The properties of rhizobacteria from tomato rhizosphere as biocontrol and biofertilizer

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Abstract. Low soil fertility and the presence of pathogen are the limiting factors in horticultural crops cultivation in Ambon City. This study aimed to isolate plant growth promoting rhizobacteria from tomato rhizosphere as a potential biocontrol, biostimulant and biofertilizer. The research consisted of two stages, namely (1) isolation of bacteria from tomato rhizosphere and purification of the isolates, and (2) characterization of colony morphology, gram reaction, hypersensitivity test and antagonist test to soil pathogens, phosphate dissolution, and phytohormones indole acetic acid (IAA) production. Forty-two isolates were found from tomato rhizosphere, 36 isolates suppressed growth of Rhizoctonia solani while 6 isolates increased the growth of those pathogen. The growth of Phytophthora sp. pathogen was decreased by 35 bacterial isolates and increased by 7 bacterial isolates. All isolates produce IAA but only 13 isolates are enabled to dissolve inorganic phosphate. Based on bacterial properties, the isolates of TT-22, TT-21, TT-12, TT-11, WT-11 and WT-332 are the promising isolates for developing the biological agent as pathogen control and biofertilizer.

1. Introduction

The presence of pathogens and low soil fertility are a major problem in optimizing horticultural and food crops production, and therefore is a major constraint in maintaining food availability in Ambon city. Horticultural and food crops are cultivated on entisol and inceptisol soils which often contain 1.67% C-organic; 0.12% N Total; 13.92 C/N Ratio; 9.08 mg / 100 g P2O5; 35 mg / 100 g K2O; 7.86 ppm P total; 0.44 cmol / kg K total; 5.86 pH (H2O) and 4.5 pH (KCl), and they are classified as soil with low nutrient oil borne [1]. In addition, the lack of using organic and inorganic fertilizers, and the presence of soil borne pathogens in the nursery phase to production has been exacerbated this condition, as indicated by the presence of damping off and leaf blight diseases such as Rhizoctonia solani, Sclerotium rolfsii, and Fusarium oxysporum [2,3].

Organic materials such as compost and biological fertilizers can be used as a source of nutrients for plants and to improve soil physical and biological [4,5]. Biological fertilizers are inoculants with beneficial living organisms active ingredients which can be used to improve crop yields productivity and quality by increasing the availability of nutrients in the soil [6].

Microbes that play a role in spurring plant growth of plants are known as Plant Growth Promoting Rhizobacteria (PGPR). In general, the function of RPTT in increasing plant growth is characterized by the following mechanisms: (1) growth promoters (biostimulants) by synthesizing and regulating the concentration of various growth regulators or phytohormones such as indol acetic acid (IAA), gibberellin, cytokinins, and ethylene in the root environment; (2) a nutrient provider (biofertilizer) by tethering N2 from the air asymbiosis and dissolving P nutrients bound in the soil; and (3) controlling...
soil-derived pathogens (bioprotectant) by producing various anti-pathogenic compounds or metabolites such as siderophore, β-1,3-glucanase, chitinase, antibiotics, and cyanide [7,8,9].

Indigenic bacteria from the genus Azotobacter, Acinetobacter Azospilium, Bacillus and Pseudomonas are types of microorganisms found in soil and plants, especially at the base of the stems and roots. These four genera of bacteria have been widely used as Plant Growth Promoting Rhizobacteria (PGPR) to increase plant growth through nitrogen fixation, phosphate dissolving, and phytohormone production [6,9,10]; and to reduce the intensity of disease attacks caused by soil pathogens [2,11,12], and they are more effective if applied to their natural environmental conditions. This indicates that the environmental conditions play important roles in nutrient supply through N tethering, phosphate dissolving, and production of phytohormones as well as producing enzymes to suppress plant pathogens and disease.

This study aimed to isolate plant growth promoting rhizobacteria from tomato as they are potentially used as biocontrol, biostimulant and biofertilizer

2. Methods
This research was conducted from June to October 2019. Bacteria were isolated from tomato rhizosphere collected from three vegetable cultivated fields in Ambon City, namely Hutumuri Village, Waiheru Village, and Tawiri Village. Research activities included isolation, selection and purification of isolates; isolate characterization; and testing the ability of the isolates to stimulate the growth of tomato seedlings.

