THE EFFECT OF BACTERIAL ISOLATES FROM THE RHIZOSPHERE OF IRON SAND PLANTS ON THE GROWTH OF CHILLI SEEDLINGS (*Capsicum annuum* L.) USING THE AXENIC SAND CULTURE ASSAY METHOD

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Abstract. Iron sand soil has low organic matter and high iron (38–59%), making it less fertile for plant growth. Chili plants (*Capsicum annuum* L.) are tolerant to harsh environments, and the use of Plant Growth Promoting Rhizobacteria (PGPR) can enhance their growth on such marginal land by improving nutrient absorption. This study aimed to test the potential of bacterial isolates PT4C5, KT6A5, and CT4A7 as PGPR, evaluate their ability to increase chili seed germination and plant growth using an axenic sand culture assay, and identify their phenotypic characteristics. The research consisted of survey and experimental methods. The experiment used a Completely Randomized Design (CRD) with five treatments: control (sterile distilled water), isolates PT4C5, KT6A5, CT4A7, consortium, and EM4, each with three replications. Results showed that isolates PT4C5, KT6A5, and CT4A7 exhibited PGPR traits by fixing nitrogen, solubilizing phosphate and potassium, producing indole-3-acetic acid (IAA), and siderophores. The isolates, especially the consortium, significantly increased chili seed germination percentage and improved vegetative growth, including plant height, root length, wet weight, number of leaves, and chlorophyll content. Based on phenotypic characterization, isolates PT4C5, KT6A5, and CT4A7 were identified as belonging to the genus *Planococcus*.

Keywords: *Axenic sand culture assay, Capsicum annuum L., PGPR*

1. Introduction

Plant Growth Promoting Rhizobacteria (PGPR) play an important role in agriculture because they have abilities such as fixing nitrogen from the air, dissolving phosphate, producing siderophores, dissolving potassium, producing the growth hormone IAA, stimulating plant growth, and assisting in the absorption of plant nutrients (Istiqomah *et al*., 2018). These PGPR bacteria are able to provide and assist in the absorption of various nutrients in the soil, synthesize, and change the concentration of growth-promoting phytohormones (Asfar *et al*., 2022). PGPR groups can be obtained from various ecosystems such as soil and iron sand.

Iron sand soil is a marginal environment that has sandy texture characteristics, iron (Fe) content of 38-59%, black color, and low organic matter content. Bacteria are generally associated with plants and help these plants obtain the nutrients they need. Microbial groups that have the ability to colonize roots and enhance plant growth are called PGPR (Rachma & Budi, 2018).

Chili plants have high tolerance to environmental conditions, one of which is marginal land (Sukmawati & Rahmah, 2022). Chili plants grow optimally at temperatures between 25-30°C with air humidity around 60-80%, and require at least 10-12 hours of sunlight per day to support photosynthesis, flower formation, and fruit ripening. The use of biological agents is

necessary to improve the growth and productivity of chili plants (Novanursandy & Rachmawati, 2023).

The diversity of PGPR isolates in the rhizosphere and iron sand soil has not been widely studied. Oedjijono *et al*. (2014) successfully isolated several PGPR isolates from the genus Azospirillum that play a role in assisting plant growth in iron sand habitats. Research on the potential of PGPR in the rhizosphere of plants growing in iron sand environments has not been widely conducted and applied to chili plants, either in the laboratory or in the field, making it an important focus of this study. This study reveals the ability of bacteria from the rhizosphere of plants growing in iron sand environments to affect the germination and vegetative growth of chili plants using the axenic sand culture assay method.

The axenic sand culture assay method is a method that interacts microorganisms with plants in a sterile sand medium with controlled environmental factors. This method is carried out using large tubes measuring 3 x 20 cm. Before use, the sand is washed with water to remove mud, clay, and organic material contained in the sand (Oedjijono *et al*., 2014).

