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Antagonistic Ability and Fungicide Tolerance of Rhizosphere Actinomycetes from the Hungayono Karst Ecosystem, Gorontalo

Nazifah R Adam*, Yuliana Retnowati, Abubakar Sidik Katili,**
Novri Youla Kandowangko, Wirnangsi D. Uno
Program of Conservation Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Gorontalo,
Jl. Prof. Dr. Ing. BJ. Habibie, Kabupaten Bone Bolango, Gorontalo, 96119Tel./Fax. +62-822-5970098, Indonesia.

Corresponding author
nazifahadam6@gmail.com*, yuliana.retnowati@ung.ac.id**

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Abstract

Actinomycetes show promise as biological control agents, reducing fungicide use in agriculture due to their bioactive compounds and ecological resilience. This study focused on the initial screening and ecological bioprospecting of actinomycetes from the Hungayono karst ecosystem in Gorontalo by determining their antagonistic activity against the pathogenic fungi *Fusarium oxysporum* and *Neocosmospora solani*, as well as their tolerance to the fungicides thiram and benomyl. Potential isolates were identified using molecular methods. Samples were collected from three locations. Rhizosphere soil samples were obtained from plant species: *Alocasia macrorrhizos, Ficus microcarpa,* and *Acrostichum aureum* L. Antagonistic activity against root-pathogenic fungi and tolerance to fungicides were used as initial screening criteria to assess their potential as biological control agents. This study successfully isolated 9 actinomycete isolates. The results showed that only 33% of the isolates exhibited inhibitory activity, with isolate RzAH-07 showing a moderate inhibition zone (5.22 mm) against *Fusarium oxysporum* and weak inhibition (4.18 mm) against *Neocosmospora solani,* while also demonstrating tolerance to the fungicide benomyl up to 1000 ppm. RzAH-07 was selected as the top candidate due to its two key advantages. Phylogenetic analysis based on 16S rRNA gene sequences indicates that RzAH-07 is closely related to the genus *Streptomyces*, with a similarity percentage ranging from 98.35% to 99.14% based on GenBank data.

Keywords: Biocontrol; Fungicide tolerance; Karst; Rhizosphere; Streptomyces.

INTRODUCTION

The rhizosphere is the ecosystem in the root zone which has a considerable impact on plant productivity and growth. The rhizosphere is an area of interaction between plants, soil and microbes. The exudates secreted from the plant roots serve as nutrients for several microbes in that region (Solomon et al., 2024). However, soil-borne fungal pathogens like *Fusarium oxysporum* and *Neocosmospora solani* pose serious challenges to plant root systems. They often cause deadly diseases such as root rot, wilting and stunting (Priyadarshini, 2025). Pandey & Saharan (2025) in their study revealed the presence of several soil-borne pathogens viz., *Fusarium, Rhizoctonia, Pythium, Phytophthora,* and *Macrophomina* causing root rot, stem rot, wilt, and other important diseases.

The presence of these fungi can lead farmers to use fungicides as a control measure (Muslim & Suwandi, 2023). Reports show that the fungicide market in Indonesia is growing steadily, at about 3.44% per year from 2026 to 2031 (CAGR) (Mordor Intelligence, 2026). This is indicative of an increasing reliance on fungicides in agriculture. Synthetic fungicides have traditionally been used to manage diseases, but their widespread use poses environmental risks. Several studies have demonstrated that the fungicide alixide inhibits the activity of important soil enzymes such as urease, cellulase, and phosphatase (Wang et al., 2025). It also negatively affects non-pathogenic soil micro-organisms (Schaeffer & Wijntjes, 2022; Edlinger et al., 2022). According to Meena et al. (2020) the use of synthetic fungicides with active ingredients such as benomyl, mancozeb, captan and Thiram can decrease populations of soil microorganisms.

This problem needs to be solved by developing effective biological control agents. Actinomycetes, especially *Streptomyces* spp. are Gram-positive bacteria that can produce a range of bioactive compounds such as antibiotics, antifungals and antibacterials (Elshafie & Camele, 2022). They also produce plant growth stimulants such as indole-3-acetic acid and siderophores (Shahrajabian & Sun, 2025). In sustainable agriculture, potential candidates for biological control should not only show antagonistic activity against pathogens, but should also be tolerant to synthetic fungicides to survive in chemically treated soils.

