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Research article

Indole Acetic Acid-Producing and Phosphate-Solubilizing Bacteria From the Rhizosphere of Clove (Syzygium Aromaticum L.) in Bali, Indonesia

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Abstract.
Clove plants are routinely fertilized with synthetic fertilizer to increase yield. The use of synthetic fertilizer reduces soil productivity. Biofertilizer can be used as an alternative for increasing soil fertility. The goal of this study was to determine the potency of bacteria isolates capable of producing indole acetic acid (IAA) hormone and solubilizing phosphate, and to identify bacteria species from the rhizosphere of clove plants. Soil samples were collected from the clove plants' rhizosphere, environmental parameters were measured, the potency of IAA-producing and phosphate-solubilizing bacteria was analyzed, and bacteria were molecularly identified. After 48 hours of incubation, isolate TCKI 5 from Karangasem produced the highest IAA hormone levels (19.64 ppm), and isolate TCBP 6 from Buleleng had the highest index of solubilizing phosphate (1.91). A compatibility test between the three best isolates of IAA hormone-producing and phosphate-solubilizing bacteria revealed that TCKI 5 was able to associate with TCBP 6. Isolate TCKI 5 was identified as *Leclercia adecarboxylata* C107 with a 99.92% similarity, and isolate TCBP 6 as *Burkholderia cepacia* GJ8 with a 99.61% similarity.

Keywords: Bacteria, clove, Indole Acetic Acid, phosphate, rhizosphere

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1. Introduction

Cloves (*Syzygium aromaticum* L.) are one of the plantation commodities that has an important role in the Indonesian foreign exchange. Synthetic fertilizer was routinely used to increase the production of clove plants. Synthetic fertilizer has a negative impact on soil productivity. Soil is a biodynamic and integral component of the ecosystem [1]. Reduce the use of synthetic chemical fertilizer in agriculture can improve the biochemical characterization of the soil. Applications of organic matter with enriched microbial fertilizer can improve respiration and enzymatic activity of soil [2]. Microbes effectively

interact in the rhizosphere zone. The chemistry of the rhizosphere is much different than the surrounding soil as a result of root exudates. These exudates are foodstuff for bacteria. This area supports more bacteria than the surrounding soil [3].

Microbes in soil have many important roles, especially in the recycling of organic matter to the cycle of phosphorus and producing Indole Acetic Acid (IAA). IAA is an endogenous auxin hormone that plays an important role in root development, inhibits the growth of lateral shoots, stimulates abscission, and forms of xylem and phloem tissues [4]. Some strains of Plant Growth Promoting Rhizobacteria (PGPR) can synthesize IAA from the precursor in root exudates [5]. IAA and nitrogenase enzyme proved to increase the dry weight of corn [5]. Groups of microbes include *Pseudomonas* sp. and *Azotobacter* sp. produced plant hormones. Whereas phosphate is an essential nutrient that plays an important role in photosynthesis and root development. Solubilizing phosphate bacteria secreting some low molecular weight organic acids such as oxalate, succinate, fumarate, and malate. The organic acid will react with phosphate binders such as Al^{3+}, Fe^{3+}, Ca^{2+}, or Mg^{2+} to form a stable organic chelate so that it can be absorbed by plants [6]. Soils in the tropics region generally have high phosphate fixation capacity. It causes the availability of phosphate for plants very low [7]. The efficiency of phosphate fertilization ranges from 10%-25% [8]. Microbes play important roles in increasing the availability of phosphate. Previous studies indicated that the use of phosphate solubilizing microbes reduces the use of SP-36 fertilizer by 25% in the SRI method [9]. It also increases the efficiency of rock phosphate used in maize and wheat cultivation [10]. The use of phosphate solubilizing microbes is one method that can accelerate the weathering process of rock phosphate. Based on research *Citrobacter intermedia* and *Pseudomonas putida* as phosphate solubilizing bacteria can improve phosphate uptake by corn plants [11]. The purpose of this research was to study the potency of bacteria isolate to produce IAA hormone, solubilizing phosphate, and identify species of bacteria from the cloves rhizosphere.

2. Methodology

2.1. Sample Collection

Composite of soil samples were taken at rhizosphere of cloves in a different location in Buleleng (previous research [12]) and Karangasem district, Bali Province, Indonesia with 5 replications. Environmental parameters such as temperature, pH, humidity, light intensity, and organic matter of soil were measured. IAA hormone-producing bacteria

isolated in Tryptic soy agar (TSA) medium [13] and phosphate solubilizing isolated in Pikovskaya medium [14]. The soil samples were made serial dilution in physiological salt solution and its spread on agar media. The culture of bacteria was incubated at room temperature for 48 days and enumerated the colony number. Each colony of bacteria was purified according to the spread plate method. The variance of data was analyzed statistically.

