Evaluation of Endophytic Bacteria Isolated from Citrus Plant on Phytohormone Production

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Abstract. The strategy for improving the productivity of fruit and vegetables in Indonesia can be done through modification of the existing cultivation system by adding certain component of healthy practice farming. One of the approaches for achieving that aim is through the usage of endophytes in cultivation system. The identified mechanisms of endophytic microbes are that the stimulant produced by them is able to suppress diseases and pests that pose a significant threat for plants. Moreover, endophytic microbes are also capable of producing antimicrobial compounds and bio stimulating compounds such as phytohormones and proteins. The purpose of this study was to determine the ability of endophytic bacteria isolates that were taken from collection of Indonesian Citrus and Subtropical Fruits Research Institute in producing IAA hormone, phosphate solvent and nitrogen fixing. The research consisted of several stages; testing for Free Nitrogen Binding Bacteria; Phosphate Solubilizing Bacteria and in Producing IAA. The results showed the three endophytic bacterial isolates isolated from different citrus varieties had the ability to fix free nitrogen. G.28-isolates and G.35_isolates had identical ability to dissolve phosphate, while G.31-isolates could not dissolve phosphate. The most effective time to produce IAA is the fourth day of incubation. G.35-isolates was the bacterial isolate that had the highest IAA production.

1. Introduction
The agricultural sector in Indonesia play a major role in supporting economic growth, such as providing jobs, providing raw materials for industry and increasing foreign exchange through exports [1]. Based on the research data published by Ariningsih [2], Indonesia has increased the volume of imported fruits by 785.68 thousand tons during 2008-2012. This data indicates that the high import values still poses a real threat to the sustainability of the agricultural sector in Indonesia, which might be due to the fact that most of the products qualities are not met with global standard. This makes it difficult to compete with the product from global markets and low productivity. In the case of low productivity, there are various factors that can cause low fruit and vegetable productivity in Indonesia such as pests or diseases, the vigor of plant, the long-time of harvesting, lack resistance to environmental stress, and water availability [3].

Increasing the production of fruits and vegetables must be followed by improvements to a more effective cultivation system. One of the approaches that could be employed is by using endophytes. Endophytes are microorganisms that naturally live inside plant tissues beneficiantly supporting the growth of a given plant [4]. Endophytic microbes were known to influence the growth and productivity of their host plants [5], through suppressing citrus plant diseases caused by pests. In addition, endophytic microbes are also capable of producing antimicrobial and bio stimulating compounds such as phytohormones and proteins that act as stimulants for the immune system in a given plant [6].

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Endophytic bacteria are known for their ability to generate phytohormones such as indole acetic acid (IAA). The IAA is a kind of growth hormone that can directly induce root growth by stimulating cell division and elongation [7]. According to Yurnaliza [8], it was shown that root growth of rice plants will be faster when it was induced by IAA-produced by endophytic bacteria. Endophytic microbes also have ability to fix nitrogen and supply it to host plants. Furthermore, Yoneyabashi et al. [9] showed that nitrogen-fixing endophytic bacteria isolated from sugarcane increased the growth rate of potato plants. Interestingly, endophytic microbes have the ability to dissolve phosphate, thus assist the host plant to utilize the phosphate. Supporting evidence was shown by Oteiono et al. [10] stated that the Pseudomonas sp. Phosphate solvent that was isolated from grass (Miscanthus giganteus) can boost the growth rate of pea plants.

With the positive role of endophytic bacteria in stimulating growth, it is necessary to evaluate the existing collection of endophytic bacteria isolated from citrus plants in ICSFRI (Indonesian Citrus and Subtropical Fruits Research Institute), especially their ability to produce IAA, phosphate and nitrogen. Thus, this research’s aim was to evaluate and determine the ability of several endophytic bacterial isolates that have previously been isolated from citrus plants in the production of IAA hormone, phosphate solvent and nitrogen-fixing.

