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Actinomycetes from Plant Rhizosphere in Gorontalo Karst Area as Plant Growth Promoting Rhizobacteria

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Abstract

This study aimed to describe Actinomycetes from the rhizosphere of plants in the Gorontalo karst area as Plant Growth Promoting Rhizobacteria (PGPR). The research method is based on a quantitative descriptive method. Soil samples from the rhizosphere were collected using a purposive sampling technique from 8 plant species at two locations in the Gorontalo karst region, namely the Tanjung Kramat Hills. The characteristics of PGPR in this study focused on phosphate solubilization ability, Indole Acetic Acid (IAA) production, antagonism against the fungus *Fusarium oxysporum*, and tolerance to fungicides. Potential PGPR isolates were analyzed for phylogenetic relationships based on 16S rRNA gene sequences. The results showed that there were 6 actinomycetes isolates (RzHKC-01, RzKO-02, RzO-03, RzAK-04, RzPK-05, and RzOC-06) from 8 plant species in the Gorontalo karst region. One actinomycetes isolate, RzPK-05, showed potential as a PGPR with the ability to solubilize phosphate and produce IAA at 0.165 mg/L and 0.133 mg/L, respectively. Isolate RzPK-05 exhibited antagonistic properties against *Fusarium oxysporum* with an inhibition rate of 82.24% and was tolerant to fungicides such as Captive, Benlate, and Thiramo up to a concentration of 100 mg/L. Molecular analysis based on the 16S rRNA gene and phylogenetic tree reconstruction indicated that the RzPK-05 isolate is closely related to the genus Streptomyces with 100% similarity.

Keywords: Actinomycetes; Karst; PGPR; Rhizosphere.

INTRODUCTION

Karst areas are formed through the dissolution of water on soluble rocks, with characteristic features such as channels, cavities, vertical holes, disappearing rivers, springs, underground waterways, and caves (Lu et al., 2014). Karst regions are classified as hydrogeological environments with high diversity, closely related to other geospheric processes, particularly the atmosphere, hydrosphere, and biosphere, and play an important role in human history and development (Goldscheider et al., 2020). These areas generally have calcium-rich soil (Ca), which affects the availability of nutrients for plants and soil organisms (Fan et al., 2019).

The karst area in Gorontalo Province covers the southern part of Gorontalo Regency, around Lake Limboto, and Bone Bolango Regency. Plants living in karst areas generally develop physiological and morphological adaptations (Meng et al., 2023) to survive in extreme environmental conditions. Some plants form symbiotic relationships with various microorganisms in their root zones (Lakshmanan et al., 2014). Some types of microorganisms found in karst areas include fungi, bacteria, Actinomycetes, and algae (Mubarak et al., 2017). According to Mubarak et al., (2017), bacteria from the Actinomycetes group are one of the most commonly found microorganisms associated with root systems in karst regions.

The rhizosphere is the zone around plant roots that serves as a habitat for various microorganisms, whose activities play an important role in supporting plant growth and development (Putra et al., 2020). The presence and activity of microorganisms in this area are greatly influenced by the exudates produced by plant roots (Dewi, 2018). Each plant species has a different composition of exudates, which influences the variation and abundance of microorganisms present in the soil. Research conducted by Syahril et al., (2023) revealed that microorganisms originating from the rhizosphere have a specific distribution influenced by the physical and chemical conditions of the soil.

Actinomycetes are capable of producing bioactive compounds such as antibiotics and the phytohormone IAA, which play a role in root growth, plant organ formation, and fruit ripening (Anwar et al., 2016; Etchells et al., 2016). Some types of Actinomycetes can solubilize phosphate by producing organic acids such as gluconate, citrate, and oxalate, which convert bound

phosphate into a form available to plants (Rajput et al., 2013; Putri et al., 2018). Additionally, Actinomycetes have potential as biocontrol agents (Ilsan, 2016). The presence of Actinomycetes in the rhizosphere is influenced by plant root exudates, which determine microbial activity in the surrounding area (Dewi, 2018). Based on the capabilities possessed by Actinomycetes, these microorganisms can be one of the alternatives to inorganic fertilizers, due to their ability as plant growth-promoting rhizobacteria (PGPR).

