Isolation and characterization of indole-3-acetic acid producing bacteria from red onion rhizosphere

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Abstract. There are many bacteria classified as growth enhancer in plants which known as Plant Growth Promoting Rhizobacteria (PGPR). PGPR can directly induce plant growth by producing of phytohormone Indole-3-Acetic Acid (IAA). The objective of this study was to obtain bacterial isolates of IAA producing rhizobacteria from red onion rhizosphere and to characterize the potential isolates. The methods used in the study were a sampling of soil, isolation of IAA producing rhizobacteria, measurement of IAA by using colorimetric assay, morphological identification of bacterial isolates, measurement of bacterial growth and IAA production, and test of hypersensitivity on tobacco leaves. There were fourteen IAA producing bacterial from red onion rhizosphere. The isolates could produce IAA by colorimetric assay detection. Three isolates produced IAA was higher than other isolates i.e. BIT 2,4 (61.72 ppm), BIS 3,4 (60.92 ppm) and BIT 2,1 (49.3 ppm). Meanwhile, isolate BIT 4,1 produced the lowest IAA as much as 3 ppm. Four isolates were gram-positive bacteria. Isolates BIS 3,4 and BIT 2,1 produced exogenous (extracellular) IAA on stationary phase. Two isolates potential (BIS 3,4 and BIT 2,1) did not cause necrotic symptoms or negative results for the hypersensitivity test on tobacco leaves.

Keywords: Allium cepa, hypersensitivity, rhizosphere, tobacco

1. Introduction

Indonesia has good potential in producing red onion, which is indicated by the enhancement of domestic red onion productivity in 2017 in the amount of 2.40% [1]. Red onion farming is still dependent on the use of chemicals as inorganic fertilizers. However, excessive use of inorganic fertilizers on agriculture results in a decrease of land productivity and serious impact on the soil such as hardening the soil and reducing the soil aggregate stability [2]. One alternate substitute for inorganic fertilizer is by using soil microorganism inoculants. Many bacteria classified as plant growth promoting rhizobacteria (PGPR) have an important role in soil fertility and plant health. PGPR can affect plant growth through different mechanism both directly and indirectly. One of the mechanisms of PGPR is producing phytohormones such as Indole-3-Acetic Acid (IAA) [3].

IAA is a major part of the auxin group that controls many physiological processes in plants [4]. The important role of IAA producing rhizobacteria includes increasing root growth and the number of lateral roots and elongation of roots to increase the absorption of water and nutrients in the soil [5]. However, high IAA concentrations could inhibit the growth of primary roots [6]. Rhizobacteria could synthesize IAA because the supply of substrate from root exudates is more than non-rhizosphere [7].
Some genera of bacteria that able to produce IAA are *Azospirillum*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Pantoea*, *Streptomyces*, and *Xanthomonas* [8]. Therefore, this study aimed to isolate and characterize of IAA producing bacteria from red onion rhizosphere.

2. Materials and methods

2.1. Material

Soil samples are collected from 2 locations i.e. soil from healthy red onion plants (A) and diseased red onion plants (B) in Brebes agricultural land, Central Java, Indonesia.

2.2. Soil sampling

Soil samples were taken from 5 points sampling around the red onion plants rhizosphere at a depth of ± 5-10 cm from soil surface. The method of sampling was used simple random sampling [9].

2.3. Isolation of IAA producing bacteria

Each sample was taken in the amount of 1 gram and diluted from $10^{-1}$ to $10^{-4}$ by using physiological saline (NaCl 0.85%). A total of 0.1 mL of soil suspension was spread using the Total Plate Count (TPC) on Nutrient Agar (NA) media and incubated for 24 hours at room temperature (± 27°C). The bacterial colonies with different morphologies have been purified using the quadrant method in NA media with the addition of 1 mM L-tryptophan [10].