2.2. The Potency Assay of IAA Hormone Producing Bacteria

Each bacteria isolate was screening to obtain candidates of IAA-producing bacteria. One loop of the pure culture of IAA producing bacteria was inoculated into 25 ml Tryptic soy broth (TSB) medium and incubated at 25^{o} C, 120 rpm for 72 h. Each bacteria culture at 0, 24, 48, and 72 hours with 0.6 optical density (10^{7} CFU/ml) was picked up 3 ml and centrifuged at 12.000 rpm for 15 minutes for measured the IAA concentration. The supernatant was adjusted at pH 2.8 then extracted used ethyl acetate. The sample evaporated used a pump temperature of 4 °C, and a water temperature of 35 °C. The sample was inserted into a rotary flask until dry, then reconstituted with 1.5 ml of absolute ethanol then injected into the HPLC. Each sample was analyzed with the standard curve. IAA hormone was quantified by integrating the area under the peak with sigma authentic IAA as standard [15].

2.3. The Potency Assay of Phosphate Solubilizing Bacteria

Each bacteria isolate was screening to obtain candidates of phosphate solubilizing bacteria used Pikovskaya medium. Bacterial isolates were inoculated into Pikovskaya medium. The sample was incubated at 28 °C for three days and a clear zone was observed around the colony [14]. The phosphate solubility index was measured.

2.4. Compatibility Test among the Bacteria Isolates

Each isolate of bacteria was a subculture in Nutrient broth medium and incubated at 25^{o} C, 120 rpm for 24 h. Each bacteria isolates with a similar cell density (0.6 optical density) was spread on the Nutrient agar medium in the Petri dish. The sterile paper disc was immersed into the liquid culture of each bacteria isolate then placed on the center of a surface of Petri dish plate that contained other bacteria isolates. The bacteria cultures

were incubated at room temperature for 5 days. Antagonism isolates will be shown with a clear zone [4].

2.5. Identification of Bacteria Isolates base on 16S rDNA Sequence

The bacteria culture with the highest potency of IAA hormone-producing was cultured into a TSB medium. Phosphate solubilizing bacteria were cultured into a Pikovskaya broth medium. It is incubated at 25^{o} C, 120 rpm for 48 hours. Chromosome DNA of bacteria was extracted used the GES method and DNA concentration was a measure used nanophotometer [16].

Bacteria DNA was amplified with a *Polymerase Chain Reaction* machine using universal primer 27F (5'GAGAGTTTGATCCTGGCTCAG3') and 1495R (5'CATCGGCTACCTTGTTACGA3') [17]. DNA sequences obtained from the sequencing results incorporated into DNA sequencer programs and Bioedit. The data of the 16S rDNA sequence of isolated and reference bacteria from the DNA sequence data bank (NCBI) were aligned. The phylogenetic tree was constructed with the Mega program based on the Neighbour-Joining algorithm and evolutionary distance matrix [18, 19, 20].

3. Result and Discussion

3.1. Environmental Parameters of Sampling Sites in Buleleng and Karangasem, Bali

The environmental parameters that were measured include moisture, pH, organic matter and temperature of the soil, altitude, and light intensity. Based on the result (Table 1) showed that soil moisture (31.94 %), soil organic matter (6.65 %), altitude (863 m), and light intensity (1527 Klux) were higher ($p < 0.05$) in the Buleleng sampling site were higher than Karangasem. Environment parameters of pH (5.025) and soil temperature (26.12 ^{o}C) in the Karangasem sampling site were higher ($p < 0.05$) than Buleleng. Soil from the Buleleng site contained higher organic matter that it was caused the higher capacity to absorb water (water holding capacity) than Karangasem. This encourages the growth of microbes to decomposed organic matter faster and leads to lower soil pH. The altitude of the Sampling site in Buleleng was higher than Karangasem, it's caused lower soil temperature, however light intensity in both places was similar.

The total number of IAA-producing bacteria (Figure 1) was higher in Buleleng sampling sites (6.4 x 10^{6} CFU / g) than in Karangasem sampling sites ($p < 0.05$). The total number

Table 1: Environment parameter.