A group of microorganisms known as Plant Growth-Promoting Rhizobacteria (PGPR) consists of bacteria and fungi that live in symbiosis with plant root systems, providing nutrients and promoting plant growth (Vocciante et al., 2022). The role of PGPR includes biostimulant functions through the synthesis and regulation of growth hormones, enhancing the availability of major nutrients, and acting as bioprotectants to control soil-borne pathogens (Marom et al., 2017). Exploration of Actinomycetes from the rhizosphere of plants in the Gorontalo karst region has the potential to produce superior isolates that support nutrient availability and plant growth. This study aims to describe the potential of Actinomycetes from this region as Plant Growth Promoting Rhizobacteria.

MATERIALS AND METHODS

Materials and equipment

This study was conducted from June to December 2024 at the Biology Laboratory of the Faculty of Mathematics and Natural Sciences, Gorontalo State University. The samples used were rhizosphere soil from plants in the Gorontalo karst area, specifically at Tanjung Kramat, Hulonthalangi District, and the Karst Mountains, Kota Barat District.

The tools and materials used in this study included a soil tester, small shovel, sample bags, labels, notebook, pencil, smartphone camera, laminar air flow, incubator, oven, autoclave, shaking incubator, analytical balance, water bath, hot plate, Bunsen burner, Petri dishes, Erlenmeyer flasks, inoculation needles, beaker glasses, stirring rods, test tubes, a 26-tube rack, micropipettes, culture bottles, a centrifuge, a spectrophotometer, cuvettes, an aluminum mortar, measuring cups, a vortex mixer, a cork borer, a UV transilluminator, PCR, BigDye® Terminator v3.1, USA, BioEdit software, Molecular Evolutionary Genetics Analysis (MEGA) 11, automatic sequencing machine, distilled water, alcohol, SCA medium (Starch Casein Agar), Agar Powder, Nystatin/Cycloheximide, Ringer's solution, PDA medium (Potato Dextrose Agar), NB medium (Nutrient Broth), ISP2 medium, Salkowski, L-tryptophan, Pikovskaya broth medium, Pikovskaya agar medium, sodium molybdate, hydrazine, KH2PO4, *Fusarium oxysporum* fungal culture (obtained from IPB Culture), glass beads, lysis buffer, proteinase, lysozyme, phenol, chloroform, ethanol, 2% agarose gel electrophoresis, MgCl2, dH_2O, eubacterial universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3').

Methods

Soil sampling from plant rhizosphere in karst areas Gorontalo

Rhizosphere soil samples were taken using purposive sampling from shrubs and herbs in Tanjung Kramat, Hulonthalangi District, and the West Mountain in Kota Barat District. Soil was collected at a depth of 10–15 cm using a small shovel, stored in sterile plastic bags, and placed in a coolbox (Maulana et al., 2022; Katili & Retnowati, 2017). Location coordinates were recorded using GPS, and environmental parameters such as pH and soil moisture were measured using a soil tester.

Isolation of Actinomycetes from plant rhizosphere

Five grams of soil samples from the karst area were mixed with 45 ml of sterile distilled water, homogenized at 225 rpm, then heated at 60°C for 15 minutes (Mangamuri et al., 2012; Retnowati et al., 2017). Serial dilutions were performed up to 10^{-5}, and 200 µl of dilutions ranging from 10^{-3} to 10^{-5} were inoculated onto SCA medium using the spread plate method, then incubated at 37°C for 14–28 days. Cycloheximide/nistatin was added to prevent fungal contamination (Baskaran et al., 2011).

