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Isolation and Characterization of Cambodian Tree Rhizosphere Bacteria (*Plumeria acuminata*) at TPU Pracimaloyo as a producer of IAA

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Abstract

Background: Public burial places (TPUs) where the decomposition of bodies actively produces soil nutrients and minerals that support the growth of surrounding living things, including bacteria and Cambodian trees. Cambodian trees are known to be resistant to biotic and abiotic stress, which may be influenced by the presence of rhizosphere bacteria as plant growth-promoting rhizobacteria (PGPR). This study aims to isolate and characterize Cambodian tree rhizosphere bacteria from burial soils that have the potential to produce IAA. **Methods:** Rizosphere soil samples were taken from TPU Pracimaloyo Surakarta, Central Java, at 5 points attached to the root surface of frangipani trees to be inoculated using the stocking dish method at dilutions of 10-5 and 10-6 in NA (nutrient agar) media. Rhizosphere bacteria as producers of IAA were carried out qualitatively and quantitatively at the age of culture 24 and 48 hours. Bacterial isolates of the rhizosphere yield potential IAA characterized macroscopically (colony morphology) and microscopic with Gram staining. **Results:** The bacterial population of the frangipani tree rhizosphere in all blocks showed no significant difference and was detected to have a population between 1.9 - 10.4 x 106. A qualitative test of the ability to produce IAA saw 34.88% of isolates made very high IAA. P37 isolates have the highest concentration of IAA. All potential isolates producing IAA are cocci-shaped gram-negative bacteria. **Conclusions:** The resulting IAA concentration was higher at culture age 48 hours compared to 24 hours, with the highest concentration by P37 isolate (83.098 ppm and increased to 113.588 ppm). P37 isolate is a Gram-negative bacterium in the form of cocci and irregular colonies.

Keywords: Bacteria; Cemetery; Frangipani tree; IAA; PGPR; Rhizobacteria.

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Introduction

A public burial place (TPU) is a place of burial of the dead regulated in the Government Regulation of the Republic of Indonesia Number 9 of 1987 concerning the Provision of land use for burial purposes (Lumombo, 2017). When a person dies, the decomposition process will begin through autolysis (Lewis, 2000), producing simple compounds that enrich soil nutrients and minerals (Laskow, 2018). The abundance of these nutritional sources greatly supports the growth of living things around them, including the group of bacteria (Jacoby et al., 2017). Soil bacteria in cemeteries were reported to be as much as 1.96 x 108 CFU/g (Yusnita, 2023, not yet published). This abundance proves the influence of the decomposition of human remains, which produce many nutrients and minerals needed for bacterial growth (Wallenstein, 2017).

Some of these bacterial groups interact with plants both outside the tissue (exofer) and inside the plant tissue (endofer) (Hayat et al., 2010). The most studied interaction of bacteria with the outside of plants is with roots, known as rhizosphere bacteria. Rhizosphere bacteria are soil bacteria that approach the seeds because they are attracted by root exudate (Santoyo et al., 2016; Gang et al., 2019) because it contains 5%-21% of photosynthetic carbon in the form of dissolved sugars, amino acids, or metabolites needed by the bacterial community in the rhizosphere (Bais et al., 2006).

Based on reference searches, no research has been found that discusses the rhizosphere bacteria of frangipani trees in burial grounds. This frangipani tree is synonymous with funerals and is known as a plant resistant to both biotic and abiotic stress. One of the factors affecting the resilience of frangipani trees is the presence of microorganisms associated with the roots of frangipani trees, known as the rhizosphere group of bacteria. Rhizosphere bacteria are widely reported to have a character as plant growth-promoting rhizobacteria (PGPR) (Jeyanthi & Kanimozhi, 2018). PGPR provides many benefits for the agricultural world in increasing crop yields, crop protection, and reducing the need for chemical fertilizers and pesticides (Vejan et al., 2016; Backer et al., 2018). Also, PGPR can improve soil texture, nutrient acquisition, and hormone secretion, resulting in better plant growth (Backer et al., 2018). Furthermore, PGPR helps plants tolerate abiotic stresses such as drought, salinity, and heavy metals (Hashem et al., 2019).