2. Methods

2.1 Subcultures. of Endophytic Bacteria
There were three isolates of citrus endophytic bacteria used in this study, namely G.28, G.31 and G.35. The three isolates were isolated from citrus plants, each of which had different varieties. G.28 was isolated from the roots of tangerine plants, G.31 was isolated from the roots of Keprok Garut and G.35 was isolated from the roots of Keprok Konde. The three isolates were sub cultured on nutrient agar (NA) media and incubated for one week at room temperature.

2.2 Isolate OD Equalization
The three isolates were inoculated by taking one loop of isolating on Erlenmeyer containing 50 ml of nutrient broth (NB) media done for each isolate. The three Erlenmeyers that had been inoculated with bacterial isolates were incubated in a rotary shaker at 100 rpm for 48 hours. Furthermore, the absorbance value of each isolate was measured using the spectrophotometric method wavelength of 600 nm. The measured OD value was equalized to 0.65 by the dilution method.

2.3 Free Nitrogen-Binding Bacteria Test
Free nitrogen fixing test using Nitrogen Free Biological (NFB) media with the composition of the media: 5 g / L calcium phosphate, 2 g / L calcium carbonate, 1 g / L K₂HPO₄, 0.5 g / L MgSO₄.7H₂O, 0.5 g / L NaCl, 2 mL 0.5% bromothymol blue, 0.005 g / L Na₂MoO₄ 2H₂O, 20 g / L glucose monohydrate, FeSO₄ and 15 g / L agar. The bacterial isolate culture (OD = 0.65) on NB media was inoculated as much as 0.1 ml on NFB media and levelled using the drigalski rods. NFB media containing isolates were incubated for 72 hours at room temperature. The presence of pellicles that formed on the surface of the media shows a positive result, indicate that bacteria can fix nitrogen in the atmosphere. The growing bacterial isolates were then transferred to new NFB media to confirm the previous test results.

2.4 Phosphate Solubilizing Bacteria Test
Phosphate solvent test by bacteria used Pikovskaya media, which its composition as follows f: 0.5 g / L peptone, 10 g / L glucose, 5 g / L calcium phosphate, 0.5 g / L ammonium sulphate, 0.2 g / L potassium chloride, 0.0001 g / L FeSO₄, 0.0001 g / L MnSO₄ HO, 0.1 g / L magnesium sulphate and 15 g / L agar. Pikovskaya media were divided into three quadrants. The bacterial culture isolates ion NB medium (OD = 0.65) was inoculated as much as 20 µl onto sterile disc paper. Disc paper that already contained bacterial isolates was placed in each quadrant of Pikovskaya media and incubated
for 120 hours at room temperature. Each isolate was done in 3 replications. The clear zone formed on each disc paper was measured and averaged.

2.5 Test of Endophytic Bacteria in Producing IAA

Each bacterial isolate was quantitatively tested for its ability to produce IAA. Bacterial isolate (OD = 0.65) was inoculated on tryptone Soy Broth Medium (TSB) with added L-tryptophan (5 mM) and incubated at room temperature in the shaker room at a speed of 100 rpm. Every 48 hours, cultures were centrifuged (5 min, 10,000 x h, at room temperature). 1.5 ml of Salkowski reagent was added to the 1.5 ml supernatant. The mixture was incubated for 20 minutes in dark conditions at room temperature. IAA levels were analysed using a spectrophotometer with a wavelength of 520 nm. Measurement of IAA levels was carried out until the concentration decreased. Each isolate was carried out 3 times repeating the standard curve of the IAA made as a determining tool to determine the concentration of IAA in the supernatant.

