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RESEARCH ARTICLE

# Selection of plant growth-promoting endophytic bacteria isolated from *Reutealis trisperma* in various Indonesian agroecosystems

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## ABSTRACT

Endophytic microbes play a crucial role in plant growth and health. Specifically, endophytic bacteria can be isolated from *Reutealis trisperma* (Blanco) Airy Shaw, a non-edible oil-producing plant prevalent across various Indonesian agroecosystems. This study aimed to select potential endophytic bacteria from *R. trisperma* in various Indonesian agroecosystems as plant-growth-promoting (PGP) through in vitro assays. Plant tissues were collected, and endophytic bacteria were isolated from five different *R. trisperma* planting areas. The bacterial colonies underwent biosafety testing; the surviving isolates were then evaluated in vitro for their ability to promote rice seedling growth, along with phytohormone analysis. The potential bacterial isolates were sequenced using 16S rRNA sequencing. The results indicated that 234 isolates passed the biosafety tests; 11 isolates were selected such as *Serratia marcescens* (BTB3, BNB2, KTA1, KTA5), *Serratia* sp. (NNB30), *Klebsiella* sp. (NKB2, SNA11, NTB22, GKB20), *Citrobacter* sp. (GKB21), and *Lysinibacillus* sp. (NNB25). However, isolates NTB22, NKB2, NNB30, and GKB20 have the highest potential as PGP.

Key words: Bacterial isolates, biosafety test, phytohormone, *Reutealis trisperma*.

## INTRODUCTION

Endophytic microbes, recognized for their colonization within plant tissues without causing pathogenic symptoms, have become pivotal in research for enhancing soil quality and plant productivity. Endophytic bacteria exhibit direct plant growth-promoting (PGP) mechanisms and indirect biocontrol capabilities. Furthermore, these bacteria produce siderophores for Fe chelation and the synthesis of phytohormones, thereby enhancing plant health and vitality (Souza et al., 2015). Endophytic bacteria are often beneficial in biofertilizer formulations due to their ability to colonize and survive within plant tissues over long periods, which provides prolonged efficacy (Reinhold-Hurek and Hurek, 2011). The colonization ability of endophytic bacteria is influenced by their interactions with host plants, involving various microhabitats within plant tissues. As a result, each strain or species possesses distinct functionalities, specializations, adaptations, and competencies tailored to its specific plant-host environment (Compant et al., 2010).

The adaptability of endophytic bacteria and their host plants can be affected by various environmental factors, including biotic and abiotic stresses. These bacteria can enhance plant resistance to challenging conditions such as drought, high salinity, heavy metal exposure, and synthetic pesticide residues (Souza et al., 2015). Their ability to support plant growth in extreme environments, such as arid regions, deserts, and ex-mining lands, highlights their resilience. Therefore, isolating endophytic bacteria from less-explored plant species, such as *Reutalis trisperma* (Blanco) Airy Shaw (Philippine tung) in various agroecosystems, offers promising prospects for future research and technological advancements.

*Reutealis trisperma*, renowned for its non-edible oil used in biodiesel production, thrives in various agroecosystems, including sub-optimal lands. Since 2010, four superior varieties (KS 1, KS 2, Kermindo 1, and Kermindo 2) have been developed across various Indonesian agroecosystems (Pranowo et al., 2015). In Indonesia, despite being cultivated in limited areas, populations of *R. trisperma* can be found in various agroecosystems. It is a perennial plant with a robust root system that can thrive in sub-optimal soil. These include the dry climate regions of East Nusa Tenggara, ex-mining areas of East Kalimantan and Bangka, poorly maintained cemetery lands in the wet climate regions of West Java, and well-maintained lands in West Java.

Endophytic bacteria obtained from plants in suboptimal soils have the potential to survive in extreme environmental conditions. The diversity of endophytic bacteria is influenced by specific factors of the bacteria, the plant, the environment, and the culture methods used (Afzal et al., 2019). Research on endophytic bacteria across diverse ecosystems presents a promising opportunity to discover novel bioproducts and resources with significant biotechnological potential (Kandasamy and Kathirvel, 2023). The varied environmental conditions and plant management practices associated with *R. trisperma* offer substantial opportunities for obtaining endophytic bacteria as biofertilizer agents. The potential of endophytic bacteria from *R. trisperma* in Sukabumi, as a biocontrol agent against *Fusarium* sp., was assessed in vitro by Amaria et al. (2019). However, further exploration is needed at various *R. trisperma* planting locations to increase bacterial diversity.

Therefore, this study aimed to select endophytic bacteria isolated from *R. trisperma* in various Indonesian agroecosystems for their potential as plant growth promoters, as determined through in vitro assays. It is crucial to gradually characterize endophytic bacterial isolates based on biosafety and phytohormone content through the in vitro assay. This process is essential for obtaining PGP endophytic bacteria, which serve as a key component of biofertilizers that can enhance plant production and health, thereby supporting sustainable agricultural productivity improvement programs. In this regard, in vitro testing enables more precise control over experimental variables, thereby leading to enhanced accuracy and reproducibility of results. This condition provides more reliable data, allowing the researchers to draw meaningful conclusions (Bernd, 2023).