Plant resistance to biotic and abiotic stress is closely related to rhizosphere bacteria capable of producing IAA (Indole-3-Acetic Acid) due to interrelated regulation with the presence of the ACC deaminase enzyme. This enzyme regulates ethylene synthesis, which is responsible for the destruction and aging of plant tissues (Orozco-Mosqueda et al., 2020). ACC deaminase in plants will suppress ethylene levels to maximize plant growth-promoting bacteria's performance (Nascimento et al., 2016; Zarei et al., 2020). The ability to produce IAA is thought to affect all characters that support the potential of rhizosphere bacteria as PGPR because IAA secretion by bacteria will loosen plant cell walls, thereby increasing root exudate as attractants and providing additional nutrients to support the growth of rhizosphere bacteria. In addition, IAA will stimulate the formation of root hairs and lateral roots, thereby increasing the surface area and root length, providing greater access for plants to soil nutrients and water absorption. This ability can replace the function of ACC deaminase, siderophore-producing bacteria, and phosphate solvent bacteria (Etesami et al., 2015).

This study aims to isolate and characterize frangipani tree rhizosphere bacteria from burial soils that have the potential to produce IAA. The study results are expected to obtain bacterial isolates that can produce IAA, which can be applied in agriculture and plantations for plant breeding.

Method

This research was conducted at the research laboratory of the Biology Education Study Program FKIP Universitas Muhammadiyah Surakarta from November 2022 to February 2023.

Sampling

Rhizosphere soil samples were taken from TPU Pracimaloyo Surakarta, Central Java, at 5 points spread over five blocks, namely blocks 4, 6, 12, 13, and 14 (Figure 1.). The soil is taken in mud attached to the surface of the roots of frangipani trees as much as 100 grams, then put in a plastic bag and labeled (Ristiari et al., 2019).

Figure 1. Sampling Floor Plan

Bacterial Isolation and Purification

Rhizosphere soils are variously diluted to a dilution degree of 10-6. Inoculated soil samples were diluted 10-5 and 10-6 on NA (nutrient agar) media using the stocking method and incubated for 24 hours at a temperature of 28 ° C (Walida et al., 2019). After 48 hours, the colony was calculated with a colony counter device to obtain the bacterial population using the formula Devi et al. (2019):

$$N = \frac{\Sigma C}{[(1 \times n1) + (0{,}1 \times n2)] \times (d)}$$

Information:

N = Number of product colonies, expressed in colonies per ml
ΣC = Number of colonies on all counted cups
n1 = Number of cups at the first diluted calculated
n2 = Number of cups at the second retailer counted
d = First diluted calculated

Different colonies were inoculated onto inclined NA media for further storage and analysis.

IAA Yield Capability Test

The ability of frangipani root rhizosphere bacteria to produce IAA is carried out qualitatively and quantitatively. The IAA qualitative test begins with preparing NA media added with 1 mg/ml of L-tryptophan. Bacterial isolates were etched on NA media in a petri dish and incubated for 48 hours at room temperature. After the bacterial colony grew, the Salkowski reagent was dripped on the bacterial colony and reincubated for 30 minutes in a dark place. If the bacterial colony turns red, the isolate can produce IAA. The more concentrated the color produced, the higher the concentration of IAA produced. The results of IAA qualitative test observations are labeled with signs "-," "++," and "+++," which indicate the degree of color redness that appears. The IAA quantitative test begins with preparing NB (nutrient broth) media with added L-tryptophan 1mg/ml. Rhizosphere bacterial isolates were inoculated into NB medium and incubated with shaking for up to 48 hours, but IAA production measurements were made at culture ages of 24 and 48 hours. After 24 and 48 hours, a 1.2 mL liquid culture of bacteria was inserted into a micro-tube and centrifuged at 10,000 rpm for 10 minutes. 1 ml of supernatant is taken and put into a test tube, and 2 mL of Salkowski reagent is added. After incubation in a dark place for 30 minutes, IAA concentrations were measured with a spectrophotometer at a wavelength of 530 nm (Ariyani et al., 2021). The concentration of IAA is calculated by the value of the equation obtained from the IAA standard curve.