Screening potential of Plant Growth Promoting Rhizobacteria (PGPR)

Phosphate solubilization activity test. Qualitative testing of phosphate solubilization activity in Actinomycetes isolates refer to Karpagam & Nagalakshmi, (2014) using Pikovskaya Agar medium Pikovskaya Agar for 7 days. Phosphate solubilization ability is indicated by the formation of a clear zone around the Actinomycetes colonies. Quantitative phosphate solubilization activity refers to Lyn et al., (2013). Actinomycetes isolates were grown on Pikovskaya Broth medium for 7 days. The supernatant was separated from the cell pellet by centrifugation at 225 rpm. Phosphate solubilization was measured spectrophotometrically by adding molybdate reagent to the supernatant and measuring at a wavelength of 840 nm. The concentration of dissolved phosphate was calculated using KH_2PO_4 standard curve.

Indole Acetic Acid (IAA) production activity test. IAA production by Actinomycetes was assessed qualitatively and quantitatively. Qualitatively, isolates were grown in NB medium supplemented with 0.1 g L-tryptophan, incubated for 7 days, then the supernatant was mixed with Salkowski reagent and observed for a pink color change as an indicator of IAA (Patten & Glick, 2002). Quantitative testing is performed spectrophotometrically at a wavelength of 535 nm. IAA concentration is calculated using a standard curve of pure

IAA (Sukmadewi et al., 2015; Gordon & Weber, 1951; Kaur & Sharma, 2013).

Antagonistic test against *Fusarium oxysporum* mold. The antagonistic ability of Actinomycetes isolates against Fusarium oxysporum was tested using the cross streak method on Potato Dextrose Agar medium for 7 days (Saha & Santra, 2014). Antagonistic ability was indicated by the formation of an inhibition zone around the Actinomycetes colony. Antagonistic activity was determined using the formula (Kurnia et al., 2014), and the inhibitory strength categories were based on the table (Zivkovikc et al., (2010); Nuraini et al., (2017) with percentages <30% (weak), 30-<50% (moderate), 50-<70% (strong), ≥70-100% (very strong).

$$I = \frac{r1 - r2}{r1} \times 100\%$$

Note: I = percentage of inhibition zone; r1 = Control; r2 = Radius of *Fusarium oxysporum* approaching Actinomycetes.

Fungicide tolerance test (Captive, Benlate, Thiramo). Actinomycetes tolerance to fungicides was tested using the poison bait method (Humaidi et al., 1999; Dotulog et al., 2019) at concentrations of 25, 50, 75, and 100 mg/L. Isolates producing IAA, phosphate solubilizers, and antagonists were inoculated using cork borers. The tolerance test results were measured based on the Relative Inhibition Level (RIL) using the formula by Kumar et al., (2007), with categories: RIL>90% (highly sensitive), 75-90% (sensitive), 60-75% (moderately resistant), 40-60% (resistant), and ≤40% (highly resistant).

$$I = \frac{C - T}{C} \times 100\%$$

Note: I = Percentage Growth of Actinomycetes; C = Control diameter (mm); T = Treatment diameter (mm).

Molecular identification based on the 16S rRNA gene

Potential PGPR Actinomycetes isolates were cultured in Starch Casein Broth medium for 7 days in a shaker incubator and then centrifuged at 500 rpm for 15 minutes to separate the cell pellets. Genomic DNA was extracted from the pellets using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). The 16S rRNA gene was amplified using universal primers 27F and 1492R along with 2X MyTaq HS Red Mix, with PCR conditions following Okolie et al., (2013). PCR products were purified using the Zymoclean™ Gel DNA Recovery Kit. Sequencing of the gene was performed in both directions (Retnowati et al., 2017) and the results were analyzed using BLAST against the NCBI database (Retnowati et al., 2023).

Data analysis

The research data were analyzed descriptively and quantitatively. The ability of Actinomycetes as Plant Growth Promoting Rhizobacteria, which includes phosphate solubilization, IAA production, antagonism against Fusarium oxysporum, and tolerance to fungicides, was presented in tables and graphs. The molecular identification data were compared with data in GenBank NCBI.

