |
| NPP                             | 3.779 | 2.518 | 0.853 | 8.74 |
| PP 2009                         | 4.279 | 2.527 | 0.545 | 6.5 |
| Fallow land                     | 4.234 | 2.134 | 1.03  | 6.109 |
| Primary Forest                  | 3.733 | 2.115 | 0.829 | 6.681 |
| Secondary forest                | 3.744 | 2.061 | 0.76  | 8.125 |
| PP 2008                         | 3.85  | 2.446 | 0.85  | 8.033 |
| PP 2010                         | 4.189 | 3.072 | 0.595 | 7.441 |
| PP 2011                         | 4.371 | 3.388 | 0.81  | 8.428 |

From Table 2, it can be known content of phytohormones IAA, zeatin, kinetin and Gibberellin from the eighth isolate of nitrogen-blocking bacteria tested using the HPLC (High Performance Liquid chromatography) method. The eight samples showed that there were phytohormonal activity of both IAA, zeatin, kinetin and Gibberellin in the eight isolates tested but, having different content. Indole-3-acetic acid (IAA) is a natural auxin that farmers need to increase agricultural productivity. The use of natural auxin is expected to be increased to replace the use of a synthetic auxiliary phytohormone that is less efficient because it will not blend with the plant and will be washed away with water currents if the rainy season arrives. The synthetic auxin is also less effective in stimulating the roots compared to natural auxin.

3.3. Bioassay test

The results of the biological test of a variety of NFB isolates to the height of the plant, the length of the root, the dry root and the dry weight of the maize plant heading for 14 days are shown in Table 3.

Table 3. Plant height and root length data

| Treatment          | Plant height (cm) | Root Length (cm) | Shoot length Increase (%) |
|--------------------|-------------------|------------------|--------------------------|
| No NFB (a₀)        | 20.2              | 11               | -                        |
High growth of plants and the length of the roots are influenced by the availability of nutrients in planting media. The presence of elevated crop height and the length of the roots is an influence of bacteria that can alter the unavailable elements to be available to plants. It can be seen in Table 3, maize plant that is in the biological test (control) or without the use of nitrogen-blocking bacteria tends to have a lower plant height than the five maize plants in the biological test using nitrogen-blocking bacteria. When sorted the high yield of the highest crop is the first 5 treatment derived from rhizosphere 2009 palm oil plant has a high average value of plant 24.02 cm, then 1 treatment derived from the primary forest rhizosphere of 23.76 cm, treatment 3 derived from the rhizosphere palm crop has not produced at 23.68 cm, treatment 2 derived from the rhizosphere of a fallow land of 23.66 cm, treatment 4 derived from the oil palm plant 2010 of 22.32 cm and which has the lowest plant height is control or not given a treatment of 20.2 cm.

The result of the highest root length is the first 3 treatment derived from rhizosphere palm oil has not produced the average value of the root length of 14.08 cm, then the treatment 5 derived from rhizosphere of palm oil plant 2009 of 13.12 cm, 2 treatment derived from the rhizosphere of a fallow land of 12.54, treatment 4 derived from rhizosphere of palm oil plant 2010 of 11.62 cm, treatment 1 derived from the primary forest of 11.8 cm and which has the lowest root length of the control treatment of 11 cm. Test results using a follow-up of Duncan showed that there was no noticeable difference in the test. Suspected because, there is not a difference in the concentration of administration of nitrogen-blocking bacteria in the biological test.

It can be seen in Table 4, when sorted by the results of the dry roots of a noticeable root that is the first 2 isolates derived from the rhizosphere of a fallow land of 142 mg, then the isolates 3 derived from the rhizosphere palm oil plants have not produced an average value of 138 mg, the 5 isolates derived from the rhizosphere of the 2009 palm oil plant amounted to 128 mg, isolates 1 derived from the primary forest of 120 mg while the one that is no different from the real one of the 4 is derived from the rhizosphere of palm oil plant 2010 of 78 mg and the control of 72 mg.

Table 4. Shoot and root dry weight data

| Treatment                           | Shoot dry weight (mg) | Root dry weight (mg) |
|-------------------------------------|-----------------------|----------------------|
| No NFB (a₀)                         | 40 a                  | 72 ab                |
| Primary Forest NFB (a₁)             | 80 ab                 | 120 abc              |
| Fallow land NFB (a₂)                | 74 ab                 | 142 c                |
| Non-producing NFB (a₃)              | 70 ab                 | 138 bc               |
| 2010 planted NFB (a₄)               | 74 ab                 | 78 a                 |
| 2009 planted NFB (a₅)               | 88 b                  | 128 abc              |

Note: The numbers followed by the same letter are not significantly different according to Duncan's Multiple Range Test at 5% significance.
3.4 Ranking

The best isolate determination is determined based on the test results of nitrogenase ability, phytohormone and biological test results (bioassay) for 14 days which include the dry root weight, the heading dry weight, the length of the plant and the length of the roots. Isolates that have the smallest score value are the best isolates.

