Screening of plant growth-promoting bacterial endophytes and rhizobacteria isolated from *Curcuma xanthorrhiza*

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**Abstract.** Bacterial endophyte and rhizobacteria were reported to associate with medicinal plants including Zingiberaceae plants and involved in growth promotion. These beneficial bacteria are promising candidates as biostimulants because of their ability in promoting plant growth. This study aims to evaluate the activity of endophytic and rhizosphere bacteria isolated from *Curcuma xanthorrhiza* (Javanese turmeric) in promoting rice seedling and Javanese turmeric growth. Fifty-seven of 150 total bacterial isolates with negative hemolysis and hypersensitivity reactions were characterized to investigate their plant growth-promoting (PGP) traits. Ten selected bacteria (two bacterial endophytes and eight rhizobacteria) with multiple PGP traits were inoculated to rice seed with seed treatment and inoculated to Javanese turmeric rhizome with seed treatment and seed treatment+soil drenching. Our results showed that bacterial isolates tested on rice seed promoted rice seedling growth significantly. A total of five, three, six, and three bacterial isolates could increase leaf number, root length, fresh shoot weight, and fresh root weight of rice seedling (*p*<0.05), respectively. In contrast, all of the bacterial isolates tested on Javanese turmeric rhizomes showed a non-significant effect on the plant growth. Further studies should be considered to investigate the effect of formulated potential bacterial isolates with different application frequencies and environmental conditions on the harvest yield of rice and Javanese turmeric as well as active compounds of Javanese turmeric.

1. Introduction

Medicinal plants including Zingiberaceae were reported to associate with rhizobacteria and bacterial endophytes. Bacterial endophytes inhabit internal plant tissue without damaging host plants, whereas rhizobacteria colonize the rhizosphere region [1,2]. Plant growth-promoting bacteria (PGPB) including endophytic bacteria and rhizobacteria enhance the growth and development of plants by direct and indirect mechanisms. PGPB facilitates the acquisition of nutrients from the environment or modulates plant growth by altering hormone levels of plants in the direct mechanism. Indirect mechanism relates to biocontrol against plant pathogens [3–5].

Several studies reported that inoculation of bacterial endophytes and rhizobacteria isolated from Zingiberaceae were able to enhance the host and non-host plant growth. Bacterial endophyte (*Bacillus cereus*) and rhizobacteria (*Psudomonas aeruginosa*) isolated from *Curcum longa* demonstrated significant enhancement to plant height and fresh rhizome yield of *C. longa* compared with untreated control [6]. Another study reported that isolated rhizobacteria from *C. longa* increased the growth of a non-host plant, tomato (root and shoot length, fresh weight and dry weight, respectively) under salinity stress condition [7]. Inoculation of bacterial endophytes from ginger to *Zea mays* were able to increase plant height, enlarge leaf area and *Z. may* biomass yield significantly (*p*<0.05) [8]. Therefore, PGPB
were potential biostimulants to be utilized for improving various plant growth in a sustainable way and eco-friendly. One of Zingiberaceae family plants was *Curcuma xanthorrhiza* (Javanesenese turmeric) and is known as a medicinal plant. The rhizomes of this plant have various pharmacological activities including antioxidant, anti-inflammatory, antimicrobial and anticancer [9]. As Zingiberaceae family plants were a potential source of PGPB for host and non-host plants, the objectives of this study were to isolate bacterial endophytes and rhizobacteria from Javanese turmeric (*Curcuma xanthorrhiza*), characterize their plant growth-promoting traits, and evaluate their activities in promoting rice seedling and Javanese turmeric plant growth.

2. Materials and Methods

2.1. Rhizobacteria and bacterial endophyte isolation

Rhizobacteria and bacterial endophyte were isolated from healthy Javanese turmeric (*Curcuma xanthorrhiza*) plants grown in three different locations in Bogor, West Java. Rhizobacteria were isolated from the rhizospheric soil of turmeric plants, whereas bacterial endophytes were isolated from roots and rhizomes followed Vinayarani and Prakash method [6]. Serial dilutions method was used to dilute suspension of rhizospheric soil and ground plant organs before plated into TSA medium. Bacterial colonies that grown from those organs and rhizospheric soil were purified into pure culture and used for further screening.