## MATERIALS AND METHODS

### Sampling locations

In this study, a purposive sampling procedure was employed across five *Reutealis trisperma* (Blanco) Airy Shaw planting areas, each representing agroecosystems: (1) Ngada (East Nusa Tenggara, Indonesia), a dry climate area at 600 m a.s.l. with an erratic rainfall pattern strongly influenced by the monsoon cycle, resulting in a concentrated rainy season and an extended dry season; (2) Bangka (Bangka Belitung, Indonesia), at 50 m a.s.l., characterized as ex-mining lands managed by private sector plantations; (3) Kutai Kartanegara (East Kalimantan, Indonesia), at 75 m a.s.l., characterized as ex-mining lands managed by private sector plantations; (4) Garut (West Java, Indonesia), at 600 m a.s.l., poorly maintained cemetery lands in the wet climate regions under smallholder plantation management; and (5) Sukabumi (West Java, Indonesia), at 450 m a.s.l., wet climate areas, serving as the Experimental Station of the Ministry of Agriculture of the Republic of Indonesia (Figure 1).

### Isolation of endophytic bacteria

Plant tissue samples from *R. trisperma* were collected from five distinct locations and isolated following the method described by Hallmann et al. (1997). The leaf, stem, and root samples were cut into smaller segments and subjected to surface sterilization. Afterward, 1.0 g each sample was finely ground using a mortar and pestle, and then suspended in sterile distilled water, creating a dilution series ranging from $10^{-2}$ to $10^{-5}$. From each dilution level, 0.1 mL was spread onto agar plates containing 20% tryptic soy agar (TSA), 20% nutrient agar (NA),

and 20% King's B medium. The bacterial colonies that emerged on these media were individually purified using 100% NA medium, preparing them for subsequent biosafety tests.

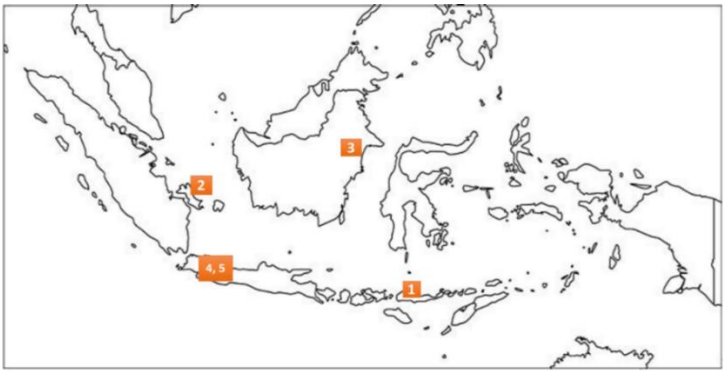


**Figure 1.** Five sampling locations. 1. Ngada, East Nusa Tenggara; 2. Bangka; 3. Kutai Kartanegara, East Kalimantan; 4. Garut, West Java; 5. Sukabumi, West Java.

### Biosafety assay

The haemolysis test involved inoculating a loopful of bacterial colonies onto a blood agar medium enriched with 5% sheep blood. A positive haemolysis reaction, indicated by a clear or dark zone surrounding the bacterial colony, suggests that the bacteria may be potential pathogens for humans and animals (Amaria et al., 2023). Meanwhile, bacterial isolates that did not exhibit such zones (negative haemolysis) were then subjected to hypersensitivity testing. Tobacco plants are used as indicator plants to categorize bacteria with potential as pathogens of hypersensitivity responses. Two millilitres of bacterial culture were injected into the lower surface of tobacco leaves using a disposable syringe, followed by an incubation period of 24-48 h. The appearance of necrotic symptoms on the tobacco leaves indicated a positive hypersensitivity reaction. Bacterial isolates that did not induce necrosis (negative reaction) were advanced for further testing.

### Biostimulation assay

This evaluation utilized 'Ciherang' rice (*Oryza sativa* L.) seeds, prepared according to the Ragdoll method specifications. A selection of 10 seeds, chosen based on their classification as submerged after water soaking, were immersed in the bacterial isolate suspensions for 24 h. Following immersion, the seeds were air-dried and then placed on moistened paper towels. This setup was then rolled and encased in plastic to maintain a consistent humidity level. The samples were incubated for 7 d at 19-21 °C. The effectiveness of endophytic bacterial isolates for rice seed germination was evaluated based on two main criteria in sequential stages. The first stage requires that seed viability reach 100%. In the second stage, the selected isolates that satisfy the first criterion must have significantly higher average shoot and root lengths than those of the control. Furthermore, after obtaining isolates that meet these two criteria, we conducted the statistical analysis to evaluate the selected isolates on the shoot and root growth, as well as measuring the phytohormone content of each isolate.

### The potential of endophytic bacteria as plant growth-promoting (PGP)

The production potential of phytohormones, including indole acetic acid (IAA), gibberellic acid ($GA_3$), and cytokinins (zeatin and kinetin), was assessed in selected bacterial isolates via rice seed germination tests. This analysis followed the method described by Linkens and Jackson (1987). For sample preparation, 10 mL 60% methanol was added to each sample in Falcon tubes, followed by centrifugation at 3000 rpm for 5 min. The resulting supernatant was purified using Whatman 42 filter paper and then analysed by high-performance liquid chromatography (HPLC) equipped with a UV-Vis detector (Shimadzu 20A, Shimadzu Corporation, Kyoto, Japan), using 10 µL each sample. To quantify each phytohormone by comparing the peak areas in the sample chromatograms to those in a standard chromatogram at specific retention times.