Actinomycetes are ubiquitously distributed in different ecosystems, such as karst environments with their particular conditions (Matalauni et al., 2025). Previous studies have found actinomycetes in the rhizosphere of different mangrove plants in the Gorontalo karst ecosystem (Katili & Retnowati, 2017). Recent studies have revealed that actinomycetes are present in the Gorontalo karst ecosystem with high population diversity (Retnowati et al., 2025). In addition, the characteristic of the karst ecosystem is a very marginal soil. For example, the area of the Hungayono Karst in Gorontalo. This provides unique ecological opportunities to identify adaptive and resilient microbes. Karst soils are characterized by high calcium (Ca) and low nutrient availability, especially phosphorus (P), and have different physicochemical properties than other soil types (Luo et al., 2023; Chen et al., 2024). The Hungayono karst also features hot springs with a water surface temperature of 54 °C (Tolodo et al., 2022).

This environmental stresses may result in the evolution of specific metabolic pathways and strong defense mechanisms of local actinomycetes. In these conditions, microorganisms are able to produce a large variety of bioactive compounds to inhibit the growth of competing organisms (Lugtenberg et al., 2017; Ghoul & Mitri, 2016). They can also form biofilms that protect them from environmental stress and other antimicrobial agents (Mirghani et al., 2022). However, it is still unclear whether the rhizosphere actinomycetes of the Hungayono karst ecosystem have antagonistic activity and fungicide tolerance.

This study aimed to address gaps in existing research from the region by isolating and selecting actinomycetes from the plant rhizosphere in the Hungayono karst area. It specifically assessed their antagonistic activity against *Fusarium oxysporum* and *Neocosmospora solani*, as well as their resistance to the common fungicides thiram and benomyl. The hypothesis is that unique selective pressures in the Hungayono karst may have shaped a community of actinomycetes with diverse traits, including potential antagonistic activity and tolerance to fungicides, making this location relevant for screening potential biocontrol agents.

MATERIALS AND METHODS

Study area

Rhizosphere soil samples were collected on August 5, 2025, from the Hungayono karst ecosystem in Tulabolo Village, East Suwawa Subdistrict, Bone Bolango District. The environmental context of this site features marginal land characterized by high vegetation diversity. Samples were collected from three different locations: Location A (0°30'19.4“N 123°17'29.5”E), location B (0°30'18.13'‘ N 123°17'29.08’‘E), and location C (0°30'14.7’‘N 123°17'32.25’E) (Figure 1). Sampling locations were determined based on soil slope (lower, middle, and upper) with distances between points of ± 40–150 m, and the elevations of each location were as follows: location A (129 masl), location B (145 masl), and location C (169 masl). Rhizosphere soil samples were collected from each point at a depth of 15–30 cm using a sterile shovel, placed in sterile plastic sample bags, labeled according to location, and stored in a cooler box (Retnowati *et al.* 2017). Sampling was conducted using purposive sampling by selecting one dominant plant species at the sapling stage at each location, for a total of three samples. Additionally, location coordinates were recorded using GPS, and environmental parameters such as temperature, pH, and soil moisture were measured using soil testing equipment.