2.2. Hemolysis and hypersensitivity reaction screening

All of the rhizobacteria and bacterial endophyte isolates were tested for hemolysis activity on blood agar. The hemolysis activity was shown by a clear zone formation surrounding the bacterial, and isolates with hemolysis activity were eliminated.

   Bacterial isolates with no hemolysis activity were screened for hypersensitivity reaction on a tobacco plant. Bacterial culture was infiltrated into the tobacco leaf using a syringe [10]. Hypersensitivity reaction (HR) shown by the presence of necrotic symptoms on tobacco leaf and this bacterial isolate will be eliminated. Bacterial isolates with no hemolysis and hypersensitivity reaction activity were screened for their plant growth-promoting traits.

2.3. Screening of the bacterial isolates for plant growth-promoting traits

The bacterial isolates were screened for IAA production by the following procedure as described by Gravel (2007) with modification [11], and the absorbance was measured using a UV-Vis spectrophotometer (Shimadzu) at 530 nm and the amount of IAA production was estimated using a standard curve for IAA [12]. The ability of bacterial isolates in solubilizing phosphate was screened by spotted inoculation of bacterial isolates on Pikovskaya (PKV) agar [13]. A loopful of bacterial culture was placed on a glass slide for catalase test, and 3% peroxide was dropped on the bacterial colony. Positive test results of tested bacterial isolates showed a rapid formation of gas bubbles [14]. Detection of ammonia production performed by inoculating 100 µL of bacterial culture into peptone water in test tubes. The tubes were incubated at 30°C for 4 days. The ammonia production was shown by yellow colour (+) and deep yellow (++) to brownish colour (+++) formation in the tubes after 0.5 mL Nessler’s reagent addition [15]. Screening of bacterial ability in fixing nitrogen was done on semisolid nitrogen-fixing bacteria (NFb) medium in test tubes [16]. The tubes containing NFb medium were inoculated with bacterial culture and incubated for 14 days at room temperature. The presence of pellicle indicated that tested bacteria were able to fix nitrogen. Determination of exopolysaccharide production was performed according to Paulo *et al.* [17]. ACC deaminase activity was performed by the method of Ali *et al.* [18], while detection of HCN was performed according to Baker and Schippers [19]. Production of hydrolytic enzymes was performed by the method described by Cappuccino and Sherman for protease, cellulase, pectinase [14], whereas chitinase assay according to Subramanian *et al.* [20].
2.4. *Plant growth assay*

2.4.1. *Rice.* The assay was designed with a completely randomized design with eight replications. Ten selected bacterial isolates were used in this assay. Rice seeds were surface sterilized and then soaked in each bacterial suspension with Optical Density (OD$_{600}$ 0.5) for 24 h. Uninoculated physiological saline solution was used as a control. Inoculated and uninoculated seeds were sown in sterile soil:compost (1:1) medium. The shoot height, root length, leaf number, shoot and root fresh weight were measured at 14 d after planting.

2.4.2. *Javanese turmeric.* The assay was designed with a randomized complete block design with 2 factors and 5 replications. Factor one was bacterial treatments (10 selected bacteria and control), Factor two was the application method (seed treatment and seed treatment + soil drenching). Javanese turmeric rhizomes were surface sterilized, washed in sterile distilled water thrice and air-dried. Dried rhizomes were soaked in bacterial culture for 5 h. and sown in sterile soil:sand:compost (1:1:1) medium. Soil drenching was given 4 weeks after planting. Plant height and leaf number of Javanese turmeric were measured at 8 and 12 weeks after planting.

2.5. *Statistical analysis*

The plant growth data were analyzed through analysis of variance (ANOVA) using R studio. Probabilities of significant difference from ANOVA were used to test the significance among treatments (P<0.05). Mean comparisons were performed by Duncan’s multiple range test (DMRT).