### Observation variables and statistical analysis

The study assessed several observed variables, including the levels of phytohormones (IAA, $GA_3$, zeatin, and kinetin) and parameters of rice seed germination such as seed viability, shoot length, and root length. The clustering of the phytohormone contents from the selected isolates was analysed hierarchically using Euclidean distance with Ward's method. Additionally, Pearson's correlation, simultaneous regression, and principal component analysis (PCA) were conducted on selected isolates to evaluate the relationships between phytohormone content and rice seed germination parameters. All data analyses were performed using the STAR version 2.0.1 (International Rice Research Institute (IRRI), Los Baños, Laguna, Philippines), SPSS version 21.0 (IBM, Armonk, New York, USA), and XLSTAT version 2019.2.2 (Lumivero, Denver, Colorado, USA).

### Morphological characterization and molecular identification

The potential of endophytic bacteria isolates was found using molecular techniques. The DNA extraction procedure was performed using the CTAB method. The DNA amplification by PCR using universal primers 27F and 1492R based on 16S rRNA. The gel was then viewed and recorded using a GelDoc Fire Reader V4 (Uvitec, Cambridge, UK) after being stained with RedSafeTM nucleic acid stain (iNtRON Biotechnology, Seongnam-si, South Korea). The PCR products (amplicons) were sequenced at the Saraswanti Genomics Institute, then aligned and edited using the BioEdit Sequence Alignment Editor (North Carolina State University, Raleigh, North Carolina, USA). The edited nucleotide sequences were compared with bacterial genomes from various countries using GenBank data from the National Center for Biotechnology Information (NCBI; Bethesda, Maryland, USA). Homology analysis of the nucleotide sequences was performed using the Basic Local Alignment Search Tool (BLAST; NCBI). Finally, a phylogenetic tree was constructed using the MEGA-X program (Institute for Genomics and Evolutionary Medicine (iGEM), Temple University, Philadelphia, Pennsylvania, USA), employing the Neighbor-Joining (NJ) method with 1000 bootstrap replications.

## RESULTS

### Biosafety selection

The result of the biosafety test shows that 80% negative haemolysis (γ haemolysis) (Figure 2A), while 20% exhibited positive haemolysis. Positive haemolysis was characterized by the clear-zone formation (β-haemolysis) around bacterial colonies on blood agar media (Figure 2B), indicating the secretion of β-toxin, which results in the complete lysis of red blood cells. In contrast, a dark green zone around the colony (α haemolysis) (Figure 2C) indicated the α-toxin production, causing partial lysis of red blood cell components. Meanwhile, a hypersensitivity reaction is characterized by necrotic symptoms following inoculation with bacterial isolates (Figure 2D). In contrast, isolates resulting in a negative response did not induce necrotic symptoms on the tobacco leaves (Figure 2E).

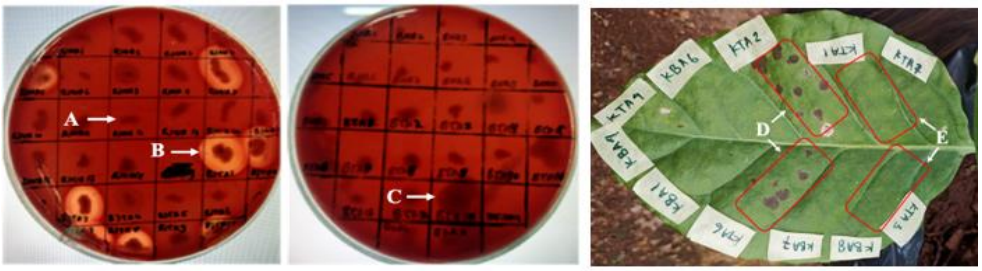


**Figure 2.** Haemolysis reaction of endophytic bacterial isolates on blood agar medium: Negative γ haemolysis (A); positive β haemolysis (B); positive α haemolysis (C). Hypersensitivity reaction to tobacco leaves: Positive, necrosis (D); negative, no symptoms (E).

Figure 3 illustrates that 421 endophytic bacterial isolates were successfully isolated and purified from leaf, stem, and root samples, including 148 isolates from Ngada, 82 from Bangka, 18 from Kutai Kartanegara, 114 from Garut, and 59 from Sukabumi. Of the 337 isolates tested for hypersensitivity, 234 isolates showed a negative hypersensitivity reaction. These 234 isolates were distributed as follows: 52.03% from Ngada, 81.71%

from Bangka, 50% from Kutai Kartanegara, 52.63% from Garut, and 35.59% from Sukabumi. Following the rejuvenation (re-culture) of the 234 endophytic bacterial isolates, 66 isolates showed slower colony growth, while the remaining 168 isolates demonstrated optimal colony growth. Consequently, only these 168 isolates (49 from Ngada, 55 from Bangka, 8 from Kutai Kartanegara, 53 from Garut, and 3 from Sukabumi) were tested for their potential to promote growth in the rice seed germination test.

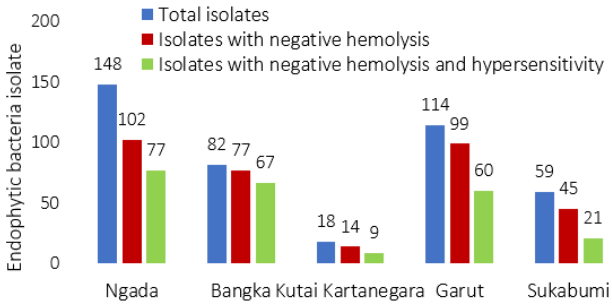


**Figure 3.** Biosafety selection (haemolysis and hypersensitive test) of endophytic bacteria isolated from *Reutealis trisperma* at five Indonesian agroecosystems: Ngada, Bangka, Kutai Kartanegara, Garut, and Sukabumi.