Environmental Parameter	Sampling Sites	
	Karangasem	Buleleng [12]
Humidity (%)	21.57a	31.94b
pH	5.025b	4.845a
Organic matter of soil (%)	2.83a	6.65b
Elevation (m)	469.8a	863b
Temperature (^{o}C)	26.12b	21.55a
The intensity of light (Klux)	12.57a	15.27a
Total plate count phosphate solubilizing bacteria (CFU/g)	8.2 x 10^{4}b	3.3 x10^{3} a
Total plate count Indole Acetic Acid (IAA) producing bacteria (CFU/g)	6.4 x 10^{6}b	4.3 x10^{3}a

of phosphate solubilizing bacteria (Figure 1) was higher ($p < 0.05$) in Buleleng sampling sites (8.2 x 10^{4} CFU / g) than Karangasem sampling sites. This was predicted because environmental parameters in Buleleng support the growth of IAA producing bacteria and phosphate solubilizing bacteria, especially organic matter and water content of soil were higher in Buleleng site than Karangasem. It makes bacteria optimal for solubilizing phosphate and producing IAA.

3.2. The potency of IAA Producing Bacteria From Clove Plant Rhizosphere

Based on the results (Figure 2) showed that TCKI 5 isolate from Karangasem produce the highest IAA hormone 19.64 ppm ($p < 0.05$) obtained at 48 hours incubation time. IAA hormone is produced abundantly by the bacteria in the stationary growth phase. IAA production will increase when the bacteria be declining growth conditions, the limited availability of carbon, and an acidic pH environment. The condition occurs when bacteria enter the stationary growth phase [21].

This is opposite to the site sampling in Karangasem. The sampling site in Karangasem has an organic matter content (2.83 %) lower than Buleleng ($p < 0.05$) and pH value 5.025 that classified as acidic soil. Environment parameters are suspected as a factor stimulate isolates TCKI 5 produce IAA hormone for surviving. In this study, analysis of IAA hormone used of 200 ppm concentration of tryptophan as a precursor and plays an important role in the biosynthesis of IAA. The highest of IAA hormone produced by TCKI 5 isolate (19.64 ppm) was lower if compared with the result of de Bashan et al. [13] research's which produced IAA hormone 51.18 $\pm$ 0.81 ng·µL^{-1} (tryptophan used 200 µg·µL^{-1}).

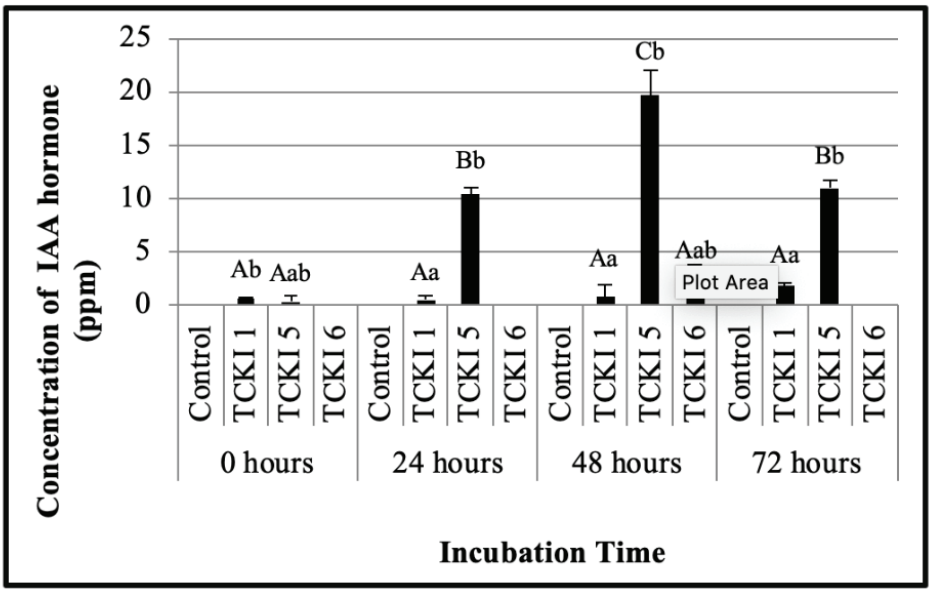

Figure 1: The potency of bacteria to produce IAA hormone.

3.3. The potency of Phosphate Solubilizing Bacteria

Bacteria isolate of TCBP from the Buleleng sampling site (Figure 2) has the highest (1.91) phosphate solubility index ($p < 0.05$) among the other isolates. Five potential isolates have high phosphate solubility index TCBP2 (1.69), TCKP3 (1.39), TCBP 11 (1.42), and TCBP 9 (1.38). The existence of phosphate solubilizing bacteria has influenced the content of organic matter in the environment [22]. This is under the sampling site in Buleleng was higher organic matter content than Karangasem site. The sampling site in Buleleng has a low temperature (21.55 °C) and pH (4,8) that the pH range is suitable for the growth of phosphate solubilizing bacteria (pH 4 to 10.6) [12].