3. *Results and Discussion*

3.1. *Isolation of rhizobacteria and bacterial endophyte*

A total of 150 bacterial isolates were isolated from rhizospheres, roots and rhizomes of Javanese turmeric from 3 locations. The bacterial isolates mostly came from the rhizosphere rather than the endosphere region (Table 1). Plant exudates contain amino acids and sugar, a rich source of energy and nutrients for the bacteria in the rhizosphere zone and result in the increment of microbial population in the zone rather than outside zone [21]. The diversity of endophytic bacteria can be determined by host plant age and growth stage, geographical location, climate condition, genotype and isolated plant tissue [22–24].

| Bacterial isolates     | Number of bacterial isolates | Number of bacterial isolates with no hemolysis and HR activity |
|------------------------|------------------------------|---------------------------------------------------------------|
| Root endophyte         | 27                           | 6                                                             |
| Rhizome endophytes     | 34                           | 8                                                             |
| Rhizobacteria          | 89                           | 43                                                            |
| Total of Isolates      | 150                          | 57                                                            |

Table 1. Number of bacterial isolates and selected bacteria with no hemolysis and hypersensitivity reaction activity.

Thirty-eight percent of total bacterial isolates did not show hemolysis and hypersensitivity reaction (Table 1). The bacteria with haemolytic reaction produce haemolysin toxins that are potentially be a human pathogen. Hypersensitivity reaction showed by the presence of necrotic symptoms on inoculated tobacco leaf and indicated the bacteria potentially to be plant pathogen. Therefore, the bacteria demonstrating hemolysis and hypersensitivity reaction activities will not be used for further study.
3.2. Plant growth-promoting traits

A total of 55 bacterial isolates were able to produce IAA and the concentrations were varied from 0.6 to 79.243 mg L\(^{-1}\). Phosphate solubilizing bacteria enable plants to uptake available phosphate from insoluble phosphate through producing enzymes that mineralize organic phosphorus [25,26]. In this study, a total of 29 bacterial isolates from 57 tested bacterial isolates could solubilize phosphate with phosphate solubilizing index ranging from 1.06 to 5.59. Almost all bacterial isolates (98%) were positive for catalase activity. The formation of bubbles was varied for each bacterial isolate. Catalase helps the bacteria to detoxify ROS [27].

A total of 51 bacterial isolates could produce ammonia. Higher ammonia accumulation was indicated by deep yellow to brownish colour as a result of Nessler’s reagent addition. Ammonia produced by bacteria will be a nitrogen source for plant growth [28]. Our results revealed that rhizobacteria as well as endophytic bacteria were able to fix nitrogen. A total of 32 bacterial isolates showed this activity. The bacteria formed a pellicle on semisolid NFb medium and the colour of the medium changed from green to blue as a result of pH increment of the medium by nitrogen-fixing bacteria [16,29]. Exopolysaccharides could be produced by 27 bacterial isolates. The bacteria produced a mucoid substance on the medium and precipitated after being mixed with absolute ethanol [17]. A total of 21 bacterial isolates were able to produce ACC deaminase. The enzyme ACC deaminase was able to decrease ethylene level by cleaving the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), into \(\alpha\)-ketobutyrate and ammonia [30]. There is no bacterial isolate that has characteristics on HCN production in this study. Bacterial antagonists produced HCN as a biological control mechanism [31]. In this study, a total of 16, 35, 29, and 11 bacterial isolates could produce cellulase, pectinase, protease and chitinase, respectively. All of the hydrolytic enzymes could be produced by rhizobacteria as well as bacterial endophytes. The activity of cellulase and pectinase enable microorganisms to penetrate plant tissue. Protease, cellulase and chitinase enzymes were involved in biological control against plant pathogens [32–34].