## Potential of endophytic bacteria in enhancing rice seed germination

Out of 168 endophytic bacterial isolates, only 11 (Ngada 4 isolates, Bangka 2 isolates, Kutai Kartanegara 2 isolates, Garut 2 isolates, and Sukabumi 1 isolate) demonstrated significant potential. The 11 isolates achieved 100% seed viability. After conducting the statistical analysis, they showed significantly improved shoot length compared to the control, although there was nonsignificant improvement in root length (Table 1).

**Table 1.** Rice seed germination tests of 11 selected endophytic bacterial isolates and a control. Numbers followed by the same letters in each column are not significantly different according to the LSD test at $\alpha = 0.05$.

| No. | Code of the isolates | Origin of the isolates | Rice seed germination parameters | | |
|---|---|---|---|---|---|
| | | | Viability[na] | Shoot length | Root length |
| | | | % | cm | cm |
| 1 | NKB2 | Ngada | 100 | $6.15 \pm 0.41^{abc}$ | $9.40 \pm 1.10^{a}$ |
| 2 | NNB25 | Ngada | 100 | $6.30 \pm 0.19^{ab}$ | $11.95 \pm 0.77^{a}$ |
| 3 | NNB30 | Ngada | 100 | $6.35 \pm 0.24^{ab}$ | $10.25 \pm 0.89^{a}$ |
| 4 | NTB22 | Ngada | 100 | $6.65 \pm 0.22^{a}$ | $10.80 \pm 0.88^{a}$ |
| 5 | BTB3 | Bangka | 100 | $4.92 \pm 0.13^{d}$ | $9.97 \pm 0.76^{a}$ |
| 6 | BNB2 | Bangka | 100 | $5.69 \pm 0.31^{bc}$ | $9.51 \pm 0.68^{a}$ |
| 7 | KTA1 | Kutai Kartanegara | 100 | $5.65 \pm 0.24^{bcd}$ | $9.80 \pm 0.74^{a}$ |
| 8 | KTA5 | Kutai Kartanegara | 100 | $5.90 \pm 0.16^{bc}$ | $10.10 \pm 1.14^{a}$ |
| 9 | GKB20 | Garut | 100 | $6.30 \pm 0.29^{ab}$ | $10.00 \pm 0.96^{a}$ |
| 10 | GKB21 | Garut | 100 | $5.65 \pm 0.26^{bcd}$ | $10.20 \pm 0.80^{a}$ |
| 11 | SNA11 | Sukabumi | 100 | $5.45 \pm 0.32^{cd}$ | $9.15 \pm 1.29^{a}$ |
| 12 | Control | - | 80 | $4.06 \pm 0.31^{e}$ | $7.50 \pm 0.97^{a}$ |

## Phytohormone content of 11 selected endophytic bacteria

In our study, we measured the production of phytohormones by 11 endophytic bacterial isolates from five different agroecosystems of *R. trisperma*. Further analysis showed that these 11 isolates contained varying amounts of IAA, $GA_3$, zeatin, and kinetin. The levels of various phytohormones fluctuate within specific ranges, with IAA content being the highest, followed by $GA_3$, zeatin, and kinetin, respectively (Figure 4).

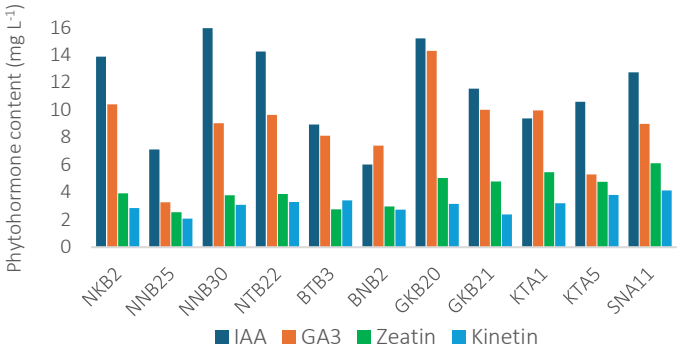


**Figure 4.** Phytohormone (IAA, $GA_3$, zeatin, and kinetin) content of 11 selected isolates. IAA: Indole acetic acid; $GA_3$: gibberellic acid.