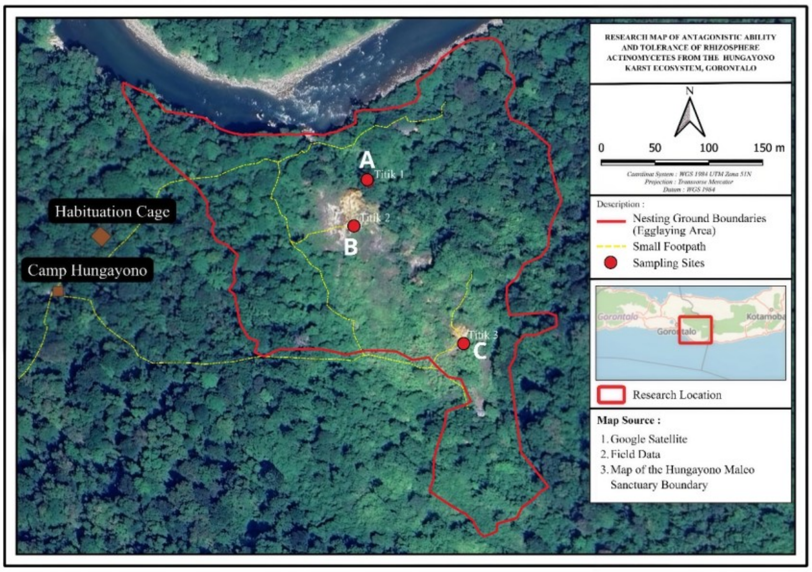


Figure 1. Rhizosphere-soil sampling location on the Hungayono karst ecosystem, Gorontalo, Indonesia: A:0°30'19.4"N 123°17'29.5"E, B: 0°30'18.13"N 123°17'29.08"E, and C: 0°30'14.7"N 123°17'32.25"E.

Procedures

Isolation and purification of Actinomycetes

Soil samples collected from the karst area were crushed using a mortar and pestle; then, 5 grams of the sample were taken and placed into an Erlenmeyer flask containing 45 ml of sterile Ringer's solution, and homogenized using a magnetic stirrer. The soil suspension was then heated in a water bath at 60°C for 15 minutes (Retnowati et al., 2024) to inactivate heat-sensitive non-actinomycete bacteria. Serial dilutions were performed up to a 10^5 dilution, and 100 µl of each dilution was inoculated into selective media for actinomycete growth, namely Starch Casein Agar (1% starch, 0.2% K_2HPO_4, 0.2% KNO_3, 0.03% casein, 0.005% $MgSO_4 \cdot 7H_2O$, 0.002% $CaCO_3$, 0.001% $FeSO_4 \cdot 7H_2O$, and 1.5% agar) using the plate spread method. Nystatin at a concentration of 50 µg/mL was added to the SCA medium as an antifungal agent (Meenakshi et al., 2024). Plates were incubated for 14 days at 37 °C. The incubation process was monitored daily to observe the development of the isolates. Isolates exhibiting distinct colony morphologies were transferred to oatmeal agar (ISP-3) for subculturing to obtain pure cultures. Isolates confirmed to be actinomycetes were then subjected to further purification using the streak plate method on SCA medium until single colonies were obtained.

In vitro Antagonistic Activity of Actinomycetes Isolates Against Pathogenic Fungi

Antagonistic assays were conducted using a modified Cross Streak method (Hossain & Rahman, 2014). The pathogenic fungal subcultures used in this test were *Fusarium oxysporum* IPBCC 880012 and *Neocosmospora solani* IPBCC 070525 (IPBCC ITB collection). Actinomycetes isolates were inoculated individually and streaked in vertical straight lines on the surface of *Potato Dextrose Agar* (PDA). The cultures were then incubated for 7 days at 37°C. After 7 days of incubation, pure cultures of the pathogenic fungi *Fusarium oxysporum* and *Neocosmospora solani* were inoculated horizontally on the left and right sides, respectively, of the Actinomycetes line on the previously prepared PDA medium. After that, they were incubated again for 3 days at 30°C to study the effects of the interaction between the pathogen isolates and the actinomycetes (Pathalam *et al.,* 2017). Antagonistic activity was measured based on the inhibition zone of fungal growth. Measurements were carried out using a digital caliper, calculated from the edge of the actinomycetes colony to the nearest front of the fungal growth. For each Petri dish, measurements were taken at 3 points along the intersection area for each fungus: 3 points on the left side for *Fusarium oxysporum* and 3 points on the right side for *Neocosmospora solani*. The average of these 3 points was used as the inhibition distance for each fungal species. The average inhibition zone was calculated separately for each fungal species in each dish. All experiments were conducted in triplicate, and results are reported as mean ± standard deviation (SD). Inhibitory activity was classified into four categories: weak (<5 mm), moderate (5–10 mm), strong (10–20 mm), and very strong (>20 mm) (Ariandi *et al.,* 2021). This classification is operational in nature and is used to compare the activity of different isolates under in vitro conditions. Each pathogen was categorized separately to ensure accuracy and precision.