3. Result and discussion

3.1 Qualitative Results of Nitrogen Binding Bacteria Test

From the observations on NFB I media, the three isolates have shown positive results by the observed of pellicles indicated that the isolate are able to produce some amount of nitrogen. The pellicles were clearly visible in G.28-isolates and G.35-isolates, while G.31-isolates were not, formed very few pellicles (Figure 1). Observations on NFB II media showed pellicle formation in all isolates. The pellicles of G.28-isolates and G.35-isolates were clearly visible with yellowish colour observed, while G.31-isolates had very few pellicles (Figure 1). According to Aryantha & Hidiyah [11], the yellowish pellicles shown due to discoloration of the dark blue colour in the media. Pellicle formation on NFB media is the indicator of bacterial growth and nitrogenase enzyme activity from non-symbiotic nitrogen-fixing bacteria. The formation of pellicle as well as discoloration of the medium from a bluish green colour to yellow was growth characteristics and nitrogenase activity of non-symbiotic nitrogen-blocking (N) bacteria [10]. Bacteria capable of living on nitrogen-free media will secrete organic acids causing the discoloration of the media [12]. In addition, the semi-solid media allow non-symbiotic nitrogen-fixer bacteria to thrive at low partial of O2 pressures that favorable this bacterium at the time of nitrogen fixation [13].

Figure 1. The evaluation for nitrogen-fixing bacteria on NFB media, a) G.28-isolates in NFB I media, b) G.31-isolates in NFB I media, c) G.35-isolates in NFB I media, d) G.28-isolates in NFB media II, e) G.31-isolates in NFB II media, f) G.35-isolates median NFB II and g) Control

3.2 Phosphate Solvent Test Quantitative Results

Based on the observation of the three isolates, the clear zone in the media was observed after 5 days of incubation in the G.28-isolates and G.35-isolates, while G.31-isolates did not form clear zone (Figure 2). The formation of a clear zone indicates that the bacterial isolate has ability to dissolve the
phosphate that contained in Pikosykava media. The mean diameters of G.28-isolates and G.35-isolates were 7.1 mm and 6.4 mm, respectively. Figure 3 showed a graph of the clear diameter formed by the three isolates.

Figure 2. The clear zones formed in Pikosvkaya media after 5 days of incubation, a) G.28-isolates, b) G.3-isolates and c) G.35-isolates

Figure 3. Graph of clear zone diameter of three bacteria isolates

The result obtained in this experiment was in line with the result in other several studies, regarding the phosphate dissolve test by endophytic bacteria, that varying clear zone of diameter will be observed. Firstly, Silitonga [14], showed that endophytic bacteria from soybean roots with the diameter of the clear zone ranged from 3-12 mm indicates the ability of endophyte to dissolve phosphate. Furthermore, Sutariati et al. [15] also showed that several bacteria isolate were able to dissolve phosphate with the observed of clear zone diameters ranging from 5-12 cm in their experiment.

There are two main factor known that involved in the formation of various sizes in the diameter of the clear zone form, which are the type of bacteria and the environment condition. Additionally, Some of the factors such as uneven concentration of phosphate sources in the media, the thickness of the pikosvkaya medium and the speed of microbial growth may lead to the formation of various sizes. Some other known factor such as physiological and biochemical characters in each strain of bacteria may also influence the variations of the diameter sizes [12]. This is because each isolate has different abilities to dissolve phosphate. The value of the phosphate dissolution index resulting from Taniwan's research is 5-12 cm [16]. Bacteria that are able to dissolve phosphate by
forming an environmental zone around the colony on Pikovskaya media added with Ca3PO4. The formation of a clear zone indicates that the phosphate has dissolved. Measurement of the ability to phosphate by bacteria is carried out to see the response, and application, in dissolving, undissolved phosphate into phosphate that can be absorbed by plants. According to Silitonga [14] the difference in the area of halozone produced in pikovskaya media depends on the type of bacteria and the area of origin. isolates isolated from the roots of soybean plants in soybean plantations produced halozone ranging from 0.3 to 1.45 cm. Sutariati et al. [15] obtained several bacteria that can dissolve phosphate with varying levels of dissolution, namely 0.15-1.25 cm. These results are not much different from the results of Suliasih and Rahmat [17] which are 0.45-1.35 cm. According to Rao [18], phosphate solvent microbes secrete a number of organic acids such as formic acids, acetic, propionic, lactonic, glycolic, fumarate, and succinate.