## Clustering 11 selected isolates and their effects on rice seed germination

The cluster analysis identified three groups based on the levels of phytohormones, including IAA, $GA_3$, zeatin, and kinetin, in each isolate. The dendrogram indicated that the first cluster comprised four isolates (NTB22, NKB2, NNB30, and GKB20) derived from Ngada and Garut; the second cluster included three isolates (KTA1, GKB21, and SNA11) from Kutai Kartanegara, Garut, and Sukabumi; and the third cluster encompassed four isolates (KTA5, NNB25, BNB2, and BTB3) from Kutai Kartanegara, Ngada, and Bangka (Figure 5A). Generally, the first cluster was identified as the highest producer of phytohormones, especially IAA and $GA_3$, followed by the second and third clusters. For this reason, we label the first cluster as the high cluster (HC). The second cluster has a relatively higher content of zeatin and kinetin than the first and third clusters, and we refer to it as the medium cluster (MC). The third cluster has a lower content of the four phytohormones, which is why we refer to it as the low cluster (LC) (Figure 5B). In this regard, the coordinates of the HC isolates are closer to IAA and $GA_3$ coordinates, the MC isolates are closer to zeatin and kinetin coordinates, and the LC isolates are farther from the four phytohormone coordinates. Furthermore, the HC is closer to the shoot length variable than the MC and LC, while all clusters are approximately equidistant from the root length variable (Figure 6A). It indicates that the isolates in the HC produce longer shoot lengths of rice seedlings than those in the MC and LC, while the root lengths show nonsignificant difference between clusters (Figure 6B). These findings can also be confirmed by the partial correlation value presented in Table 2. The increased shoot length in HC is attributed to higher levels of IAA and $GA_3$ compared to MC and LC (Figure 5B), with their correlation values of 0.32 and 0.24, respectively. Other correlations indicated a strong relationship between IAA and $GA_3$ ($r$ = 0.97) and a perfect correlation between zeatin and kinetin ($r$ = 1.00) (Table 2). Consequently, to avoid multicollinearity effects, two variables were removed from the simultaneous regression model. Finally, we presented four valid simultaneous regression models, each with two independent variables. The IAA and $GA_3$ positively affect the shoot length of rice seedlings; on the contrary, zeatin and kinetin (Table 3).

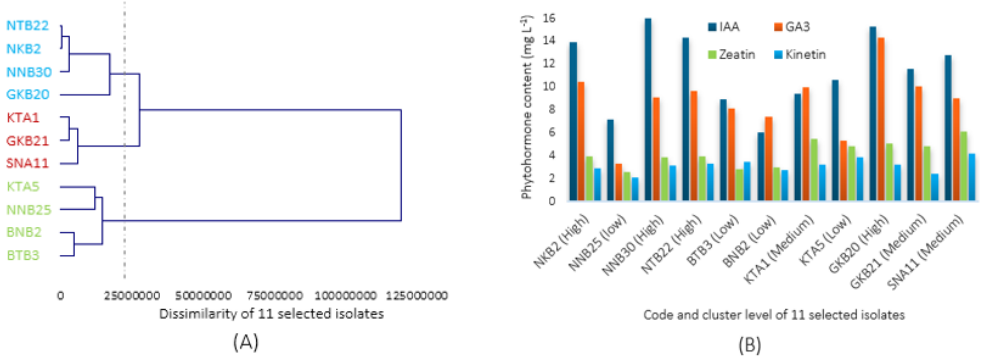


**Figure 5.** Dendrogram (A) and clustering of 11 selected isolates (B) based on four phytohormones (IAA, $GA_3$, zeatin, and kinetin) content. IAA: Indole acetic acid; $GA_3$: gibberellic acid.

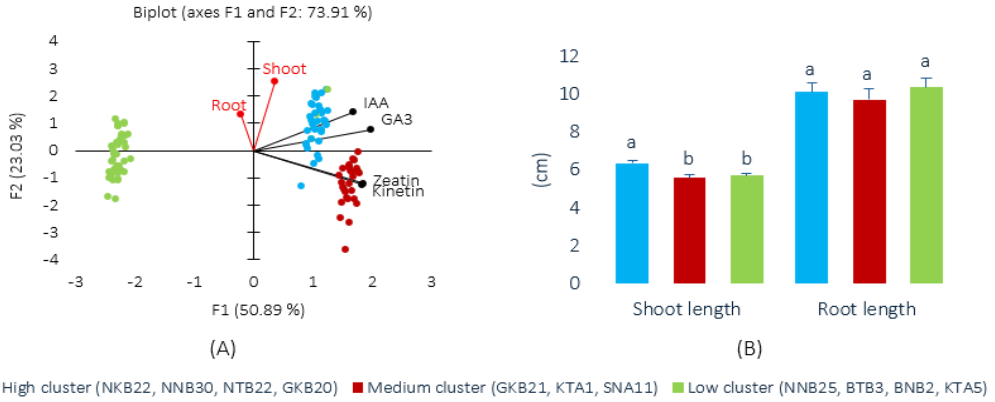


**Figure 6.** Biplot between three clusters formed and variable observed based on PCA (A) and comparison of shoot and root length between the three clusters based on Tukey's test at α = 0.05 (B). IAA: Indole acetic acid; $GA_3$: gibberellic acid.

**Table 2.** Partial correlation between phytohormone content and the shoot and root length. IAA: Indole acetic acid; $GA_3$: gibberellic acid. $^{*}p < 0.05$; $^{**}p < 0.01$.

| Variables | IAA | $GA_3$ | Zeatin | Kinetin | Shoot length | Root length |
|---|---|---|---|---|---|---|
| IAA | - | 0.97** | 0.49** | 0.38** | 0.32** | -0.04 |
| $GA_3$ | | - | 0.65** | 0.64** | 0.24* | -0.06 |
| Zeatin | | | - | 1.00** | -0.06 | -0.09 |
| Kinetin | | | | - | -0.07 | -0.09 |
| Shoot length | | | | | - | 0.15 |
| Root length | | | | | | - |