The objectives of this study were to test the potential of bacterial isolates PT4C5, KT6A5, CT4A7, and the consortium as PGPR quantitatively, to determine the ability of bacterial isolates PT4C5, KT6A5, CT4A7, and the consortium on the germination and growth of chili plants, and to determine the identity of bacterial isolates PT4C5, KT6A5, CT4A7, and the consortium based on phenotypic characteristics.

2. Methods

The materials used were PT4C5 isolate, KT6A5 isolate, CT4A7 isolate, Bangkok cayenne pepper seeds, sterile sand, Caceres medium, Ashby medium, Alexandrov medium, Pikovskaya medium, Nutrient Agar medium, Nutrient Broth medium, Salkowski reagent, L-Tryptophan, Chrome Azurol Sulfate (CAS) medium, Hoagland Nutrient Solution, Nitrogen-free Bromthymol Blue (Nfb) medium, Sulfide Indole Motility (SIM A) medium, Skim Milk Agar (SMA) medium, Starch Agar (SA) medium, Carboxy Methyl Cellulose (CMC) medium, Simmon's citrate medium, sugar medium, and Indole-3-Acetic Acid.

The equipment used includes a lux meter, centrifuge, spectrophotometer, Millipore 0.2 µm, 3 x 20 cm glass tubes, test tubes, Bunsen burner, Eppendorf tubes, syringe, pH meter, micropipette and tips, lamp, and Whatman No. 1 filter paper.

Research to determine the characteristics of bacterial isolates as PGPR was conducted descriptively. The potential of isolates to enhance the vegetative growth of chili plants was determined experimentally using the Axenic Sand Culture Assay method with a completely randomized design (CRD). The treatments for the experiment consisted of 5 treatments, namely, control using sterile distilled water, isolate PT4C5, isolate KT6A5, isolate CT4A7, consortium, and EM4. The experiment was repeated 3 times, resulting in 18 experimental units.

2.1 Sterilization of tools and materials (Istini, 2020)

Tools made of glass and heat-resistant medium are placed in an autoclave, then sterilized using an autoclave at a temperature of 121°C for 15-20 minutes.

2.2 Subculture of bacterial isolates (Nur'ainy *et al*., 2020)

A total of 1 ose of bacterial isolates is taken and inoculated in a zig-zag pattern on slanted NA medium. The isolates are then incubated at room temperature for 24 hours.

2.3 Preparation of Standard Curves and Bacterial Growth (Rosmania & Fitri, 2020)

Bacterial isolates were recultured on slanted NA for 24 hours, then gradually inoculated into NB to 100 mL and incubated (120 rpm, room temperature, 24 hours). Absorbance was measured at 600 nm and a series of 0.1–0.9 was created to calculate the number of cells

using the TPC method, thus obtaining a standard curve (absorbance vs. number of cells). A growth curve was created by measuring absorbance every 2 hours for 24 hours, converted to CFU/mL using a regression equation. The growth phase and highest specific rate (μ) were determined from the results of this curve.

2.4 Nitrogen fixation ability test (Nur'ainy *et al*., 2020)

Qualitative nitrogen fixation ability testing was conducted by inoculating one bacterial isolate ose in 9 mL of semi-solid NFB medium and incubating it for 7 days at room temperature. If the semi-solid Nfb medium changed color from green to blue or a white pellicle ring formed on its surface, it indicated that the bacterial isolate was capable of fixing nitrogen.

2.5 Qualitative and Quantitative Phosphate Solubility Test (Larasati *et al*., 2018)

Bacterial isolates from the rhizosphere were inoculated onto Pikovskaya medium using the spot method and incubated at room temperature for 7 days. Phosphate solubilization activity was indicated by a clear zone, and the PSI value was calculated using the formula:

$$\text{PSI} = \frac{clear\ zone\ diameter\ (mm) - colony\ diameter\ (mm)}{colony\ diameter\ (mm)}$$

PSI: Phosphate Solubility Index

For the quantitative test, 1 mL of inoculum (10^8 CFU/mL) was inoculated into 9 mL of Pikovskaya Broth and incubated for 7 days. The filtrate is filtered, centrifuged, and the supernatant is reacted with KH_2PO_4 and measured for absorbance at 693 nm. The concentration of dissolved phosphate is determined based on the regression equation from the standard curve.