2.1. Isolation of Bacteria Tomato Rhizosphere
100 g of soil was taken from the rhizosphere of the fruiting tomato plant. Five very healthy plants were selected from each of the sampling locations. 500 g of soil was mixed and 10g was taken and added to 90 ml of sterile water in erlemeyer and was shaken using a hotplate stirrer for 15 minutes. A total of 1 ml of this solution was taken to 9 ml test tube and shaken using a vortex for 1 minute until a 10-5 dilution series was obtained. The 0.5 ml was taken using pipette and distributed in petridis on a petri dish (Ø 9 cm), and added with nutrient broth (NB) media while shaking it with a rotary petridis. The sample was then incubated at room temperature for 24-48 hours. Single colonies of growing bacteria were separated based on colony color and shape, grown and purified using NA media. Pure colonies were maintained on NA media in test tubes (slanted media) to be used as stock cultures.

2.2. Characteristics Bacterial Isolates
Bacterial characterization was determined based on motile, size, shape, periphery, and colony color on nutrient agar (NA) media; gram reaction test; phosphate dissolution test; Indole Acetic Acid (IAA) phytohormone test; hypersensitivity test; and antagonist test.

2.2.1. Gram Reaction Test
Stored-bacterial colonies on NA media in a test tube were transferred to a glass object using a loop needle, added with two drops of 3% KOH solution and mixed for 5-10 seconds. If the sample looks slimy and unbroken when lifted up, it shows a positive reaction and is classified as gram-negative, conversely, if the sample is not slimy and removeable, it shows a negative reaction and is classified as gram-positive [13,14].

2.2.2. Phosphate Dissolution Test
Phosphate dissolution was tested using the Pikovskaya method [9,15]. The 72 hour old suspension isolated-bacteria on NA media was taken aseptically 1 ose and innoculated on solid Pikovkaya media containing tricalcium phosphate (Ca₃PO₄), then incubated for 72 hours at room temperature (27-28 °C). Phosphate dissolution activity is indicated by a clear zone around the bacterial colony. The phosphate dissolution index (IP) is measured as follows: IP = (clear zone diameter - colony diameter) / (colony diameter)

2.2.3. IAA phytohormone test
The cultured-bacterial on NB media containing 10 ml tryptophan was centrifuged at 3,000 rpm for 30 minutes. The 2 ml supernatant from centrifugation was taken and added with 2 drops of ortho phosphate
solution and 4 ml of Salkowsky’s reagent (50 ml 35% H₂SO₄ + 1 ml M FeCl₃), incubated for 20 hours at room temperature without light. The sample was measured by spectrophotometry at wavelength (λ) = 353 nm. The resulted-absorbance were then entered into the standard curve equation of IAA 0-60 ppm to obtain the final concentration of IAA [16].

2.2.4. Hypersensitivity Test
The isolated-bacteria obtained after screening were carried out by the HR (Hyhipersensitive Reaction) test on tobacco plants and tomato seeds. Isolates were grown on Lurial Bertani (LB) media for 3 days while shaking with a shaker at a speed of 150 rpm at room temperature. A total of 1 ml isolated-bacterial suspension with a cell density of 10⁷ cfu / ml was injected into tobacco leaves and observed for 5 days. The present of pathogenic bacteria is indicated by necrotic symptoms. Hypersensitivity test was carried out as follows: 50 tomato seeds were immersed in the bacterial isolate suspension with a cell density of 10⁷ cfu/ml for 5 and then planted in sterile soil in the seed boxes. Observations were made from the seeds germinating until 10 days old.

2.2.5. Antagonist Test
The isolated-bacteria obtained after hypersensitivity test were tested for antagonism against the fungal pathogens Rhizoctonia sp and Phytophthora sp. The antagonism testing of each isolated-bacteria against pathogens was conducted by using the dual culture method on PDA media. The antagonism of each isolate against pathogens was calculated by measuring the area of the pathogenic colony as control and compared to the growing area of the pathogenic colony with isolated-bacteria using the formula:

\[ PIRG = \frac{l_{12} - l_{11}}{l_{11}} \times 100\% , \]

where \( l_1 \) = area of pathogenic colonies without bacteria (control) and \( l_2 \) = area of pathogenic colonies with bacteria [17].

3. Results and discussion
The screening of the isolated-bacteria from tomato rhizosphere results in 42 isolates, and most of the bacterial colonies are white, circular shape, flat elevation, and entire margin (Table 1). Thirty three of them are motile, 26 are gram positive, 16 isolates are gram negative, while 13 isolates can be active as phosphate solvents, however all isolates can produce the IAA hormone and negative hypersensitivity test (Table 2).