**Table 3.** Four valid simultaneous regression models for predicting shoot length variables. IAA: Indole acetic acid; $GA_3$: gibberellic acid. $^{*}p < 0.05$; $^{**}p < 0.01$.

| Regression model | | Unstandardized coefficients | | Standardized coefficients | | |
|---|---|---|---|---|---|---|
| | | B | Std. error | Beta | t | Sig. |
| 1** | (Constant) | 11.514 | 2.989 | - | 3.853 | 0.000 |
| | IAA | 0.129 | 0.031 | 0.399 | 4.117 | 0.000** |
| | Kinetin | -2.283 | 1.002 | -0.221 | -2.279 | 0.025* |
| 2** | (Constant) | 5.411 | 0.448 | - | 12.072 | 0.000 |
| | IAA | 0.131 | 0.032 | 0.404 | 4.131 | 0.000** |
| | Zeatin | -0.237 | 0.104 | -0.223 | -2.279 | 0.025* |
| 3** | (Constant) | 16.067 | 3.463 | - | 4.640 | 0.000 |
| | $GA_3$ | 0.207 | 0.050 | 0.479 | 4.117 | 0.000** |
| | Kinetin | -3.883 | 1.202 | -0.374 | -3.213 | 0.002** |
| 4** | (Constant) | 5.749 | 0.421 | - | 13.666 | 0.000 |
| | $GA_3$ | 0.211 | 0.051 | 0.468 | 4.131 | 0.000** |
| | Zeatin | -0.404 | 0.126 | -0.380 | -3.213 | 0.002** |

## Morphological characterization and molecular identification

The DNA amplification through PCR using universal primers 27F and 1492R, which target the 16S region, successfully produced amplicons of approximately 1200 bp for all 11 potential endophytic bacterial isolates. According to BLAST analysis, the 11 bacterial sequences show an identity percentage ranging from 96.19% to 100% and a query cover of 100% when compared with the sequence data of related bacterial species from GenBank.

According to the phylogenetic tree analysis (Figure 7), the 11 potential endophytic bacterial isolates clustered into four groups representing the genera *Klebsiella*, *Serratia*, *Citrobacter*, and *Lysinibacillus*, alongside the

outgroup species *Alicyclobacillus acidocaldarius*. The *Klebsiella* cluster, isolates SNA11, NKB2, GKB20, and NTB22, were closely related to *K. oxytoca*, *K. pasteurii*, and *K. michiganensis*. Therefore, all four isolates were known as *Klebsiella* sp. The *Serratia* genus cluster showed that the NNB30 was identified as *Serratia* sp., because it is closely related to *S. nematodiphila* and *S. marcescens*. Isolate BNB2, KTA1, BTB3, and KTA5 formed a group and were most closely related and identified as *S. marcescens*. In the *Citrobacter* cluster, isolate GKB21 was closely associated with two species, namely *C. murliniae* and *C. freundii,* and was therefore identified as *Citrobacter* sp. Other clusters determined that isolate NNB25 was related to *Lysinibacillus macrolides*, *L. xylanilyticus*, and *L. sphaericus,* and NNB25 was *Lysinibacillus* sp. The sequences of 11 endophytic bacteria from the *R. trisperma* have been registered and deposited in the GenBank database (NCBI https://www.ncbi.nlm.nih.gov) with accession numbers LC887956, *Klebsiella* sp. SNA11, LC887953 *Klebsiella* sp. NKB2, LC887949 *Klebsiella* sp. GKB20, LC887957 *Klebsiella* sp. NTB22, LC887958 *Serratia* sp. NNB30, LC887955 *S. marcescens* BNB2, LC887952 *S. marcescens* KTA1, LC887954 *S. marcescens* BTB3, LC887951 *S. marcescens* KTA5, LC887950 *Citrobacter* sp. GKB21, and LC887948 *Lycinibacillus* sp. NNB25 (Figure 7).

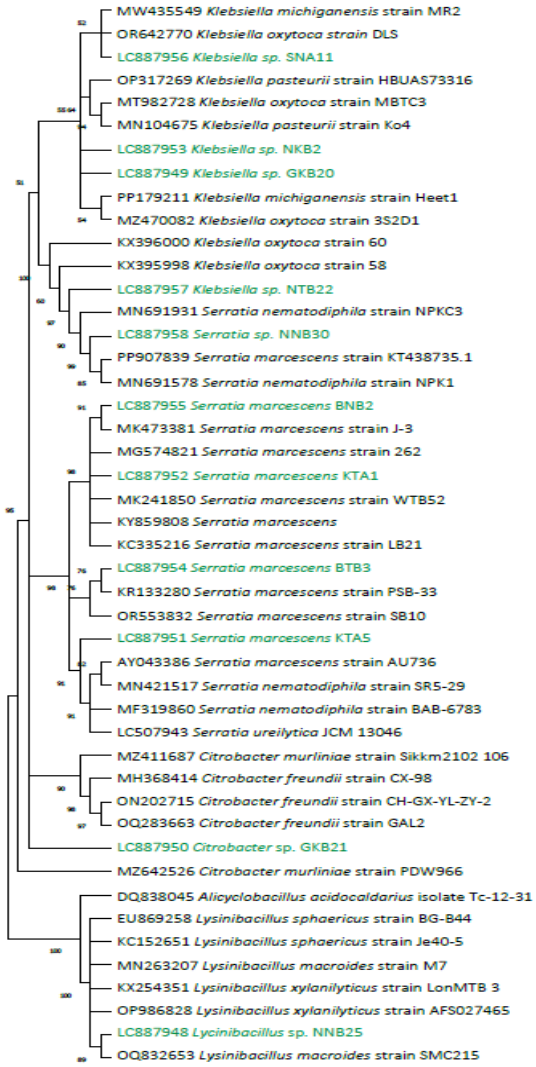


**Figure 7.** Construction of a phylogenetic tree of 11 selected isolates based on 16S rRNA sequences using the Neighbor-Joining (NJ) method with bootstrapping 1000 times. The green typed indicates the 11 selected isolates.