In vitro Tolerance Assay of Actinomycetes Isolates to Fungicides

Tolerance assay of actinomycetes to fungicides was conducted using the Poisoned Food technique, referring to Sari *et al.* (2021), which has been modified. Actinomycetes isolates were grown for 14 days on SCA medium, then each of the isolates with a 5 mm of diametre were taken using a cork borer. Each actinomycete isolate was then placed into 90mm diameter Petri dishes containing fresh SCA medium mixed with the synthetic fungicides Benlate® (Benomyl 50%, benzimidazole) and Thiramo 80WP® (Thiram 80%, dithiocarbamate) at various concentrations (100, 200, 300, 400, and 500 ppm). Fungicide stock solutions were prepared by mixing powdered fungicides with sterile distilled water until homogeneous using a magnetic stirrer. These were then added to SCA medium that had been previously autoclaved and cooled. Once the SCA medium containing the fungicide stock solution was homogeneous, it was poured into Petri dishes. The plates were incubated at 30°C for 7 days. The tolerance ability of actinomycetes isolates were showed by the growth of actinomycetes isolates on the medium. A control treatment was prepared for each isolate and then placed in a Petri dish containing SCA medium without the addition of fungicide solution. The fungicide tolerance was stated as a percentage of Relative Inhibition Rate (RIR) (Indra *et al.* 2015), where K_1 and K_2 represent the total diameters of the control and treated colonies, respectively, including the initial inoculum plug. All experiments were performed in triplicate, with the following categories: <40 (Very strong), 40-60 (Strong), 60-75 (Moderate), >75-90 (Weak), and >90 (Very weak) (Mahendra *et al.,* 2022). The actinomycetes isolated showed a very strong category to fungicide were used to further assay at higher concentrations of fungicides (600, 700, 800, 900, and 1000 ppm).

$$Relative\ Inhibiton\ Rate\ (\%) = \left(\frac{K_1 - K_2}{K_2}\right) \times 100$$

Note: RIR = Relative Inhibition Rate; K_1= Control diameter (mm); K_2 = Treatment diameter (mm).

Molecular identification of potential Actinomycetes isolates

The selected actinomycetes isolated (RzAH-07) exhibiting the highest antagonistic activity and fungicide tolerance was identified based on the molecular character of 16S rRNA gene sequences. The actinomycetes isolates were cultured in Starch Casein Broth medium for 7×24 hours at 28-30°C on a 200 rpm shaker incubator. The pellet cells were separated from the supernatant by centrifugation for 15 minutes at a centrifugal force of approximately 4,000–6,000 ×g (equivalent to ±5,000–6,000 rpm) to pellet the cells. The resulting cell pellet was subsequently used for genomic DNA extraction using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research). The 16S rRNA gene was amplified using universal primers 27F and 1492R together with 2X MyTag HS Red Mix. *Polymerase Chain Reaction* (PCR) conditions followed Okolie *et al.* (2013). PCR (Polymerase Chain Reaction) conditions were adjusted according to the kit's standard protocol, including an initial denaturation step, followed by cycles of denaturation, annealing, and extension. The PCR products were then purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research) before sequencing (Retnowati *et al.,* 2017), and the results were analyzed using Basic Local Alignment Search Tool (BLAST) against the National Center for Biotechnology Information (NCBI) database. The reconstruction of the phylogenetic tree was based on the neighbor-joining algorithm on MEGA XI software with 1000x of bootstrap (Retnowati & Katili, 2023).

Data analysis

The data were analyzed using descriptive and quantitative methods. Data antagonist activity data is presented in a table showing the average ± standard deviation (SD). Meanwhile, the data on fungicide tolerance tests are presented in a table along with their descriptions. The 16S rRNA sequence data of actinomycetes strains were analyzed by comparing them with the NCBI GenBank database.