2.6 Qualitative and Quantitative Potassium Solubility Test (Rajawat *et al*., 2014)

Bacterial isolates were inoculated on Alexandrov + bromthymol blue medium using the spot method and incubated for 3 days at room temperature. Potassium solubility activity was indicated by a clear zone around the colony.

$$\text{PSI} = \frac{clear\ zone\ diameter\ (mm) - colony\ diameter\ (mm)}{colony\ diameter\ (mm)}$$

PSI: Potassium Solubility Index

For the quantitative test, 1 mL of isolate was inoculated into 9 mL of Alexandrov Broth and incubated (140 rpm, 30°C, 5 days). The culture was centrifuged (13,000 rpm, 5 minutes), the supernatant was reacted with 12.5% sodium cobalt nitrate, incubated for 45 minutes, then washed with distilled water and ethanol. The precipitate was added to HCl, incubated for 15–20 minutes, and its absorbance was measured at 693 nm. The concentration of dissolved potassium was calculated using the regression equation from the standard curve.

2.7 IAA Production Test (Asra *et al*., 2020)

Bacterial isolates were tested on NB medium containing 0.2 mL of 100 ppm L-Tryptophan and incubated for 48 hours in a shaker incubator (150 rpm, 30°C). The culture was then centrifuged (5000 rpm, 20 minutes), and 1 mL of supernatant was mixed with 2 mL of Salkowski reagent. After being stored in a dark room for 30 minutes, a pink-red color change indicated the presence of IAA. The IAA concentration was measured using a spectrophotometer at a wavelength of 530 nm.

2.8 Siderophore production test (Ariyani *et al*., 2021)

One ose isolate of PT4C5, KT6A5, and CT4A7 was inoculated on CAS agar medium using the spot inoculation method and incubated for 3x24 hours. The clear zone calculation formula was calculated based on the following formula:

$$\text{SPI} = \frac{clear\ zone\ diameter\ (mm) - colony\ diameter\ (mm)}{colony\ diameter\ (mm)}$$

PSI: Siderophore Production Index

2.9 Phenotypic identification of bacterial isolates (Nur'ainy *et al*., 2020)

Macromorphological characterization of PT4C5, KT6A5, and CT4A7 isolates began with purification of the isolates on Caceres and Ashby media. The colony characteristics observed included size, pigmentation, transparency, shape, surface, elevation, and colony edges. Macromorphological characterization of bacterial isolates included Gram staining, endospore staining, and motility tests. The biochemical characterization of bacterial isolates included catalase tests, oxidase tests, proteolytic tests, amylolytic tests, cellulolytic tests, sugar fermentation tests, and citrate fermentation tests. The biochemical characterization of bacterial isolates included physiological pH tests, physiological temperature tests, and physiological salinity tests.

2.10 Testing the Ability of Bacterial Isolates to Improve Chili Seed Germination (Dey *et al*., 2004)

Chili seeds were sterilized using 5.25% NaOCl (3 minutes) and 70% alcohol (2×2 minutes), then rinsed with sterile distilled water (3×30 seconds). After draining on moist filter paper, 10 seeds were immersed in a bacterial suspension (10^8 CFU/mL) for 10 minutes, while the control used sterile physiological NaCl. The seeds were incubated at room temperature for 7 days. The germination percentage was calculated using the formula:

$$\text{Viability } \% = \frac{Number\ of\ germinated\ seeds}{Number\ of\ seeds\ planted}\ x\ 100\%$$

2.11 Seedling Bioassay using the Axenic Sand Culture Assay Method (Wuryanto *et al*., 2016)

The potential of rhizosphere bacterial isolates to enhance chili growth was tested using the axenic sand culture assay method. Sand was filtered, washed, dried, and then sterilized twice (121°C) after adding 8 mL of Hoagland nutrient solution (40 g sand/tube). Chili seeds were prepared as in the previous test and incubated for 7 days until germination. Two seedlings were placed in each tube, then given 2 mL of bacterial inoculum (10^8 CFU/mL) around the roots. The plants were exposed to light for 12 hours per day (06.00–18.00 WIB, 8125 lux) for one month, then the roots were carefully cleaned for further analysis.