**Table 1. Morphology of bacteria isolated from tomato rhizosphere**

| No | Isolate Code | Origin | Color     | Shape     | Elevation | Margin   |
|----|-------------|--------|-----------|-----------|-----------|----------|
| 1  | WT-332      | Waiheru| Yellow    | Circular  | Umbonate  | Entire   |
| 2  | WT-321      | Waiheru| White     | Circular  | Convex    | Lombate  |
| 3  | WT-31       | Waiheru| White     | Circular  | Flat      | Entire   |
| 4  | WT-15       | Waiheru| Bright yellow | Circular  | Flat      | Entire   |
| 5  | WT-11       | Waiheru| White     | Circular  | Umbonate  | Lombate  |
| 6  | WT-16       | Waiheru| White     | Circular  | Flat      | Entire   |
| 7  | WT-13       | Waiheru| White     | Irregular | Umbonate  | Lombate  |
| 8  | WT-14       | Waiheru| White     | Circular  | Flat      | Entire   |
| 9  | WT-12       | Waiheru| Bright yellow | Circular  | Flat      | Entire   |
| 10 | WT-33       | Waiheru| Bright yellow | Irregular | Flat      | Lombate  |
| 11 | WT-331      | Waiheru| White     | Irregular | Umbonate  | Lombate  |
| 12 | WT-322      | Waiheru| White     | Circular  | Flat      | Entire   |
| 13 | HT-11       | Hutumuri| White    | Irregular | Umbonate  | Lombate  |
| 14 | HT-322      | Hutumuri| White    | Irregular | Umbonate  | Lombate  |
| 15 | HT-22       | Hutumuri| White    | Circular  | Flat      | Entire   |
| 16 | HT-331      | Hutumuri| Bright yellow | Circular  | Flat      | Entire   |
| 17 | HT-321      | Hutumuri| White    | Irregular | Convex    | Lombate  |
Table 2. Motile test, gram reaction, phosphate dissolving ability, production of IAA phytohormones, and hypersensitivity tests on tobacco and tomato seeds

| No | Kode  | Motile | Gram reaction | phosphate dissolving ability | IAA concentration (ppm) | hypersensitivity tests |
|----|-------|--------|---------------|----------------------------|--------------------------|------------------------|
| 1  | WT-332| +      | +             | 1,15                       | 1,000                    | -                      |
| 2  | WT-321| +      | +             | -                          | 0,843                    | -                      |
| 3  | WT-31 | +      | +             | -                          | 0,865                    | -                      |
| 4  | WT-15 | +      | +             | -                          | 0,973                    | -                      |
| 5  | WT-11 | +      | +             | 1,20                       | 0,865                    | -                      |
| 6  | WT-16 | -      | +             | -                          | 0,834                    | -                      |
| 7  | WT-13 | -      | +             | -                          | 0,816                    | -                      |
| 8  | WT-14 | +      | +             | -                          | 0,987                    | -                      |
| 9  | WT-12 | +      | +             | -                          | 1,000                    | -                      |
| 10 | WT-33 | +      | +             | 1,23                       | 1,031                    | -                      |
| 11 | WT-331| -      | +             | -                          | 0,861                    | -                      |
| 12 | WT-322| -      | -             | -                          | 0,901                    | -                      |
| 13 | HT-11 | +      | +             | -                          | 0,883                    | -                      |
| 14 | HT-322| +      | +             | -                          | 0,847                    | -                      |
| 15 | HT-22 | +      | -             | 1,22                       | 1,175                    | -                      |
| 16 | HT-331| +      | +             | -                          | 0,829                    | -                      |
| 17 | HT-321| -      | +             | -                          | 0,924                    | -                      |
| 18 | HT-121| +      | -             | 1,90                       | 0,996                    | -                      |
| 19 | HT-32 | +      | +             | -                          | 0,861                    | -                      |
| 20 | HT-222| -      | -             | -                          | 1,211                    | -                      |
| 21 | HT-221| +      | -             | -                          | 1,391                    | -                      |
Forty two isolated bacteria were tested for pathogenicity against the pathogens of the fungi *Rhizoctonia solani* and *Phytophthora* sp. The result shows that not all isolates can suppress pathogen development, but some can increase pathogen growth. Tables 3 shows that HT-11, HT-32, HT-21, TT-22, TT-212, and TT-33 isolates can help increase the growth of the pathogen *R. solani*, while isolates WT-31, WT-15, WT-16, WT-12, HT-22, HT-13, and TT-13, can increase the growth of pathogens *Phytophthora* sp.