RESULTS AND DISCUSSION

Description of research location

The rhizosphere soil sampling was conducted in the Hungayono karst ecosystem in Tulabolo Village, East Suwawa Subdistrict, Bone Bolango District. Rhizosphere soil samples from the three locations exhibited varying physicochemical conditions (soil pH, temperature, and moisture) as well as vegetation (Table 1). The data showed that the highest humidity was recorded at site A (20%), while sites B and C showed similar values (10%). The soil acidity tends to similar on three sites about neutral conditions. The soil temperatures varied from 29°C to 31°C at sites A and B, up to a maximum threshold of 37°C at site C. The soil sampling site showed varying dominant vegetation. The vegetation components in the karst ecosystem at the 3 sampling locations vary. There are 3 dominant species, which include *Alocasia macrorrhizos*, *Ficus microcarpa*, and *Acrostichum aureum* L.

Table 1. Physicochemical characteristics and vegetation of the environment in the Hungayono karst ecosystem

Location site	Coordinate	Environmental parameters			Dominant Vegetation
		Temperature	pH	Humidity (%)	
A	0°30'19.4"N 123°17'29.5"E	29°C	6.8	20	*Alocasia macrorrhizos*
B	0°30'18.13"N 123°17'29.08"E	31°C	6.8	10	*Ficus microcarpa*
C	0°30'14.7"N 123°17'32.25"E	37°C	7	10	*Acrostichum aureum* L.

Description of Actinomycetes Isolates from the Rhizosphere of Plants in the Hungayono Karst, Gorontalo

The isolation and purification process of actinomycetes successfully yielded 9 pure isolates derived from 3 plant species (Table 2). These actinomycetes isolates exhibited distinct morphological characteristics based on colony shape, colony margins, colony texture, and colony color. However, the numbers suggest that the isolation was relatively limited in success, indicating that the diversity of actinomycetes is low. The actinomycetes isolates were then tested for their antagonistic activity against pathogenic fungi and their tolerance to fungicides.

Table 2. Source and host plant identification of actinomycetes isolates from the Hungayono karst ecosystem.

Isolate Code	**Sampling Site**	**Coordinate**	**Host Plant**	**Colony Shape**	**Color of The Mycelium**		**Source Sample**
					Areal	**Substrat**	
RzAM-01	A	0°30'19.4"N 123°17'29.5"E	*Alocasia macrorrhizos*	Circular	Gray	Red	Rhizosphere
RzPP-02	C	0°30'14.7"N 123°17'32.25"E	*Acrostichum aureum* L.	Irregular	White	White	Rhizosphere
RzOO-03	A	0°30'19.4"N 123°17'29.5"E	*Alocasia macrorrhizos*	Irregular	Orange	Orange	Rhizosphere
RzPH-04	C	0°30'14.7"N 123°17'32.25"E	*Acrostichum aureum* L.	Irregular	White	Black	Rhizosphere
RzHH-05	A	0°30'19.4"N 123°17'29.5"E	*Alocasia macrorrhizos*	Circular	Black	Black	Rhizosphere
RzUU-06	A	0°30'19.4"N 123°17'29.5"E	*Alocasia macrorrhizos*	Circular	Purple	Purple	Rhizosphere
RzAH-07	B	0°30'18.13"N 123°17'29.08"E	*Ficus microcarpa*	Irregular	Gray	Black	Rhizosphere
RzPC-08	C	0°30'14.7"N 123°17'32.25"E	*Acrostichum aureum* L.	Circular	White	Cream	Rhizosphere
RzPC-09	C	0°30'14.7"N 123°17'32.25"E	*Acrostichum aureum* L.	Circular	White	Cream	Rhizosphere

Antagonistic Activity of Actinomycetes Against Pathogenic Fungi

The antagonistic activity of actinomycetes isolates against *Fusarium oxysporum*-IPBCC 88 0012 and *Neocosmospora solani*-IPBCC 07 0525 was determined based on the formation of inhibition zones around the actinomycetes colonies. The test results showed significant variation in the ability to inhibit the growth of the pathogenic fungi *Fusarium oxysporum* and *Neocosmospora solani* (Table 3). Among the nine actinomycetes isolates tested, only three (RzPP-02, RzHH-05, and RzAH-07) exhibited antagonistic activity against the pathogenic fungi *Fusarium oxysporum* and *Neocosmospora solani.* The RzAH-07 isolate showed relatively higher inhibition zones of 5.22 mm against *Fusarium oxysporum*, classified as moderate, and 4.18 mm against *Neocosmospora solani*, classified as weak. Although RzAH-07 exhibited the highest activity among the other isolates, its antagonistic effect remained in the moderate-to-weak range under in vitro conditions. The other isolates (RzPP-02 and RzHH-05) produced smaller inhibition zones, ranging from 2.25 mm to 4.46 mm for *Fusarium oxysporum* and 0.82 mm to 1.49 mm for *Neocosmospora solani*, indicating overall limited antagonistic potential.