2.12 Observation of the Number of PGPR Isolate Colonies on Chili Plant Roots using the Total Plate Count (TPC) Method (Wuryanto *et al*., 2016)

Chili roots were cleaned of sand, then 1 g of roots was placed in 9 mL of sterile distilled water and diluted in stages to 10^{-6}. A total of 1 mL of the 10^{-5} and 10^{-6} dilutions was planted using the pour plate method on Caceres and Ashby media. The number of colonies was counted using the TPC method, with the following calculation:

$$\sum \text{bakteri (CFU/mL)} = \text{average number of colonies x } \frac{1}{Dilution\ factor} \text{ x } \frac{1}{Pour\ plate}$$

3. Results And Discussion

3.1. Testing of Bacterial Isolates with Characteristics as Plant Growth Enhancers

Qualitative confirmation tests for nitrogen fixation on bacterial isolates PT4C5, KT6A5, and CT4A7 showed positive results, marked by the formation of a white pellicle on the surface of the NfB medium and a change in the color of the medium from green to blue, indicating nitrogenase enzyme activity. These results are consistent with the research by Oedjijono *et al*. (2025), reinforcing the validity that the three isolates are potential nitrogen-fixing bacteria for improving soil fertility naturally (Zega & Lase, 2025).

The phosphate solubility index values of isolates PT4C5, KT6A5, and CT4A7 were 2.14 ± 0.11; 3.11 ± 0.00; and 2.09 ± 0.01 (Oedjijono *et al*., 2025), while the confirmation test results showed values of 2.39 ± 0.07; 2.63 ± 0.32; and 2.57 ± 0.11. The increase in IP values in PT4C5 and CT4A7 indicates better phosphate solubilization activity than before,

while KT6A5 experienced a slight decrease. Based on the classification by Alfiansyah *et al*. (2023), the three isolates are included in the moderate category (IP 2.00–3.00). Quantitatively, the dissolved phosphate concentrations produced by isolates PT4C5, KT6A5, and CT4A7 were 20.95 ± 0.61 ppm; 22.29 ± 0.37 ppm; and 21.77 ± 0.21 ppm, respectively, indicating their potential as biofertilizers due to their ability to increase phosphate availability in the environment. According to Wuryanto *et al*. (2016), the ability of bacteria such as *Azospirillum* sp. to dissolve phosphate is influenced by media conditions, incubation time, and biological activity.

Potassium solubility testing using the spot inoculation method on Alexandrov medium showed that isolates PT4C5, KT6A5, and CT4A7 were capable of dissolving potassium, as indicated by clear zones around the colonies. The potassium solubility indices were 4.48 ± 0.34, 4.03 ± 0.34, and 4.11 ± 0.23, respectively, which are classified as high (Setiawati & Mutmainnah, 2016). Quantitatively, the dissolved potassium concentrations were 28.78 ± 4.42 ppm; 24.26 ± 2.12 ppm; and 24.02 ± 2.27 ppm, respectively. These results show a positive correlation between qualitative and quantitative tests, confirming the potential of the three isolates as effective potassium solvents.

IAA production tests using NB medium with the addition of L-tryptophan showed that all three isolates were capable of producing IAA. The IAA concentrations of isolates PT4C5, KT6A5, and CT4A7 in the confirmation test were 29.19 ± 0.53 ppm, 28.02 ± 2.22 ppm, and 29.71 ± 0.93 ppm, respectively, which were higher than the previous results except for CT4A7, which decreased slightly. Based on the classification by Huda *et al*. (2014), all isolates were classified as high (>20 ppm) in their ability to produce IAA, indicating great potential as plant growth promoters.