2.4. Potential isolate selection

Selection of potential isolates was carried out by qualitative and quantitative methods (colorimetric method) [11]. Potential bacterial isolates were measured by IAA production. One loop of each isolate was inoculated into 25 mL Nutrient Broth (NB) media with the addition of 1 mM L-tryptophan then incubated at room temperature (±27°C) and shaken at 120 rpm. The content of IAA in culture media was tested by taking 1.5 mL of culture and put in the microtube, then centrifuged at 10,000 rpm for 10 minutes. A total of 1 mL of supernatants was reacted with 4 mL of Salkowski reagent [12].

2.5. Gram staining and characterization

Characterization of bacteria was carried out based on the morphological properties of bacterial colonies and the shape of a cell. Gram staining was conducted to identify the type and shape of bacterial cell form selected isolates [13].

2.6. Bacterial growth curve and synthesis IAA

Two selected isolates were used as models to determine the IAA production. One 24-hours-old bacterial loop was inoculated into 25 mL of Nutrient Broth (NB) then shaken at 120 rpm to reach $\pm 10^6$ cells/mL. One mL of culture was taken and put into 200 mL of Nutrient Broth (NB) media with the addition of 1 mM L-tryptophan. Bacterial density was measured by using a spectrophotometer with a wavelength of 620 nm. The cell number of bacterial culture and the IAA production is calculated every 4 hours from a total of 48 hours.

2.7. Hipersensitivity test on tobacco leaves

One loop of bacterial isolate inoculated into 25 mL of Nutrient Broth (NB) media supplemented with 1 mM L-tryptophan and shaken at 120 rpm to reach $\pm 10^6$ cells/mL. *Xanthomonas oryzae* isolate used as a positive control. Aquades and Nutrient Broth (NB) media used as a negative control. A total of 0.1 mL each culture was injected into the abaxial portion of tobacco leaves using a 1 mL syringe (without needle), then observation was carried out up to 48 hours after the injection [14].
3. Results
Isolation of IAA producing bacteria from red onion rhizosphere was conducted using Nutrient Agar (NA) media with the addition of 1 mM L-tryptophan. A total of 14 isolates were successfully obtained from the two soil samples, consisting of 5 isolates which obtained from location A (soil from healthy red onion plants) and 9 isolates from location B (soil from red onion plants has a disease). The results of the density of bacterial colonies using the Total Plate Count (TPC) method showed a cell count of more than $10^6$ CFU/mL (table 1).

Table 1. The density of bacterial colonies in each soil samples have been taken

| No | Soil samples                     | Average bacterial colony density (CFU/mL) |
|----|---------------------------------|------------------------------------------|
| 1  | Healthy red onion              | $1.5 \times 10^6$                        |
| 2  | Red onion has a disease        | $6.0 \times 10^6$                        |

All isolates obtained were tested for their ability to produce IAA using qualitative and quantitative methods. A total of 14 isolates produced IAA qualitatively which identified as pink colonies when tested by Salkowski reagent on Nutrient Agar (NA) media containing 1 mM L-tryptophan (figure 1). All of 14 isolates were tested for their ability to produce IAA quantitatively by colorimetric method. The supernatant of culture reacted with Salkowski reagent formed a mixture of red to purple solution which indicated of indole production at a wavelength of 520 nm spectrophotometry. Isolates of BIS 3.4, BIT 2.4, and BIT 2.1 were able to produce the highest concentration of IAA in Nutrient Broth (NB) media with the addition of 1 mM L-tryptophan while the lowest concentration was produced by BIT 4.1 isolate (table 2).

Figure 1. Appearance of bacterial colonies before reaction with Salkowski reagent (a) and after dropped with Salkowski reagent (b).

The appearance of isolates colonies is mostly yellow and the other part is white, the form of the colonies are varied, the edges are slippery, and the elevations vary. Gram staining results showed 10 isolates were Gram positive and 4 other isolates were Gram negative. Two selected isolates (BIT 2.1 and BIS 3.4) are Gram positive which indicated by the colors of blue or purplish-blue cells with the rod shape and single arrangement and clustering (figure 2).
Figure 2. Gram staining of isolates. (a) BIT 2.1 and (b) BIS 3.4.