Morphological Characterization

Microscopic and macroscopic observations carry out morphological characterization. Microscopic observations were made after Gram staining to obtain data on Gram groups, cell shape, and cell arrangement (Hengkengbala et al., 2021). Macroscopic observations are carried out by observing the morphology of bacterial colonies consisting of the colony's shape, edges, elevation, and color.

Result

The bacterial population of the Cambodian tree rhizosphere from the Pracimaloyo public burial ground (TPU) was isolated and characterized for its ability to produce IAA.

Isolation of rhizosphere bacteria

The bacterial population of the rhizosphere of frangipani trees sampled from five blocks (Blocks 4, 6, 12, 13, and 14) at TPU Pracimaloyo is presented in Table 1. The population of frangipani tree rhizosphere bacteria in all sampling blocks showed no significant difference and was detected to have a population between 1.9 - 10.4 x 106. Sequentially, the bacterial population from low to high is block 4, block 13, block 14, block 6, and block 12, each as much as 1.9 x 106; 4.4 x 106; 5.4 x 106;7.4 x 106; and 1.04 x 107(Table 1.).

Table 1. Cambodian Tree Rizosphere Bacterial Population

Block	Average Population of Rhizosphere Bacteria (CFU/g)
4	$1,9 \times 10^6$
6	$7,4 \times 10^6$
12	$1,04 \times 10^7$
13	$4,4 \times 10^6$
14	$5,4 \times 10^6$

IAA yield capability test

The ability to produce IAA by isolates of rhizosphere bacteria obtained is carried out qualitatively and quantitatively, whose results can be seen in Table 2.

Table 2. Results of Qualitative and Quantitative Tests of the Ability to Produce IAA by Isolates of Frangipani Tree Rhizosphere Bacteria

Isolate Code	IAA Qualitative Test		IAA Concentration (ppm)	
	Qualitative IAA	Colony Color After Dripping Salkowski Reagent	24 Hours	48 Hours
P1	-	Unchanged	n. a.	n. a.
P2	++	Pink	6,43	11,87
P3	-	Unchanged	n. a.	n. a.
P4	-	Unchanged	n. a.	n. a.
P5	-	Unchanged	n. a.	n. a.
P6	-	Unchanged	n. a.	n. a.
P7	-	Unchanged	n. a.	n. a.
P8	-	Unchanged	n. a.	n. a.
P9	-	Unchanged	n. a.	n. a.
P10	-	Unchanged	n. a.	n. a.
P11	-	Unchanged	n. a.	n. a.
P12	-	Unchanged	n. a.	n. a.
P13	-	Unchanged	n. a.	n. a.
P14	-	Unchanged	n. a.	n. a.
P15	-	Unchanged	n. a.	n. a.
P16	-	Unchanged	n. a.	n. a.
P17	-	Unchanged	n. a.	n. a.
P18	-	Unchanged	n. a.	n. a.
P19	-	Unchanged	n. a.	n. a.
P20	-	Unchanged	n. a.	n. a.
P21	-	Unchanged	n. a.	n. a.
P22	-	Unchanged	n. a.	n. a.

P23	-	Unchanged	n. a.	n. a.
P24	+++	Purplish red	64,76	55,69
P25	-	Unchanged	n. a.	n. a.
P26	+++	Deep pink	9,81	70,10
P27	+++	Deep pink	24,81	20,64
P28	-	Unchanged	n. a.	n. a.
P29	++	Pink	1,23	0,54
P30	++	Pink	10,94	7,75
P31	+++	Purplish red	28,14	24,66
P32	++	Pink	0	9,22
P33	+++	Red	n. a.	n. a.
P34	-	Unchanged	n. a.	n. a.
P35	-	Unchanged	n. a.	n. a.
P36	++	red orange	12,21	35,50
P37	+++	Purplish red	83,09	113,58
P38	+++	Purplish red	65,50	77,95
P39	++	Pink	4,32	12,41
P40	++	Pink	6,92	18,49
P41	+++	Red	10,79	12,70
P42	-	Unchanged	n. a.	n. a.
P43	-	Unchanged	n. a.	n. a.