RESULTS AND DISCUSSION

Description of research location

The study was conducted at two locations, namely Tanjung Kramat in Hulonthalangi District and the Karst Mountains in Kota Barat District, with vegetation dominated by *Catharanthus roseus, Mesosphaerum suaveolens, Indigofera tinctoria, Dolichos oliverii, Jatropha gossypifolia, Imperata cylindrica, Lantana montevidensis, Leucaena leucocephala, and Pneumatopteris pennigera*. Environmental conditions were characterized by acidic soil pH and low humidity (Table 1).

Table 1. Physical and chemical characteristics of the environment.

Location	Coordinate point	pH	Moisture
Tanjung Kramat	0°30'20.0"N 123°03'02.8"E	5,43	4,2%
Pegunungan Karst	0°32'37.5"N 123°01'57.3"E	6,6	1,67%

Description of Actinomycetes as Plant Growth Promoting Rhizobacteria

The results showed that there were six isolates obtained from eight types of plant rhizosphere and one isolate, RzPK-05, which had potential as Plant Growth Promoting Rhizobacteria with the ability to phosphate solubilazing, produce Indole Acetic Acid, antagonistic to Fusarium oxysporum (Table 2), and tolerant to fungicides (Table 3).

Table 2. Results of phosphate solubility testing by Actinomycetes isolates.

Isolate	Qual.	Quant.
RzHKC-01	-	-
RzKO-02	+	0,22 mg/L
RzO-03	-	-
RzAK-04	-	-
RzPK-05	+	0,165 mg/L
RzOC-06	-	-

Table 3. Actinomycetes isolates producing Indole Acetic Acid (IAA).

Isolate	Qual.	Quant.
RzHKC-01	-	-
RzKO-02	-	-
RzO-03	+	0,312 mg/L
RzAK-04	-	-
RzPK-05	+	0,133 mg/L
RzOC-06	-	-

Table 4. Results of Actinomycetes antagonistic testing.

Isolate	Qual.	Quant.	Categories
RzHKC-01	-	-	-
RzKO-02	-	-	-
RzO-03	-	-	-
RzAK-04	-	-	-
RzPK-05	+	82,24%	Very strong
RzOC-06	-	-	-

Table 5. Result of RzPK-05 isolate tolerance testing against fungicides.

Fungicides	C	RIL	Categories
Captive	25	-9,45%	Highly resistant
	50	17,45%	Highly resistant
	75	9,63%	Highly resistant
	100	16,72%	Highly resistant
Benlate	25	46,45%	Resistant
	50	14,43%	Highly resistant
	75	-4,52%	Highly resistant
	100	24,27%	Highly resistant
Thiramo	25	-23,24%	Highly resistant
	50	-23,24%	Highly resistant
	75	3,26%	Highly resistant
	100	38,70%	Highly resistant

Description: (C): concentration; (RIL): Relative Inhibition Level

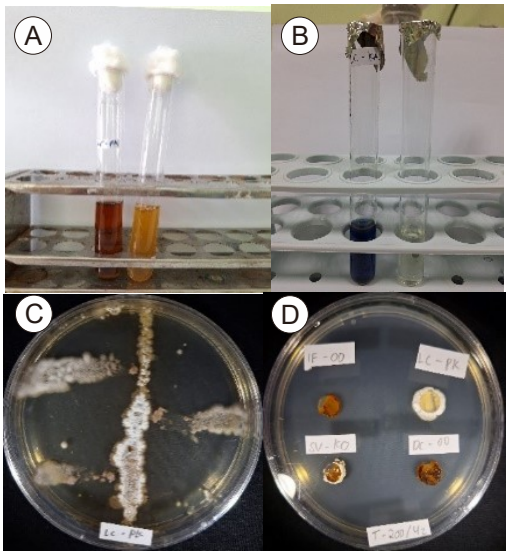

Figure 1. A: Indole acetic acid producing, B: Phosphate solubilazing, C: Antagonistic to *Fusarium oxysporum*, D: Tolarence of fungicide.