Two selected isolates (BIS 3.4 and BIT 2.1) produced IAA during the incubation period of 48 hours of growth. The concentration of L-tryptophan added to Nutrient Broth (NB) media is 1 mM. BIS 3.4 isolates showed the end of the log phase at the 12th hour. BIT 2.1 isolates had the end of the log phase at the 16th hour. BIS 3.4 isolate produced the highest IAA at the 36th hour with a concentration of 59.84 ppm. BIT 2.1 isolate produced IAA at 16th hour with a concentration of 8.81 ppm. BIS 3.4 isolate produced the highest IAA in the stationary phase of bacterial growth. The highest IAA production isolates BIT 2.1 at 12th and 16th hour when entered the end of the log phase (figure 3a and 3b).

Table 2. IAA concentration was produced by bacteria from red onion rhizosphere.

| Isolates | Production of IAA (ppm) | NB | NB + 1 mM L-tryptophan |
|----------|-------------------------|----|------------------------|
| BIS 3.4  | 10.32                   | 60.92 |
| BIS 3.5  | 4.76                    | 9.89  |
| BIS 4.2  | 8.44                    | 8.88  |
| BIS 4.3  | 5.62                    | 6.39  |
| BIS 4.4  | 3.16                    | 6.16  |
| BIT 1.4  | 4.63                    | 15.36 |
| BIT 2.1  | 3.54                    | 49.30 |
| BIT 2.2  | 6.27                    | 16.36 |
| BIT 2.3  | 4.45                    | 5.45  |
| BIT 2.4  | 22.09                   | 61.72 |
| BIT 2.6  | 7.18                    | 33.18 |
| BIT 3.5  | 3.09                    | 8.63  |
| BIT 4.1  | 2.81                    | 4.27  |
| BIT 4.5  | 9.81                    | 3.72  |

Note: BIS (soil from healthy red onion) and BIT (soil from red onion plants has a disease). Measurement of IAA was carried out on bacterial suspensions of 24 hour isolates.
phase, the nutrient content available in the media begins to decrease that the secondary metabolites are assumed that the IAA synth end of the log phase compared to BIS 3.4 isolates in the stationary phase. Based on these results, it can be seen that IAA began to b

tryptophan, tryptamine, indole pyruvate acid, and indole acetamide (IAM) [20]. The pattern of IAA synthesis curve is in line with bacterial cell growth. Based on the growth curve results are different from tobacco leaves injected with Xanthomonas oryzae inoculum as a positive control that shows symptoms of necrosis, which produced yellowish to brownish spots on tobacco leaves. Tobacco leaves injected with aquades and NB media as a negative controls showed no symptoms of necrosis (figure 4).

4. Discussion
In our study, soil samples from location B (diseased plant) had bacterial colony densities were higher than those from location A (healthy plant). The density of bacterial population can be influenced by soil properties, such as soil pH and nutrient contents could affect the microbial population in the soil. The soil pH of location A was 6.92 while the soil pH of location B was 5.33. This is not in accordance with the statement that fungi are predominant in acidic soil while bacteria are abundant in neutral conditions [15]. In addition, microbial populations are also affected by root exudates from plants [16]. It was possible that bacterial community came from the specific composition of rhizodeposits, dying-off of roots, and other plants residues, all then serving as source of carbon, nitrogen, and energy. Plants have sensitivity in optimizing their growth to overcome various problems regarding soil fertility. When plants suffer from diseases, plants will try to interact with soil microbes to make soil microbes produce the anti-pathogenic compounds and growth-promoting substances. For this purpose, the plants emit as much root exudate as possible to invite the desired microbes to increase the diversity of microbes in the rhizosphere of the red onion. Root exudates affect the formation of microbial populations in the rhizosphere because they contain source of carbon and organic compounds as energy source for soil microbes.