Information: + (Does not produce), ++ (Yield high IAA), +++ (Yields very high IAA), n.a (Not available)

From the qualitative test of the ability to produce IAA, 65.11% were detected with a score of - (not producing IAA), 16.28% with a score of ++ (resulting in high IAA) (Figure 2.), and 18.60% with a score of +++ (resulting in very high IAA) (Figure 2.).

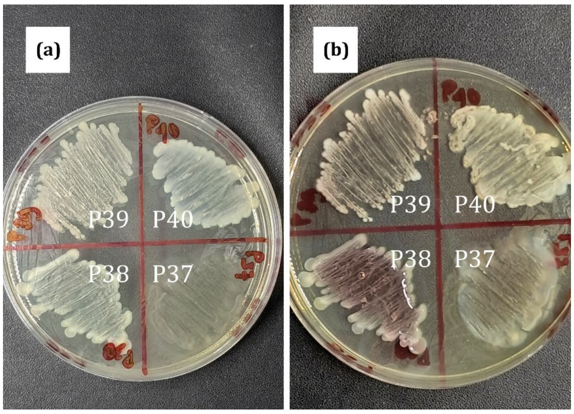

Figure 2. IAA qualitative Test Results. (a) before the Salkowski reagent is dripped, (b) after the Salkowski reagent drip (P37, P38, P39, and P40 isolates turn purplish red)

The IAA qualitative test has positive bacterial isolates, followed by quantitative tests that begin with making IAA standard curves, which can be seen in Figure 3.

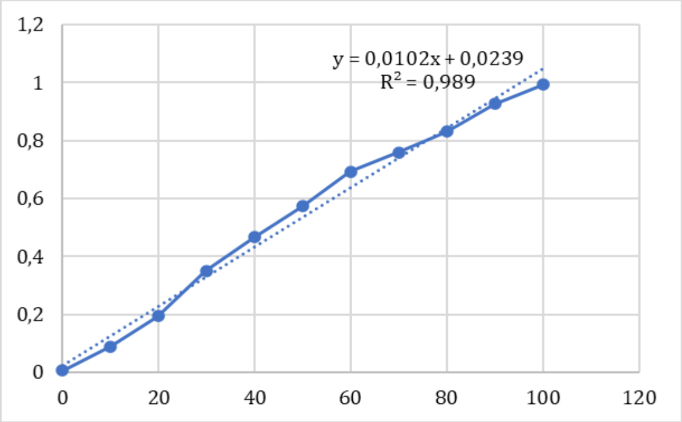

Figure 3. Standard curve of IAA solution

Standard curve measurements aim to obtain a regression equation that will be used in calculating IAA levels. Figure 3 shows that the regression equation obtained is $y = 0.0102x + 0.0239$ with a regression value of 0.989. The concentration of IAA produced by isolates of frangipani tree rhizosphere bacteria can be seen in Table 2. The highest concentration of IAA was produced by P37 isolates, followed by P38 and P24 at 24 and 48 hours isolates, respectively at 83.09/113.58 ppm, 65.50/77.95 ppm, and 64.76/55.69 ppm (Table 2). P26 isolates showed a significant increase in IAA concentrations at 48 hours of culture age, reaching 70.10 ppm from 9.81 ppm. The concentration of IAA produced at the culture age between 24 and 48 hours increased (Figure 4).