**Table 3.** Antagonist test of isolated bacterial to suppress in vitro growth of pathogens *Rhizoctonia solani* and *Phytophthora* sp.

| No | Isolate code | Percentage of suppress growth of pathogen |
|----|--------------|-----------------------------------------|
|    |              | *Rhizoctonia solani*                     | *Phytophthora* sp |
| 1.  | WT-332       | 62.57                                   | 86.67            |
| 2.  | WT-321       | 47.93                                   | 86.67            |
| 3.  | WT-31        | 11.66                                   | (-31.81)         |
| 4.  | WT-15        | 56.77                                   | (-45.17)         |
| 5.  | WT-11        | 61.77                                   | 86.67            |
| 6.  | WT-16        | 33.68                                   | (-8.70)          |
| 7.  | WT-13        | 62.41                                   | 86.67            |
| 8.  | WT-14        | 21.45                                   | 86.67            |
| 9.  | WT-12        | 71.28                                   | (-27.19)         |
| 10. | WT-33        | 28.25                                   | 86.67            |
| 11. | WT-331       | 59.31                                   | 86.67            |
| 12. | WT-322       | 21.45                                   | 83.54            |
| 13. | HT-11        | (-25.21)                                 | 77.47            |
| 14. | HT-322       | 51.82                                   | 86.67            |
The data in Table 2 and Table 3 is used as references for selecting potential isolates to be used as plant biostimulants, nutrient providers (biofertilizers), and plant disease control (biocontrol / bioprotectant). The ability of isolates to stimulate plant growth and yield is known by testing the ability of isolates to produce phytohormones, including Indol Acetic Acid (IAA) or Indol Acetic Acid. Table 2 shows that all isolates have the ability to produce IAA phytohormones. IAA is the active form of the auxin hormone [18], it plays a role in increasing plant growth and yield [19,20]. The main function of auxin hormone in plants is to promote the elongation of developing buds, affect stem and root growth, while zeatin is a natural cytokinin that can affect root growth and differentiation, promote cell division and general growth, promote germination and delay aging [21]. The action of this hormone is synergistic with gibberellin and cytokinins, therefore it can also regulate physiological processes, such as growth, division and differentiation of cells and protein synthesis [22].

The phosphate dissolution test was carried out to ensure the ability of the isolates to dissolve the phosphate in vitro. The results show that of the 42 isolates, 13 isolates have the ability as a phosphate solvent, namely isolates WT-332, WT-11, WT-33, HT-22, HT-121, TT-33, TT-21, TT-131, TT-14, TT-11, TT-12, TT-22, and TT-232 (Table 2). These results indicate that if the isolate can be used as an agent in the process of making biological fertilizers. Phosphate is a macro nutrient that plays a major role in increasing plant growth and yield. In the soil, phosphate is found as inorganic phosphate such as calcium phosphorus (Ca-P), aluminum phosphate (Al-P) and iron phosphate (Fe-P), and as organic phosphates.
such as phytin compounds, nucleic acids, and phospholipids [23,24]. Inorganic phosphates are difficult to dissolve in water. The presence of microbes through biological fertilizers will increase the availability of phosphate in plants. The activities of phosphate solubilizing microorganisms will produce organic acids including lactic, citric, succinic, glutamate, oxalate, malate, glyoxalate, and fumarate. The increasing of these acids can decrease soil pH and followed by the dissolution of the phosphate bound by Ca, Al [15,25].

In vitro hypersensitivity and antagonistic testing against pathogens *Phytophthora* sp and *Rizoctonia solani* will further strengthen the selection of isolates that have the potential to be developed as biological fertilizers. Although an isolate can produce phytohormones and dissolve phosphate but it has potency to be a pathogen or as a trigger of pathogens development, therefore this isolate cannot be recommended as biological fertilizer. Hypersensitivity test results also show that all isolates have no potential to be pathogens (Table 2). However, the results of invitro antagonistic testing against pathogens *Phytophthora* sp and *Rizoctonia solani* show that six isolates (HT-11, HT-32, HT-21, TT-222, TT-212, and TT-33) can help increase the growth of the pathogen *R. solani*, while seven isolates (WT-31, WT-15, WT-16, WT-12, HT-22, HT-13, and TT-13) can increase the growth of the pathogen *Phytophthora* sp (Table 3).

### 4. Conclusion
A total of 42 bacteria are isolated from the tomato rhizosphere. All isolates produce IAA but only 13 isolates are able to dissolve inorganic phosphate. Among all isolates, 36 isolates can suppress the growth of the pathogen *Rizoctonia solani*, and 6 isolates can increase the growth of the pathogen. 35 isolates could suppress the growth of the pathogen *Phytophthora* sp, and 7 isolates can increase the growth of the pathogen. Based on the characteristic of bacteria, isolates TT-22, TT-21, TT-12, TT-11, WT-11 and WT-332 are promising isolates to be developed as biological agents both as pathogen controllers and as biological fertilizers to increase plant growth.

### 5. References
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