Production testing of siderophores using the spot inoculation method on CAS agar medium showed that isolates PT4C5, KT6A5, and CT4A7 were capable of producing siderophores, as indicated by the formation of clear zones around the colonies. The siderophore production index in the confirmation test was 2.02 ± 0.05, 1.97 ± 0.01, and 1.99 ± 0.03, respectively, with isolate PT4C5 showing the highest value. Based on the classification by Setiawati and Mutmainnah (2016), the three isolates fall into the moderate category (IS 2.00–4.00). These results indicate that isolates PT4C5, KT6A5, and CT4A7 have the potential to be effective PGPR bacteria in enhancing plant growth.

3.2. The Ability of Bacterial Isolates to Support Germination and Growth of Chili Plants

Based on the DMRT test, all treatments were significantly different from the control. Isolate KT6A5 showed the best performance in increasing the percentage of germination, plant height, root length, wet weight, number of leaves, and leaf chlorophyll A content. The superiority of KT6A5 is in line with its ability to dissolve phosphate and potassium and produce high IAA, which plays an important role in plant metabolism and photosynthesis optimization (Asra *et al*., 2020). However, the results of single isolate treatments were still lower than those of the consortium and EM4. Overall, all isolates were proven to be effective in improving the initial growth of chili plants compared to the control.

Table 3.1 Average values of germination and growth parameters of chili plants

Treatment	Seed Germination Rate (%)	Plant Height (cm)	Root Length (cm)	Wet Weight (g)	Number of Leaves	Chlorophyll A	Chlorophyll B	Total Chlorophyll
Control	61,67^{a} ± 2,88	15,67^{a} ± 0,69	5,56^{a} ± 0,81	0,93^{a} ± 0,09	4,34^{a} ± 0,57	2,15^{a} ± 0,18	13,35^{a} ± 0,83	10,47^{a} ± 0,64
PT4C5	80,00^{b} ± 0,00	18,53^{b} ± 0,45	7,46^{b} ± 0,56	1,31^{b} ± 0,04	6,34^{b} ± 0,57	2,62^{ab} ± 0,11	16,75^{ab} ± 0,43	13,17^{ab} ± 0,34
KT6A5	83,34^{bc} ± 2,88	19,43^{c} ± 0,98	9,73^{c} ± 0,25	1,64^{c} ± 0,11	8,34^{d} ± 0,57	3,37^{cd} ± 0,05	17,03^{ab} ± 2,96	13,21^{ab} ± 2,45
CT4A7	81,67^{b} ± 2,88	18,26^{b} ± 0,21	8,16^{b} ± 0,31	1,32^{b} ± 0,06	7,34^{c} ± 0,57	2,96^{bc} ± 0,14	18,23^{ab} ± 3,27	14,32^{ab} ± 2,69
Consortium	86,67^{cd} ± 2,88	20,43^{d} ± 0,43	10,51^{c} ± 0,43	2,39^{d} ± 0,14	7,34^{c} ± 0,57	3,74^{de} ± 0,26	24,18^{d} ± 3,52	19,02^{c} ± 2,85
EM4	88,34^{d} ± 2,88	21,06^{d} ± 0,36	10,63^{c} ± 0,32	2,71^{e} ± 0,14	7,34^{c} ± 0,57	4,02^{e} ± 0,53	20,99^{c} ± 3,49	16,31^{bc} ± 2,86

Note: Numbers followed by the same letter are not significantly different in the 5% DMRT test