Description of phylogenetic relationship from Actinomycetes as Plant Growth Promoting Rhizobacteria

Actinomycetes isolates with potential as Plant Growth Promoting Rhizobacteria, namely RzPK-05, were molecularly identified based on the 16S rRNA gene, and the sequence data matching results showed that the RzPK-05 isolate was related to the Streptomyces genus (Table 6).

Table 6. The closest phylogenetic relative of the RzPK-05 isolate based on 16S rRNA gene sequencing.

Nearest phylogenetic neighbor	Percent identity	E-value	Genus
Streptomyces albus strain NRRL B-1811 (NR_118467)	100	0.0	*Streptomyces*
Streptomyces albus strain NBRC 15415 (NR_041180)	100	0.0	*Streptomyces*
Streptomyces albus strain NBRC 13078 (NR_041110)	100	0.0	*Streptomyces*
Streptomyces albus strain NBRC 13015 (NR_112342)	100	0.0	*Streptomyces*
Streptomyces albus strain NBRC 13014 (NR_112341)	100	0.0	*Streptomyces*

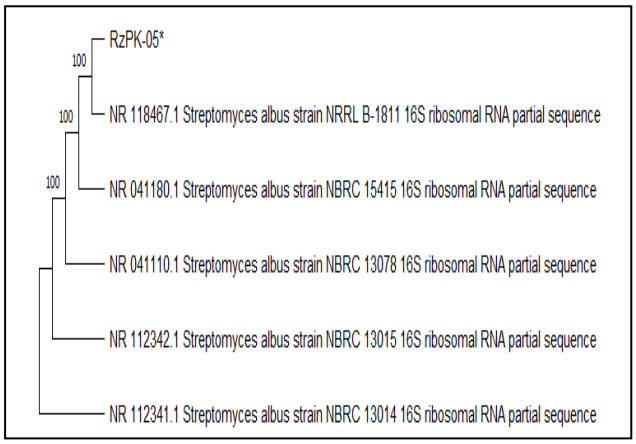

Figure 2. Reconstruction of the phylogenetic tree of the RzPK-05 isolate.

Discussion

The results of the study revealed that only one Actinomycetes isolate, RzPK-05, out of six isolates was capable of solubilizing phosphate, producing IAA, exhibiting antagonistic activity against Fusarium oxysporum, and being tolerant to fungicides. According to the research by Oksana et al., (2020), bacteria are capable of solubilizing phosphate by forming a clear zone through the secretion of organic acids. These acids interact with calcium ions (Ca) from $Ca_3(PO_4)_2$ in the Pikovskaya medium, thereby releasing phosphate ions (H_2PO_4) and producing a transparent or clear area. The ability of Actinomycetes isolates to solubilize phosphate is likely due to the presence of phosphatase enzymes in these isolates. Putri et al., (2018) stated that the formation of a clear zone around Actinomycetes colonies is influenced by the activity of phosphatase enzymes and the production of organic acids or polysaccharides. Phosphatase enzymes act as catalysts in hydrolytic mineralization, converting insoluble phosphorus into soluble forms (Baloe et al., 2023). A study by Hutagaol

et al., (2022) reported that the mechanism of phosphate dissolution by bacteria occurs chemically. Bacteria can dissolve phosphate by producing various organic acids, such as citric acid, succinic acid, glutamic acid, oxalic acid, lactic acid, malic acid, fumaric acid, glyoxylic acid, tartaric acid, and α-ketobutyric acid. The accumulation of these organic acids lowers the pH of the environment, thereby triggering the release of phosphorus (P) that was previously bound to calcium (Ca). Research by Nisa (2018) adds that differences in the ability of phosphate-solubilizing bacteria to dissolve phosphate are also influenced by the genetic factors of each microorganism, particularly in producing organic acids that play a role in the phosphate dissolution process.