The measurement of IAA concentration using the colorimetric method on the supernatant from isolate culture as used to detect indole compounds with the technique of reacting Salkowski reagent [17]. The indole ring is formed after the isolate supernatant is reacted with the Salkowski reagent. The Reddish color change reaction of the isolates that have been dripped with the Salkowski reagent indicated that the isolates were able to metabolize L-tryptophan into IAA [18]. L-tryptophan is an auxin precursor [19]. Salkowski can detect intermediate compounds in the synthesis of IAA such as tryptophan, tryptamine, indole pyruvate acid, and indole acetamide (IAM) [20]. The pattern of IAA synthesis curve is in line with bacterial cell growth. Based on the growth curve and synthesis of IAA from the two isolates, it can be seen that IAA began to be synthesized at the beginning of the log phase even in a small amount. BIT 2.1 isolates produced the highest IAA at the end of the log phase compared to BIS 3.4 isolates in the stationary phase. Based on these results, it can be assumed that the IAA synthesized by the bacteria is a secondary metabolite. At the end of the log phase, the nutrient content available in the media begins to decrease that the secondary metabolites are

![Figure 3](image-url) Bacterial growth curve and IAA synthesis of isolate(a) BIS 3.4 and (b) BIT 2.1.

Both isolates, BIS 3.4 and BIT 2.1 showed negative hypersensitivity reactions indicated by no symptoms of necrosis during the 48-hour test. These results are different from tobacco leaves injected with Xanthomonas oryzae inoculum as a positive control that shows symptoms of necrosis, which produced yellowish to brownish spots on tobacco leaves. Tobacco leaves injected with aquades and NB media as a negative controls showed no symptoms of necrosis (figure 4).
produced in response to the nutrient restrictions and are produced at the end of the log phase and during the stationary phase [21].

![Image](image.jpg)

**Figure 4.** Reaction of hypersensitivity test on tobacco leaves injected with bacterial culture (a) BIS3.4, (b) BIT 2.1. (c) *Xanthomonas oryzae* as a positive control, (d) aquades and (e) *Nutrient Broth* (NB) media as s negative control.

IAA concentration produced by BIT 2.1 isolates is not as high as BIS 3.4 isolates because it depends on the number and activity of cells and the availability of nutrients. In addition, the biochemical and genetic characters of each isolate and the ability of bacteria to convert L-tryptophan to IAA [22]. The IAA concentration produced by the isolates of BIT 2.1 at the beginning is different from the final results of the IAA synthesis curve. This is not reasonable because the result of the concentration is very low and decreased during the stationary phase. It is different from BIS 3.4 that able to produce stable IAA. The same matter with the result of this study had also been reported in the previous study. Widayanti [23] reported that rhizobacterial isolates as a result of isolation from the rice rhizosphere were known to be able to produce IAA with an initial concentration of 67.2 ppm after induction with 0.5 mM L-tryptophan, while IAA concentration in IAA production curve of the isolate was the amount of 53.9 ppm.

Biofertilizer candidates are required not pathogenic especially for plants. Hypersensitivity test in tobacco plants is an commonly initial stage used to determine the potential pathogenicity of a microbe interacted with plants. Hypersensitivity reactions are fast test and localized cell death programs. This reaction arises in infected plants when the introduction of pathogens and includes the efforts of plants to inhibit pathogens [24]. In accordance with the results of the hypersensitivity test, the two BIS 3.4 and BIT 2.1 isolates from red onion rhizosphere were not included in the pathogenic bacteria in the plant that they could be applied as biological fertilizer inoculums.

5. **Conclusion**

A total of 14 isolates Indole-3-Acetic Acid (IAA) producing bacteria were successfully isolated from rhizosphere of red onion of agricultural land in Brebes. The two highest of IAA-producing isolates were BIS 3.4 and BIT 2.1. BIS 3.4 isolate produced the highest IAA as much as 59.84 ppm in the stationary phase.
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