Table 5. Isolate ranking

| Parameters          | Nitrogenase | IA A | Plant height | Root length | Dry Shoot Mass | Dry Root Mass | Rank sum | Rank |
|---------------------|-------------|------|--------------|-------------|----------------|---------------|----------|------|
| Isolate             |             |      |              |             |                |               |          |      |
| A₁                  | 4           | 5    | 2            | 4           | 2              | 4             | 21       | 4    |
| A₂                  | 3           | 2    | 4            | 3           | 3              | 3             | 18       | 3    |
| A₃                  | 1           | 4    | 3            | 1           | 4              | 1             | 14       | 2    |
| A₄                  | 5           | 3    | 5            | 5           | 3              | 5             | 26       | 5    |
| A₅                  | 2           | 1    | 1            | 2           | 1              | 2             | 9        | 1    |

Isolates which have a characteristic value of the smallest nitrogenase is the 3 isolates derived from the oil palm plants have not produced, while the isolates that have the highest value of nitrogenase characteristics are 4 isolates derived from the rhizobiome of the 2010 palm oil plant. The smallest value of phytohormones (IAA) is 5 isolates derived from the rhizobiome of the 2009 palm oil plant, whereas isolates that have the highest characteristic phytohormone (IAA) value are isolates derived from the primary forest.

The high characteristic value of the smallest plant is the 5 isolates derived from the rhizobiome of the 2009 oil palm plant, while the highest characteristic value of the plant is 4 isolates derived from the rhizobiome of the palm oil Plant 2010. Isolates that have the smallest characterization value of the root of 3 isolates derived from the oil palm plants have not produced, whereas the highest is 4 isolates derived from rhizobiome 2010 palm oil crop.

Isolates that have a characteristic value of dry weight of the smallest header is the 5 isolates derived from rhizobiome 2009 oil palm plant, while isolates that have characteristic value of dry weight of the highest heading are 3 isolates. Characteristic value of dry weight of the smallest root is 3 isolates derived from palm oil plants have not produced, while the isolates that have a characteristic value of dry roots of the highest is 4 isolates derived rhizobiome palm oil plant 2010.

The inoculation of the NFB isolates provides different influences on the header, root and total dry weights. Overall the isolates of NFB 2 and 5 have a noticeable effect on the dry weight of the header, roots and dry weight of plants. The dry weight of the plant at NFB isolates 2 and 5 is higher at about 92.8% compared to the control.

4. Discussion

In general, the fixation capability of bacterial nitrogen can be measured among them with the measurement of ammonium which accumulates in its growth medium or by measuring acetylene reduction activity (FIG). The FIG value shows the activity of the nitrogenase enzyme in catalyses the reaction of double bond changes on an analogy substrate (acetylene) into a single bond (in ethylene). Accuracy of acetylene reduction Analysis to measure the ability of nitrogen fixation is higher compared to the accumulated ammonium. This is because ammonium in the medium or culture can be used as a source of nitrogen for bacterial growth.
Although, the analysis of the effects of NFB isolation did not occur a noticeable difference between the fifth treatment of NFB isolates on the height of the plant and the length of the roots but, NFB 3 tends to have a high increase of the root length of 28% and NFB by 19.3%. The NFB isolate used is able to produce a growing hormone that is a functioning IAA in the root extension. Although there is no noticeable difference between treatment, visually the root of the plant given isolates is longer than the control shown visually in Figure 1.

![Figure 1. Root length at 14th days after planting](image)

Figure 1. Root length at 14th days after planting

Figure 1 shows the activity of nitrogen-blocking bacteria against the root length visually. Noticeable difference between the control and the plant is given the isolates of nitrogen-blocking bacteria. It is suspected that the activity of nitrogen-blocking bacteria is more likely to work in removing the growing hormone i.e. IAA that functions in the extension of the roots, in addition to the alleged presence of other growth hormone activity such as gibberellin so that isolates applications are more inclined at the roots. The root length is more decisive than the root weight in absorbing nutrients, because the long roots will easily absorb nutrients in the soil with a wide range.

5. Conclusions

The best nitrogen fixing bacteria isolates from the based selection process ability to test nitrogenase, phytohormones, growth results plants and the yield of plant dry weight were each isolate from primary forest, fallow land, non-producing plantation, 2010 planted, and 2009 planted rhizobiome.

Bioassay test with maize plant result using isolates of nitrogen fixing bacteria has a relatively higher capability compared to the control. Thus, the use of isolates of nitrogen-blocking bacteria has good potential to be developed into the active ingredient of biological fertilizers.

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