# DISCUSSION

Based on biosafety tests (haemolysis and hypersensitivity tests), our study successfully selected 234 bacterial isolates (55.58%) from a total of 421 isolates obtained from *Reutealis trisperma* plantation across five Indonesian agroecosystems. A previous study using this method, Amaria et al. (2023) successfully tested haemolysis and hypersensitivity on 95 bacterial isolates from *R. trisperma*, rubber, cocoa, patchouli, coffee, black pepper, and weed. These findings demonstrate the importance of biosafety testing of bacterial isolates before they are further characterized or tested for their potential as plant-growth-promoting (PGP) for biofertilizer use. Keswani et al. (2020) emphasized the growing use of PGP bacteria as biofertilizers, yet cautioned about the risks posed by opportunistic pathogens/allergens to human and animal health. In this context, Keswani et al. (2019) identified 16 PGP bacteria strains with potential negative impacts on human and animal health.

The results of germination tests showed that only 11 significantly improved the germination process of rice seeds. These 11 isolates achieved 100% seed viability and enhanced the shoot growth compared to the controls. The findings demonstrated that soaking rice seeds in cultures of these endophytic bacterial isolates notably improved seed viability and increased shoot length during germination. These results are consistent with other studies that reported similar effects, showing that soaking rice seeds in endophytic bacterial suspensions leads to promoted plumula growth and enhanced lateral root formation (Sudewi et al., 2021). Other studies have also indicated that the shoot of rice seeds treated with endophytic bacteria was significantly higher than that of the control (Saengsanga, 2018).

Our cluster analysis demonstrated that the grouping of isolates into three clusters did not reflect the type of agroecosystem from which isolates were obtained, indicating that the agroecosystem is not the sole factor influencing the phytohormone content in the isolate, which is consistent with Afzal et al. (2019). For example, the high cluster (HC) consists of isolates obtained from two niche agroecosystems, Ngada and Garut. As well as with the isolates in medium cluster (MC) and low cluster (LC). Another essential aspect to consider is individual selection, which can better reveal the interactions among microbes, soil, and plants, as these interactions are directly related to the level of bacterial colonization. Hence, it is essential to accurately assess the soil's physical, chemical, and biological properties when surveying endophytic bacteria. It is also important to reveal the growth and health of the plants. These elements are interconnected to form complex and niche microbial-soil-plant interactions that will affect the level of bacterial colonization. The more diverse the biophysical environment and plant characteristics are in the survey of endophytic bacteria, the greater the likelihood of finding the expected bacteria with a high level of colonization. Conversely, limited information about these factors will lead to undesired results and unnecessary wastage of resources, energy, and time. Compant et al. (2010) stated that some failures of endophytic bacteria during field experiments were due to limited knowledge about the bacterial colonization process. Future studies proposed the utilization of metagenomics and other cultivation-independent techniques to identify and acquire the targeted bacteria.

Further analysis showed that isolates in HC increased the shoot length of rice seedlings compared to isolates in MC and LC. This increase resulted from the high levels of indole acetic acid (IAA) and gibberellic acid ($GA_3$) in HC, as both hormones showed a positive correlation with shoot length. Isolates in HC included NTB22, NKB2, and NNB30 from Ngada and GKB20 from Garut, which have relatively higher IAA and $GA_3$ content. Therefore, in our study, these four isolates have the highest potential as PGP. In the plant-bacteria interaction process, IAA and ethylene are more important plant hormones than other hormones, such as abscisic acid, cytokinins, and gibberellins (Afzal et al., 2019). A previous study demonstrated that 35 isolates of endophytic bacteria producing IAA improved both the number and length of shoots in rice seedlings (Duangpaeng et al., 2012). Likewise, eight isolates producing IAA improved the viability and growth of rice seedlings (Gholamalizadeh et al., 2017). Additionally, the endophytic bacteria producing IAA can increase both vegetative and generative growth of various rice cultivars (Krishnamoorthy et al., 2020). The other crops also demonstrated that IAA could enhance shoot growth of sorghum (Kochar et al., 2011), soybean (Khan et al., 2014), and tomato plants (Khan et al., 2016). On the other hand, $GA_3$ through brassinosteroids could increase the percentage of germination and growth of rice seeds by enhancing the metabolism of stored proteins (Xiong et al., 2021). Another study has demonstrated that $GA_3$ promotes the germination of rice seeds and plays an antagonistic role to abscisic

acid (ABA) (Gong et al., 2022). Additionally, during the generative phase, $GA_3$ signals can enhance spikelet fertility (Kwon and Paek, 2016).