Several previous studies reported that some microbes in the rhizosphere increase the phosphate solubilizing process so that their availability increases. Phosphate solubilizing microbes have the potential to increase phosphate solubilizing in the range of 1%-50% [23]. Several mechanisms are thought to increase phosphate solubilizing by microbes, including the production of organic acids and the production of phosphatase and phytase enzymes by microbes [23]. Diep and Hieu [24] reported that microbes present in the rhizosphere play an important role in the phosphate cycle in nature as well as in the phosphate solubilizing process. Phosphate solubilizing bacteria also could increase plant growth. This was indicated by a significant increase in plant height (45%), plant dry weight (40%) in the treatment of *Pseudomonas tolaasii* IEX [25].

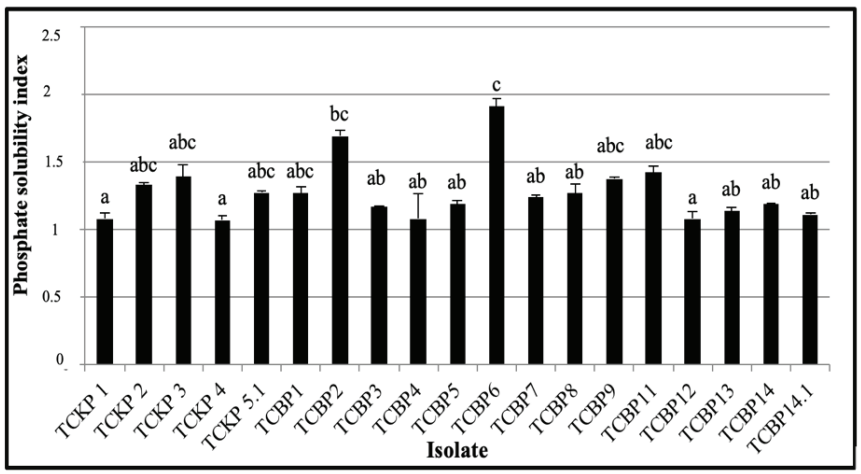

Figure 2: Phosphate solubilizing index clove plant rhizosphere bacteria.

3.4. Association among Bacteria Isolates

Compatibility test among the three best isolates of IAA hormone-producing and phosphate solubilizing bacteria (Table 2) showed that the isolates TCKI 5 which highest produced IAA hormone can associate with TCBP 6 which highest solubilized phosphate. Isolates that have a high potency to produce IAA hormone and solubilize phosphate and are capable of associating each other for the future can be used as a consortium formula as biofertilizer.

Table 2: Compatibility among IAA hormone-producing and phosphate solubilizing bacteria.

Isolate	TCKI 1 (A)	TCKI 5 (B)	TCKI 6 (C)	TCKP 3 (D)	TCBP 2 (E)	TCBP 6 (F)
TCKI 1 (A)		+	-	+	-	+
TCKI 5 (B)	+		-	+	+	+
TCKI 6 (C)	-	-		-	-	-
TCKP 3 (D)	+	+	-		-	-
TCBP 2 (E)	-	+	-	-		+
TCBP 6 (F)	+	+	-	-	+	

3.5. The species of IAA hormone-producing and phosphate solubilizing bacteria

Based on the phylogenetic tree (Figure 3) showed the TCKI 5 isolates have the highest 99.92 % of 16S rDNA sequence similarity with *Lecrecia adecarboxylata* C107. At first

Leclercia adecarboxylata was promoted to belong to the family of Enterobacteriacae formerly known as *Escherichia adecarboxylata*. *Adecarboxylata Leclercia* phenotypically different from all other species of Enterobacteriaceae [26]. Recent studies found that *Leclercia* sp. QAU-66 was first obtained from the rhizosphere of *Vigna Mungo*. Furthermore, it is known that these bacteria have a significant role in promoting the growth of *Phaseolus vulgaris*.