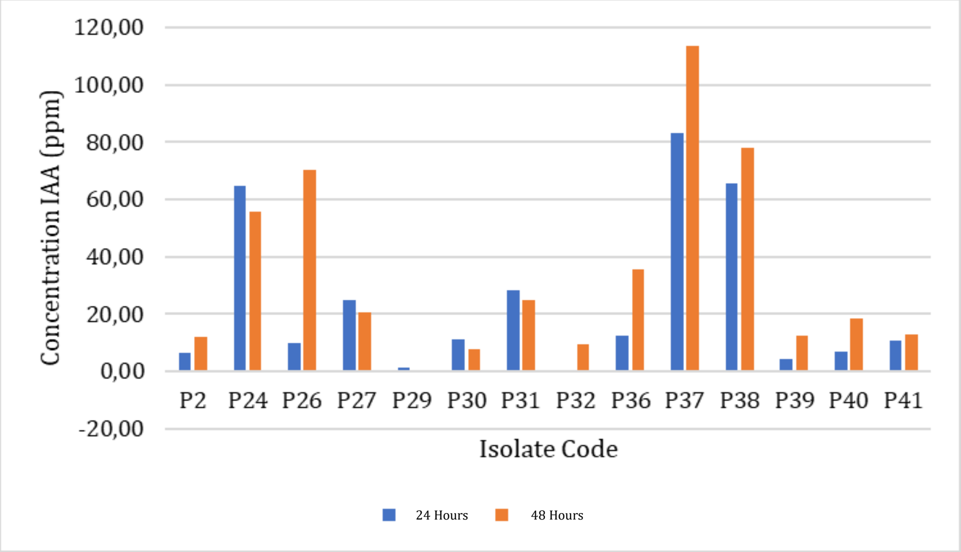

Figure 4. Histogram of IAA concentration of bacterial isolates of the rhizosphere of Cambodian trees culture age 24 and 48 hours

Identification of Isolates of Cambodian Tree Rhizosphere Bacteria that Have the Potential to Produce IAA

After obtaining potential isolates, microscopic and macroscopic observations were carried out, which can be seen in Table 3.

Table 3. Morphological Characterization of Colonies (Macroscopic) and Microscopic Bacterial Rhizosphere

Isolate	Colony Morphology (Macroscopic)				Microscopic		Gram
	Shape	Edge	Elevation	Color	Cell Shape	Order	
P2	Irregular	Undulate	Flat	White	Coccus	Dc	Negative
P24	Irregular	Lobate	Raised	Yellowish	Coccus	C	Negative
P26	Irregular	Lobate	Flat	White	Coccus	C	Negative
P27	Irregular	Lobate	Flat	White	Coccus	Sc	Negative
P29	Circular	Undulate	Umbonate	White	Coccus	C	Negative
P30	Punciform	Entire	Umbonate	Yellow	Coccus	C	Negative
P31	Circular	Entire	Convex	Yellow	Coccus	Dc	Negative
P32	Circular	Entire	Convex	Yellow	Coccus	C	Negative
P36	Irregular	Undulate	Flat	White	Coccus	Dc	Negative
P37	Irregular	Entire	Raised	White	Coccus	Sc	Negative
P38	Irregular	Lobate	Raised	Blueish	Coccus	Dc	Negative
P39	Circular	Entire	Convex	Red	Coccus	Dc	Negative
P40	Circular	Undulate	Umbonate	White	Coccus	C	Negative
P41	Circular	Entire	Convex	Orange	Coccus	Dc	Negative

Information: C (Coccus), Dc (Diplococcus), Sc (Streptococcus)

All isolates that have the potential to produce IAA are cocci-shaped gram-negative bacteria.

Discussion

The lowest Cambodian tree rhizosphere bacteria population at TPU Pracimaloyo was 1.9 x 106 cfu/ml in block four and the highest at 1.0 x 107 cfu/ml in block 12 (Table 1). Rhizosphere bacteria in Block 4 have the lowest population, thought to be due to the influence of abiotic factors in the form of soil water content (SWC), where the soil tends to be dry and hard. In Block 12, the roots of frangipani trees were the most compared to other Cambodian trees sampled, so the root exudate released was also the most abundant, and the population of rhizosphere bacteria was more. The population of rhizosphere bacteria from Cambodian trees in TPU Pracimaloyo is more than rhizosphere bacteria from rambutan trees (Nuraini et al., 2020), durian trees (Fitri, 2018), and oil palm trees (Lubis, 2021) were 4.75 x 104, 3.27 x 104, and 41 x 103 CFU/ml, respectively. These results corroborate the suspicion that burial soil affects the population density of Cambodian tree rhizosphere bacteria.