### Table 2. Plant growth-promoting traits of selected bacteria.

| Isolate | N\(_2\)-Fix | Cellu | IAA (mg/L) | PSI | Catal | Prot | NH\(_3\) | EPS | Pec | ACC | HCN | Chit |
|---------|------------|-------|------------|-----|-------|------|---------|-----|-----|-----|------|------|
| 4RH22   | +          | +     | 10.8       | 1.1 | -     | -    | ++      | -   | -   | +   | -    | -    |
| 4EA10.1 | +          | +     | 9.2        | 1.2 | +     | +    | +++     | +   | +   | +   | -    | -    |
| TAT2    | -          | -     | 10.2       | 5.6 | +     | -    | -       | -   | -   | -   | -    | -    |
| TA2     | +          | -     | 3.8        | 1.3 | +     | -    | ++      | +   | -   | -   | -    | -    |
| YAT19   | +          | -     | 5.5        | 1.4 | +     | +    | +       | +   | -   | -   | +    | -    |
| YAT9    | -          | -     | 9.0        | 1.1 | +     | +    | -       | +   | -   | -   | -    | -    |
| TAT19   | +          | -     | 7.6        | 1.2 | +     | +    | +       | -   | -   | -   | +    | -    |
| TAT12   | -          | -     | 6.5        | 1.2 | +     | +    | +       | +   | -   | -   | -    | -    |
| TAT17   | +          | +     | 10.0       | 1.3 | +     | +    | +       | +   | -   | -   | -    | -    |
| 4RH8    | +          | +     | 71.1       | -   | -     | +    | +       | +   | +   | +   | -    | -    |

\(^{a}\)N\(_2\)-Fix= nitrogen fixation; Cellu= cellulase production; IAA= indol acetic acid production; PSI= phosphate solubilizing index= ratio clear zone and colony diameter; Catal= catalase production; Prot= protease production; NH\(_3\)= ammonia production; EPS= exopolysaccharide production; Pec= pectinase production; ACC= ACC deaminase production; HCN= HCN production; Chit= chitinase production.

PGP traits of bacteria were used to select high potential bacteria as biostimulants. In the present study, we selected ten bacterial isolates for plant growth assay, eight rhizobacteria (RH22, TAT2, YAT19, YAT9, TAT19, YAT12, TAT17, and 4RH8) and 2 endophytes (4EA10.1 and TA2). The selected bacteria had multiple PGP traits ranging from 4 to 10 characteristics (Table 2). We selected bacterial isolates which represented all of PGP traits except HCN production. A previous study reported that the ability of bacteria in promoting plant growth and suppressing disease due to multiple modes of action [35].
3.3. Effect of selected bacteria and application method on rice seedling growth

The results showed that selected bacterial isolates were able to enhance the rice seedling growth. A total of three, five, six and three bacterial isolates significantly increased root length, leaf number, shoot and root fresh weight of rice seedling, respectively. However, bacterial isolates did not give a significant effect on increasing rice seedling shoot height compare with the control plant (Figure 1).

![Figure 1](image1.png)

**Figure 1.** Effect of bacterial treatments on shoot height of rice seedling. Bars with same letter are not significantly different according to DMRT (p<0.05).

The response of rice seedling root length to bacterial isolates treatment was varied from 5.2 to 8.54 cm. Rice seedling root length of YAT12, TAT17, and 4EA10.1 (8.54, 7.34, and 7.1 cm) were significantly longer than of control (4.85 cm) (Figure 2a). Bacterial isolates 4RH22, YAT12, TAT17, 4RH8 and 4EA10.1 significantly increased leaves number of leaves of rice seedlings. The leaves number average was 2.75, 2.75, 2.75, 2.63 and 2.75, respectively (Figure 2b).

![Figure 2](image2.png)

**Figure 2.** Effects of bacterial treatments on root length (a) and leaf number (b) of rice seedling. Bars with same letter are not significantly different according to DMRT (p<0.05).

Bacterial isolates TAT19, YAT12, 4RH8, YAT9, 4RH22 and YAT19 increased shoot fresh weight of rice seedling, in a significant way (p<0.05) by 93%, 100%, 110%, 105%, 184% and 130%, respectively when compared with the control (Figure 3a). Root fresh weights of rice seedling that were inoculated by bacterial isolates TAT2, TAT19 and TA2 were significantly increased root fresh weight (by 37%, 39%, and 33%, respectively) with the negative control (p<0.05) (Figure 3b). In this present study, bacterial isolates increased shoot fresh weight did not increase root fresh weight, except rhizobacteria TAT19 was able to increase both of them.