Our regression analysis revealed that the IAA and $GA_3$, as well as zeatin and kinetin, could not be included in the simultaneous model due to the multicollinearity effects. Finally, only four regression models are valid for predicting the shoot length of rice seedlings: (i) IAA with zeatin, (ii) IAA with kinetin, (iii) $GA_3$ with zeatin, and (iv) $GA_3$ with kinetin, and both variables in each model have an opposite coefficient (Table 3). It suggests that IAA is antagonistic to both zeatin and kinetin, as is $GA_3$. Furthermore, we observed the potential for substitution between IAA and $GA_3$, as well as zeatin and kinetin. Zeatin and kinetin are both types of growth hormones known as cytokinins. A previous study has demonstrated antagonistic effects between IAA and cytokinins, as well as between $GA_3$ and cytokinins. Understanding the interaction between hormones and plant performance is complex and also involves multiple factors. The plant hormone IAA does not function alone but interacts with numerous other hormones, and this interaction is complex and diverse (Mazzoni-Putman et al., 2021). The interaction between auxin and cytokinins can either be antagonistic or synergistic, depending on the specific context and the relative levels of each hormone (Jones and Ljung, 2011). Meanwhile, $GA_3$ and cytokinins interact antagonistically in various growth processes (Jasinski et al., 2005), like in different developmental and molecular processes during tomato plant growth (Fleishon et al., 2011).

Future study is needed to better understand the complex interactions between hormones that influence plant growth, particularly through in vivo and in planta studies. Our testing focuses on endophytic bacteria based on our study results, combined with external sources of $GA_3$ and cytokinins (zeatin and kinetin). It is crucial to uncover more details about the interaction between hormones. Additionally, studying the use of mycorrhiza or other soil fungi will provide more information about this interaction process. Glick (2012) suggested that for the successful commercialization of PGP bacteria, it is crucial to ensure their effectiveness across different environments (e.g., greenhouse *vs*. field) and to understand their interactions with mycorrhiza and other soil fungi. Keswani et al. (2019) noted that mycorrhiza enhances the growth of over 90% of all plants, making it essential to study the interactions between mycorrhiza and PGP bacteria. On the other hand, studies by Chompa et al. (2024) indicated that using PGP bacteria isolates in a consortium yields better rice growth compared to single applications under salt stress conditions.

Subsequent analysis revealed that the 11 selected isolates belonged to the genera *Serratia*, *Klebsiella*, *Citrobacter*, and *Lysinibacillus*. A previous study has shown that endophytic bacteria from these genera are beneficial as PGP in several medicinal, food, and horticultural plants. The endophytic bacterium *S. marcescens* has the potential to act as a PGP in *Achyranthes aspera* due to its ability to produce IAA, solubilize phosphate, fix N, and act as a siderophore (Devi et al., 2016). Asaf et al. (2017) reported that *S. marcescens* produces phytohormones that positively enhance physiological characteristics such as shoot and root length, fresh and dry weight, and chlorophyll content in soybean plants. The endophytic bacteria *Klebsiella* spp. demonstrated PGP capabilities in *Vigna unguiculata*, supported by, among other things, phosphate solubilization activity, ammonia production, N fixation, and high IAA production (Biswas et al., 2023). *Citrobacter farmeri* isolated from *Thymus vulgaris* roots also exhibits IAA, $GA_3$, ABA, benzyl, quintene, and ziaten activity, which can stimulate plant growth (Jamal and Ahmad, 2022). Several species of *Lysinibacillus* possess not only antimicrobial potential but also PGP activity. This activity arises from their ability to uptake nutrients, produce phytohormones that enhance plant chlorophyll content, and increase the activity of antioxidant enzymes (Abdel-Hamid et al., 2021).

## CONCLUSIONS

This study assessed 421 endophytic bacteria isolated from *Reutealis trisperma* in five Indonesian agroecosystems. We revealed that 11 isolates demonstrated potential as plant-growth-promoting (PGP), such as *Klebsiella* sp. (SNA11, NKB2, GKB20, NTB22), *Serratia* sp. (NNB30), *S. marcescens* (BNB2, KTA1, BTB3, KTA5), *Citrobacter* sp. (GKB21), and *Lysinibacillus* sp. (NNB25). However, NTB22, NKB2, NNB30, and GKB20 have the highest potential as PGP, due to the highest producers of indole acetic acid (IAA) and gibberellic acid ($GA_3$), and produced the highest shoot length in rice seedlings. The IAA and $GA_3$ are antagonistic to zeatin and kinetin. The IAA and $GA_3$ can substitute for each other, as can zeatin and kinetin. Future studies will focus on testing the endophytic bacteria from this result, combined with external sources of phytohormones. We will also explore the effects of mycorrhizae and other soil fungi that enhance the soil's physical, chemical, and biological properties.

## Author contribution

Conceptualization: N.K.F., W.A., K.D.S., E.W., D.P., M.H., M.F.O. Methodology: N.K.F., W.A., K.D.S., E.W., M.F.O. Validation: M.H., G.G., W.W. Formal analysis: E.W., D.P., M.M. Investigation: N.K.F., W.A., K.D.S., M.M., M.F.O. Data Curation: N.K.F., W.A., K.D.S. Writing-original draft: N.K.F., W.A., K.D.S., E.W. Writing-review & editing: W.A., K.D.S., A.A., E.W., A.P.P. Visualization: M.S., G.G., W.W. Supervision: D.P., M.H. Project administration: A.A. Funding acquisition: K.D.S. All co-authors reviewed the final version and approved the manuscript before submission.


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