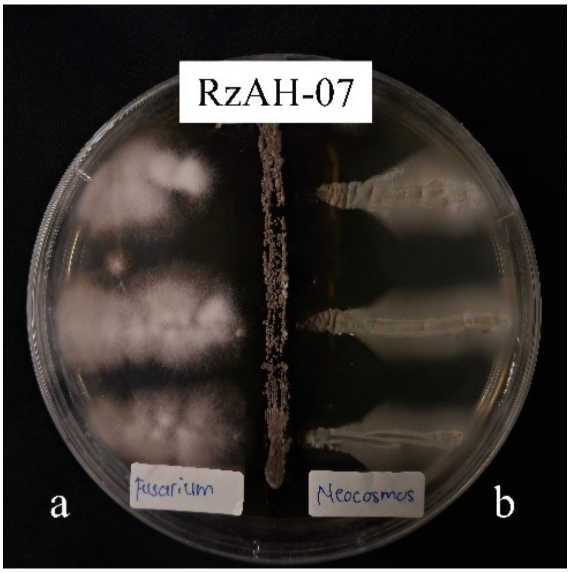


Figure 2. Antagonism test of RzAH-07 isolate against the growth of pathogenic fungi: (a) *Fusarium oxysporum* and (b) *Neocosmospora solani* using a modified Cross Streak method.

Table 3. Antagonistic Activity of Actinomycetes Against the Fungi *Fusarium oxysporum* and *Neocosmospora solani.*

Actinomycetes isolates	**Diameter of the inhibition zone (mm) (Mean ± SD)**			
	Fusarium oxysporum	**Category**	***Neocosmospora solani***	**Category**
RzAM-01	0.00 ± 0,00	-	0.00 ± 0,00	-
RzPP-02	2.25 ± 1,84	Weak	1.49 ± 0,38	Weak
RzOO-03	0.00 ± 0,00	-	0.00 ± 0,00	-
RzPH-04	0.00 ± 0,00	-	0.00 ± 0,00	-
RzHH-05	4.46 ± 3,43	Weak	0.82 ± 0,72	Weak
RzUU-06	0.00 ± 0,00	-	0.00 ± 0,00	-
RzAH-07	5.22 ± 2,59	Moderate	4.18 ± 2,17	Weak
RzPC-08	0.00 ± 0,00	-	0.00 ± 0,00	-
RzPC-09	0.00 ± 0,00	-	0.00 ± 0,00	-

Notes : These values are the means of three replicates ± SD.

Fungicide Tolerance of Actinomycetes

The tolerance of actinomycetes to fungicides was tested using two types of fungicides, namely Thiram and Benomyl, at concentrations of 100, 200, 300, 400, and 500 ppm. Tolerance was expressed as a percentage of RIR (Relative Inhibition Rate). The results showed that tolerance to fungicides varied among the 9 actinomycetes isolates (Table 4). There were four actinomycetes isolates (RzOO-03, RzUU-06, RzPC-08, and RzPC-09) that showed tolerance to Thiram up to 500 ppm in varied categories. The RzOO-03 showed 14% RIR in the very strong category, while the RzUU-06, RzPC-08, and RzPC-09 achieved RIR values of 42% to 57 in the strong categories. There were eight actinomycetes isolates (RzAM-01, RzOO-03, RzPH-04, RzHH-05, RzUU-06, RzAH-07, RzPC-08, and RzPC-09) that showed tolerance to Benomyl up to 500 ppm on varied categories. There were six actinomycetes isolates (RzAM-01, RzOO-03, RzPH-04, RzHH-05, RzUU-06, and RzAH-07) s showing a RIR percentage of 24% to 40% with a very strong category, while RzPC-08, and RzPC-09 were in strong categories with RIR values on 44%. The negative RIR values indicate that the growth of these isolates on the treated medium was greater than their growth on the control medium.