Rice seedling growth promotion in this study can be caused by PGP traits of bacteria. All of the bacterial isolates had properties for the direct and indirect mechanisms of plant growth promotion. However, our study showed that higher activity on PGP properties did not always match with the
excellent enhancement of plant growth. Plant growth promoting bacteria enhance plant growth by increasing nutrients availability, releasing phytohormone or protecting the plant from various pathogens [3,36].

![Figure 3](image-url). Effects of bacterial treatments on the shoot (a) and root (b) fresh weight of rice seedling. Bars with same letter are not significantly different according to DMRT (p<0.05).

Phosphate solubilization, nitrogen fixation and the production of IAA, ACC deaminase and ammonia are among the mechanisms exhibited by bacteria that improve plant growth [37-39]. The application of phosphate solubilizing bacteria was reported could increase phosphorus uptake by the plant and crop yield as well [40]. Nitrogen fixing bacteria could provide nitrogen for plants in available form by producing nitrogenase enzymes to convert dinitrogen to ammonia [41]. IAA producing bacteria were reported to increase root length and surface area of plants [3]. A different study reported that the effect of IAA or ACC deaminase producing bacteria on root elongation of canola seeds were exhibited under normal as well as saline conditions [38]. The enhancement of root and shoot growth and biomass production is also exhibited by ammonia producing bacteria through nitrogen accumulation [42].

Exopolysaccharide, catalase and hydrolytic enzyme production by rhizobacteria and bacterial endophytes are also important in promoting plant growth. Exopolysaccharides have an important role in protection from desiccation and surface attachment [25]. EPS-producing bacteria were reported to increase plant antioxidants in order to ameliorate the adverse effects of ROS. During colonization, catalase was important for endophytes to survive from oxidative burst [27].

Catalase, pectinase, and cellulase activities are essential for bacterial endophytes to survive and colonize into the seed endosphere [43]. Furthermore, hydrolytic enzymes also play a role to disrupt cell wall components of the phytopathogens and insect pests. Cellulase enzymes also reported to increase and stimulate seedling growth as a result of the transport of water, mineral and nutrient substances into the seed [44].

### 3.4. Effect of selected bacteria on Javanese turmeric growth

Bacterial isolates YAT19 and YAT12 significantly increased plant height of Javanese turmeric at 8 weeks after planting (WAP) but the same activity was not found at 12 WAP (Figure 4a). There are no bacterial isolates that increase the leaf number of Javanese turmeric at 8 and 12 WAP (Figure 4b).

Bacterial isolates TAT2 and YAT12 applied by seed treatment+soil drenching to Javanese turmeric showed the highest response to the plant height and were significantly different with seed treatment control at 8 WAP (Figure 5). In the response of leaf number, bacterial isolates TAT2 and YAT19 showed the highest response to the leaf number and were significantly different with seed treatment control at 8 WAP (Figure 6). There are no bacterial isolates and the application method significantly increased plant height and leaf number at 12 WAP.
The decrease of bacterial activity on 12 WAP can be caused by various factors. Inoculum density, inoculation method, root colonization, plant physiological state, pH, temperature, host plant, and root exudates are numerous factors that have been reported to affect the success of microbial inoculation [45]. Soil microbiome is very sensitive to abiotic stress including soil pH, temperature, aeration,
physico-chemical properties, moisture, and UV radiation. The tolerance of bacteria towards abiotic stress will be different for different types of bacteria [46].

4. Conclusions
Bacterial endophytes and rhizobacteria associated with Curcuma xanthorrhiza have plant growth-promoting traits including production of IAA, ammonia, ACC deaminase, catalase, exopolysaccharide, hydrolytic enzymes (cellulase, pectinase, protease and chitinase), phosphate solubilization, and nitrogen fixation. Selected rhizobacteria and endophytic bacteria isolates hold great promise as biostimulants to enhance rice plant growth. The application of selected bacteria on Javanese turmeric with seed treatment and seed treatment+soil drenching did not give a significant effect on the plant growth. The ability of bacteria in promoting plant growth decreased after 12 weeks after planting. Further studies should be considered to investigate the effect of formulated potential bacterial isolates with different application frequencies and environmental conditions on the harvest yield of rice and Javanese turmeric as well as active compounds of Javanese turmeric.

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