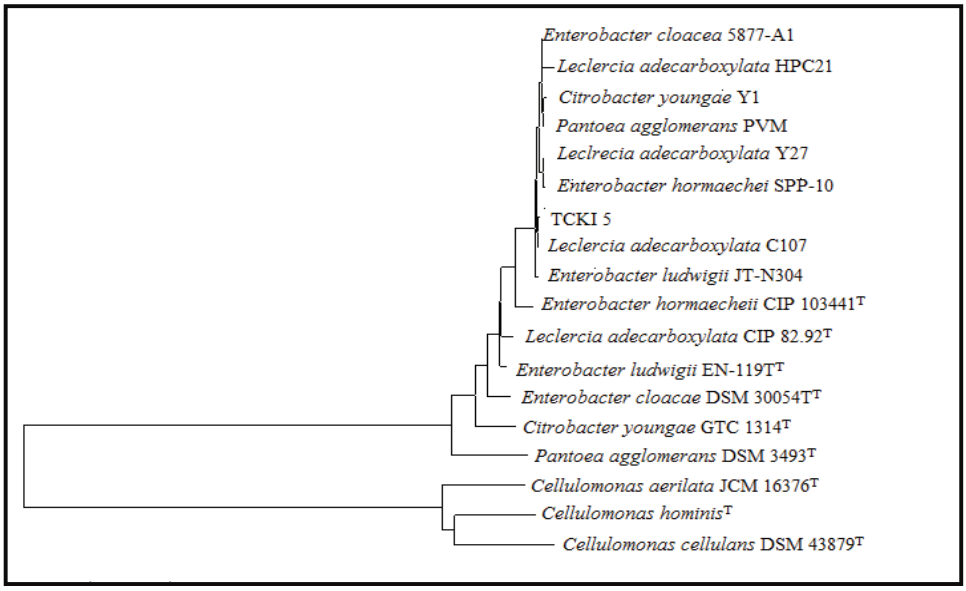

Figure 3: Phylogeny tree isolate TCKI 5 with reference isolate.

Based on the phylogeny tree (Figure 4) showed the TCBP 6 isolates have the highest 99.85 % of 16S rDNA sequence similarity with *Burkholderia cepacia* GJ8. Isolates TCBP 2 has a similarity of 99.61 % of 16S rDNA sequence with *Burkholderia stabilis* SPP-21. Naturally, the genus of *Burkholderia*, such as *Burkholderia cepacia* was found in the rhizosphere soil [5]. Based on previous studies found that the *Burkholderia cepacia* isolated from soil can solubilizing phosphate [27]

4. Conclusion

Isolate TCKI 5 from Karangasem has the highest 19,64 ppm producing IAA at 48 hours incubation and isolate TCBP 6 from Buleleng has the highest index solubilizing phosphate (1.91). Isolate TCKI 5 was identified as *Leclercia adecarboxylata* C107 with a similarity of 99.92 % and isolate TCBP 6 was identified as *Burkholderia cepacia* GJ8 with a similarity of 99.61 %.

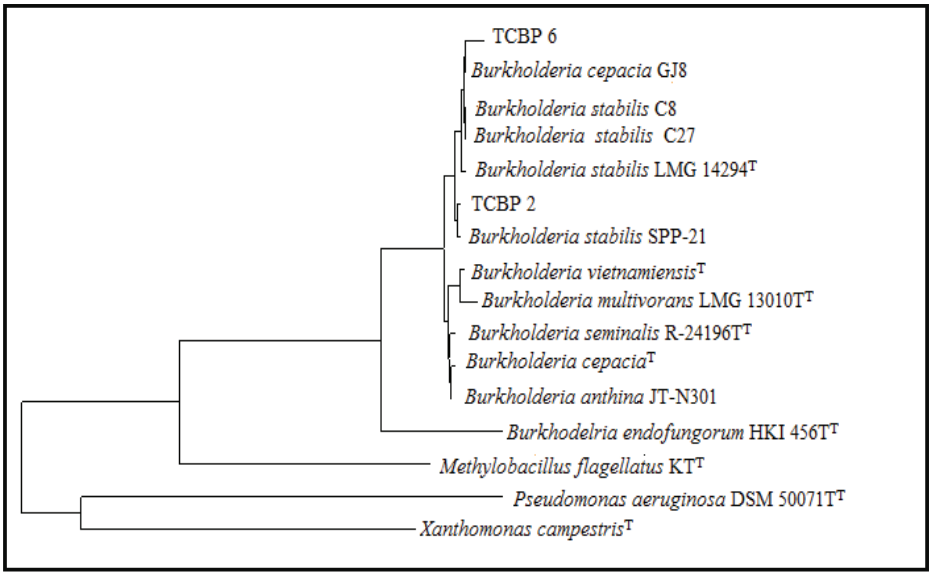

Figure 4: Phylogeny tree isolate TCBP 2 and TCBP 6 with reference isolate.

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