The ability to produce Indole Acetic Acid (IAA) is influenced by the physical and genetic characteristics of bacteria, including the presence of genes encoding metabolic enzymes (Passalacqua et al., 2016; Louis et al., 2007). Genetic differences between bacteria affect the availability of enzymes and IAA biosynthetic pathways (Patten & Glick, 2002; Lata et al., 2024). Rhizosphere bacteria generally produce IAA through the tryptophan pathway, such as IAM, TAM, and IPyA (Zhang et al., 2019; Zhang et al., 2021). The presence of the ipdC gene in some species supports the role of the IPyA pathway in IAA biosynthesis. Therefore, the ability of Actinomycetes isolates to produce IAA is likely related to the presence of genes encoding enzymes involved in that pathway.

Isolate RzPK-05 showed antagonistic activity against Fusarium oxysporum, indicating its ability to produce antifungal compounds. Zhang et al., (2021) reported that only 17 of 60 Actinomycetes isolates were active against Fusarium oxysporum. This ability is thought to be influenced by the presence of antifungal genes such as Polyketide Synthase-I and Non Ribosomal Peptide Synthetase (Nurjasmi et al., 2009). Other factors that may influence the antagonistic activity exhibited against the pathogen Fusarium oxysporum are thought to be due to the fact that Actinomycetes isolates have different physiological mechanisms, enabling them to respond to different types of pathogens. Queendy and Roza (2019) state that a microorganism's ability to produce secondary metabolites is influenced by its individual physiological characteristics. The study conducted by Queendy and Roza (2019) reported that out of 32 Actinomycetes isolates, only 4 potential isolates (C1.15, B1.07, D2.31, C2.21) inhibited Fusarium oxysporum, and 3 isolates (B1.02, C2.24, B3. 14) inhibited Ganoderma boninense.

The tolerance shown by the RzPK-05 isolate to various types of fungicides is likely due to its ability to degrade the active ingredients of fungicides. Degradation is the process of breaking down unstable compounds into more stable forms (Atlas, 1992; Lumantouw et al., 2013), allowing bacteria to survive in environments exposed to fungicides. Therefore, the RzPK-05 isolate's continued tolerance to the fungicides Captive, Thiramo, and Benlate is likely due to its ability to modify the active ingredients of fungicides into non-toxic forms.

Based on the ability of the RzPK-05 isolate as a PGPR and molecular identification showing that RzPK-05 is closely related to the genus Streptomyces. The presence of this genus in karst areas reinforces the potential of the RzPK-05 isolate obtained from the rhizosphere of plants as Plant Growth Promoting Rhizobacteria. Research shows that Actinomycetes of the genus Streptomyces are capable of solubilizing phosphate (Jog & Colleagues, 2014), producing IAA (Lin et al., 2013), and exhibiting antagonistic activity (Kanini et al., 2013). Furthermore, Actinomycetes, particularly from the genus Streptomyces, are known as bacteria capable of producing antibiotics, biosurfactants, volatile compounds, and toxins, which have the potential to be used as biocontrol agents to combat plant pathogens (Vurukonda et al., 2018).

CONCLUSIONS

Based on the research, it can be concluded that only one isolate, RzPK-05, out of six isolates has potential as a PGPR with the ability to dissolve phosphate (0.165 mg/L), produce IAA (0.133 mg/L), antagonistic against *Fusarium oxysporum* (82.24%), and tolerant to fungicides (highly resistant). Molecular identification results indicate that the RzPK-05 isolate is closely related to the genus Streptomyces with a similarity percentage of 100%, thus identifying the RzPK-05 isolate as *Streptomyces albus* strain RzPK-05.

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Competing Interests: The authors declare that there are no competing interests.

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