3.3 Test the ability of endophytic bacteria to produce IAA in-vitro

The level of IAA in each isolates were measured for 6 consecutive days, each isolate resulted different amount of IAA in different day of measurement was taken as shown in figure 4. On the second day, the isolate of G.35 produced the highest concentration of IAA in comparison to other isolates approximately 168.99 ppm (Figure 4). On the other hand, IAA concentrations in G.28-isolates and G.31-isolates were produced only 56.34 and 25.63 ppm, respectively.

On the fourth day, the IAA concentration in the three isolates was observed the significant increment. The highest IAA concentration was still produced by G.35-isolates followed by G.28-isolates and G.31-isolates respectively. However, a significant increases of IAA concentration had occurred only in isolate G.31 approximately almost 4 times compared to the production of IAA in the second day. This shows that G.31 isolate is very effective in producing IAA on the fourth day.

On the sixth day, the IAA concentration in all three isolates was plunged (Figure 4). The IAA concentrations of isolates G.28, G.31 and G.35 were 48.49 ppm, 59.65 ppm and 51.57 ppm, respectively. This shows that the IAA production on the sixth day is no longer effective to be produced by all isolates.

At the early stages of the bacterial growth the difference in IAA concentration between each isolates shown in this experiment was expected to be due to the difference in growth speed between isolates. However, over the span of 6 days, if the differences was because of the type of isolates, the

![Figure 4. Measurement of IAA concentrations on TSB + L-tryptophan media at 2, 4 and 6 days](image-url)
level of IAA measured would be consistently high level up to the end of the observation, but this was not the case. The availability of nutrient content in the media was suspected to be involved as it may also deplete over time greatly affects the productivity of IAA by bacteria. Bacterial cells are still able to undergo cell division even though there is no source of nutrient to support their normal growth, but as a consequence the bacteria will consume the IAA produced if the nutrients in the media are not available [19].

IAA will be broken down further by the bacteria if the source nutrients in the environment are still not available. IAA will be used for protein biosynthesis or other physiological activities in cells to sustain their existence. To tackle this issue, it is suggested that the addition of L-tryptophan precursor greatly may maintain the level of IAA production because L-tryptophan is the most effective precursor. This is evidenced by the results of research by Yurnaliza [8] which showed that the concentration of IAA in the media by adding L-tryptophan was higher than the media without the addition of L-tryptophan.

In Munthe's experiment [20], there were 11 bacterial isolates that constantly experience an increase of IAA production even over the period of the third day of the highest IAA concentration measured was 48 ppm. On the other hand Herlina et al. [21] with utilizing endophytic bacteria isolated from the roots of peanut plants (Arachis hypogea) the IAA concentration in 11 isolates increased on the fourth day, but started to fall down when entering the sixth day of observation that was applied for almost all isolates. After the fourth day of incubation, the highest and the lowest of IAA amount were 69.68 (mg L\(^{-1}\)) and 8.50 (mg L\(^{-1}\)) respectively. Isolates that produce high IAA levels are applied to mung beans, it affects the number of lateral roots but it does not have effect on the length of the sprouts.

4. Conclusion
Some of endophytic bacteria in citrus plants in this experiment were identified for their abilities to produce certain stimulant that most likely will be beneficial to support the growth of the host plant. The three endophytic bacterial isolates obtained from citrus plants of different varieties showed their potential to fix free nitrogen. Only the G.28-isolates and G.35-isolates had ability to dissolve phosphate, while G.31-isolates could not dissolve phosphate. The fourth day of incubation was identified as the most effective time span for bacteria to produce IAA. The G.35-isolates was a bacterial isolate that produce the highest level of IAA.

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