Auxin products in Indole-3 Acetic Acid (IAA) are L-tryptophan products metabolized by bacteria. These bacteria around the roots depend on tryptophan in the plant root exudate (Gang et al., 2019). The presence of IAA is indicated by changing the color of the colony to pink after being reacted with Salkowski reagents (Sudewi et al., 2020). In this study, there were seven positive isolates producing IAA with the label "+++" and seven positive isolates producing IAA with the title "++" (Table 2). IAA production is inseparable from the influence of several factors, including root exudate, tryptophan levels, temperature, pH, growth phase, cell density, and nutritional limitations (Kochar et al., 2013). This result is also supported by research by Suliasih & Widawati (2020), which explains that adding tryptophan as much as 250 µg / ml will produce the best IAA production. In addition, the best-recommended conditions for incubation are 96 hours at 35°C, pH 8, with sucrose as the carbon source and tripton as the nitrogen source.

The concentration of IAA produced by isolates of rhizosphere bacteria as a whole showed an increase in culture age of 48 hours compared to 24 hours (Figure 4). At 24 hours of culture age, the lowest IAA concentration was P32 isolate at 0 ppm, and the highest was 83.098 ppm by P37. At culture age 48 hours, the lowest concentration was 0.549 ppm by P29, and the highest was 113.588 ppm by P37. This measurement showed that 64.29% of isolates increased and 34.71% decreased. This dynamic difference in levels is influenced by various factors, including bacterial strains, root exudate, tryptophan levels, nutrient availability, pH, temperature, cell density, oxygen availability, and interactions between plants and microbes (Kochar et al., 2013). Fu et al. (2015) stated that the pH value would directly affect cell growth, affecting IAA production.

A total of 43 isolates were isolated from the rhizosphere of Cambodian trees, and 34.88% were positive for producing IAA hormones. Hutapea's (2023) research obtained 30 isolates from rubber plants' rhizosphere; only 16.66% were able to produce IAA. The levels of IAA produced ranged from 14,707-37,279 ppm with an incubation time of 72 hours. Another study reported that 54.55% and 36.26% of isolates from Kluthuk banana roots produced IAA from Nanggulan District, Kulon Progo Regency, and Sleman District, Sleman Regency, Yogyakarta with the highest IAA concentration of 73.50 ppm (Rahayu et al., 2021). In this study, the concentration of IAA produced by isolates of Cambodian tree rhizosphere bacteria reached 113,558 ppm (Table 2), so it can be stated that frangipani tree rhizosphere bacteria in burial grounds have the potential to produce very high IAA.

All isolates potentially producing IAA belong to the cocci-shaped Gram-negative group (Table 3). Most Gram-negative bacteria groups have motility structures such as flagella to move closer to roots (Xu &; Liu, 2014) and produce polysaccharides and fimbriae to attach to the root surface (Kandel et al., 2017). The structure of bacterial cells is likely to cause the dominance of rhizosphere bacteria, especially in Cambodian trees. Another study also found that rhizosphere bacteria from agricultural land were dominated by gram-negative. This has become very beneficial in increasing the availability of good nutrition on agricultural land (Cruz et al., 2021).

The results of macroscopic observations showed six circular-shaped isolates, seven irregular-shaped isolates, and one punctiform-shaped isolate. The color that often appears is white, but there are also other colors, such as yellow, orange, red, and blue. Isolates with the code P37 are potential isolates producing IAA with morphological characteristics of irregular shape, edge of entire, flat elevation, and white color. Microscopically the shape is coccus with a streptococcus arrangement.

Conclusions

Populasi bakteri rizosfer pohon kamboja di TPU Pracimaloyo berkisar antara 1,9-10,4 x 106 CFU/g dan 34,88% mempunyai kemampuan menghasilkan IAA. Konsentrasi IAA yang dihasilkan lebih tinggi pada umur kultur 48 jam dibanding 24 jam dengan konsentrasi tertinggi oleh isolat P37 (83,098 ppm dan meningkat menjadi 113,588 ppm). Isolat P37 merupakan bakteri Gram negatif berbentuk kokus dan koloni irregular.

Declaration statement

The authors reported no potential conflict of interest.

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