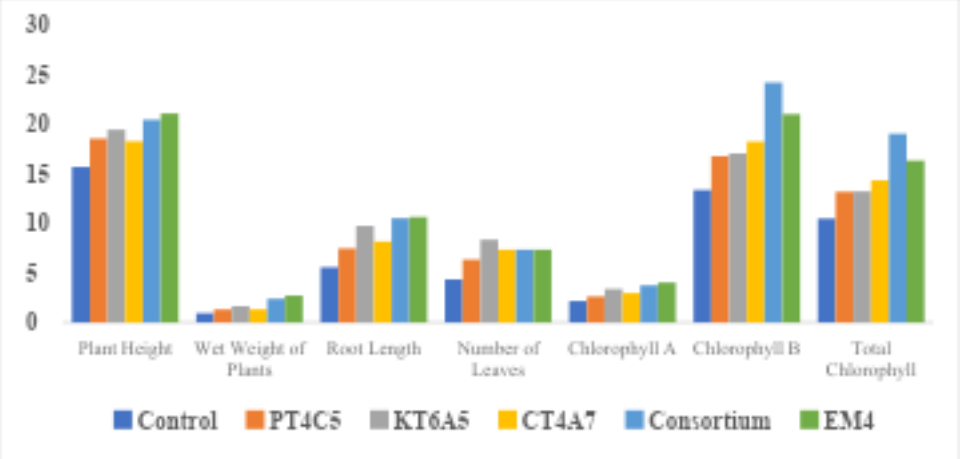

Figure 3.1. Effect of treatment on chili plant growth at 30 days after planting.

Bacteria that are capable of fixing nitrogen, dissolving phosphate and potassium, and producing plant hormones such as IAA play an important role in enhancing plant growth. Nitrogen fixation converts atmospheric nitrogen into ammonium that can be absorbed by plants, thereby increasing the availability of N for protein and nucleic acid synthesis (Situngkir *et al*., 2021). The dissolution of phosphate and potassium by bacteria also helps convert these two nutrients into forms that are easily absorbed by roots, supporting metabolism and energy production in plants. Meanwhile, IAA production stimulates cell division and elongation, contributing to increased root length, number of leaves, and plant height (Harefa & Lase, 2024).

Based on the results of total bacterial counts on the roots of 30-day-old chili plants, the bacterial population in the consortium and EM4 treatments was higher (10^{8}–10^{9} CFU/mL) than in the single isolates (10^{7}–10^{8} CFU/mL) in both Ashby and Caceres media. Among the single isolates, KT6A5 had the highest population in the plant roots, in line with the growth curve results which showed a longer exponential

phase (2 to 20 hours) compared to PT4C5 and CT4A7 (2 to 18 hours). In addition, the specific growth rate of KT6A5 did not show a decline until hour 24, indicating better adaptability and root colonization.

Based on the results of morphological, biochemical, physiological, and nutritional characterization, isolates PT4C5, KT6A5, and CT4A7 showed compatibility with the characteristics of the Planococcus genus bacteria as listed in the 9th edition of Bergey's Manual of Determinative Bacteriology. The genus Planococcus consists of Gram-positive cocci that are tolerant to pH 5–10 and temperatures of 5–65°C. They exhibit cellulolytic and proteolytic activity, are capable of fermenting various carbohydrates, utilize citrate as a carbon source, and are catalase-positive and oxidase-negative. These characteristics are in line with the research by Gagola *et al*. (2013), which reported that Planococcus has small white colonies, is motile with one to two flagella, and is thermotolerant up to 65°C. In addition, this bacterium is halotolerant and can grow at a salinity of 1–15% NaCl (Remijawa *et al*., 2020). Planococcus is also known to ferment glucose, lactose, fructose, maltose, and mannitol, and produce extracellular enzymes such as amylase, protease, cellulase, and lipase, which play a role in the degradation of complex organic materials and adaptation to extreme environments (Hastuti *et al*., 2017).

4. Conclusion

Based on the results of the research conducted, it can be concluded that:

1. Isolates PT4C5, KT6A5, and CT4A7 have PGPR characteristics because they are able to fix nitrogen, dissolve phosphate and potassium quantitatively, produce IAA growth hormone quantitatively, and produce siderophores.
2. Isolates PT4C5, KT6A5, CT4A7, and their consortium are able to increase chili seed germination.
3. Isolates PT4C5, KT6A5, CT4A7, and their consortium are able to increase plant height, wet plant weight, root length, number of leaves, and leaf chlorophyll content. Isolate KT6A5 showed superior ability compared to isolates PT4C5 and CT4A7 in helping to improve chili plant growth.
4. The identity of isolates PT4C5, KT6A5, and CT4A7 based on phenotypic characteristics includes members of the Planococcus genus.

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