Table 4. Fungicide Tolerance Ability 100-500 ppm.

Actinomycetes isolates	% Relative Inhibition Rate Actinomycetes at various fungicide concentrations									
	Thiram (ppm)					Benomyl (ppm)				
	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
RzAM-01	2	5	12	39	100	-6	9	15	18	24
RzPP-02	17	100	100	100	100	45	46	48	49	100
RzOO-03	15	-10	9	14	14	2	18	19	27	32
RzPH-04	16	100	100	100	100	17	17	23	24	34
RzHH-05	-22	-3	17	23	100	-2	1	9	16	25
RzUU-06	12	25	26	43	57	31	32	36	39	40
RzAH-07	5	9	17	73	100	13	33	19	24	29
RzPC-08	11	16	24	44	42	32	36	36	25	44
RzPC-09	30	28	26	41	52	35	39	49	41	44

Note: nd = Not detected; Very Strong Tolerance (RIR <40%); Strong Tolerance (40–60%); Moderate Tolerance (60–75%); Weak Tolerance (>75–90%); and Very Weak Tolerance (>90%).

The one actinomycetes isolate (RzOO-03) showed lowest RIR value to Thiram, and six actinomycetes isolates lowest RIR value to Benomyl, then further assays on 600 up to 1000 ppm of both fungicides. The result showed that the RzOO-03 actinomycetes isolate had no tolerance to 600 up to 1000 ppm of Thiram fungicide. Meanwhile, for the fungicide Benomyl, there were 2 isolates (RzHH-05 and RzAH-07) among 6 actinomycetes isolates that showed tolerance up to 1000 ppm, but between two isolates, the RzAH-07 maintained a very strong tolerance category at 1000 ppm benomyl, with an RIR of 27% (Table 5).

Table 5. Tolerance to Fungicides at 600–1000 ppm.

Actinomycetes isolates	% Relative Inhibition Rate of Actinomycetes at Various Concentrations of the Fungicide Thiram				
	600 ppm	700 ppm	800 ppm	900 ppm	1000 ppm
RzOO-03	100	100	100	100	100

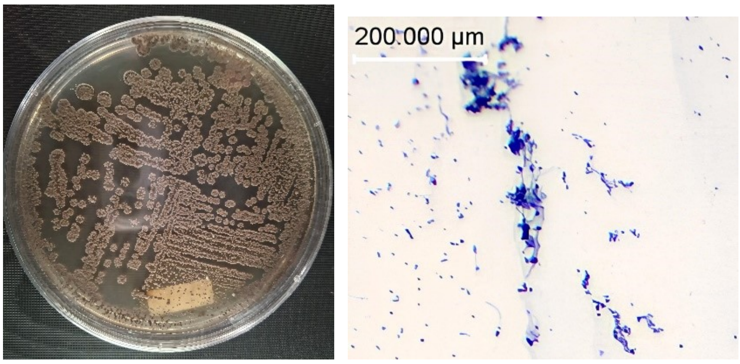
Actinomycetes isolates	% Relative Inhibition Rate of Actinomycetes at Various Concentrations of the Fungicide Benomyl				
	600 ppm	700 ppm	800 ppm	900 ppm	1000 ppm
RzAM-01	11	13	18	26	100
RzOO-03	14	26	33	33	100
RzPH-04	35	39	34	42	100
RzHH-05	4	16	16	18	28
RzUU-06	14	32	36	39	42
RzAH-07	20	21	26	25	27

Note: nd = Not detected; Very Strong Tolerance (RIR <40%); Strong Tolerance (40–60%); Moderate Tolerance (60–75%); Weak Tolerance (>75–90%); and Very Weak Tolerance (>90%).

Phylogenetic Analysis of Actinomycetes Isolates

Based on data on antagonistic activity against pathogenic fungi and their tolerance to synthetic fungicides, there is one potential actinomycetes isolates, namely RzAH-07. Because this isolate has a dual ability and is able to tolerate up to the highest concentration of benomyl. The

RzAH-07 isolate was then subjected to further microscopic observation of cell morphology. The actinomycetes isolate RzAH-07 exhibits morphological characteristics consisting of filaments of cell morphology and a purple color of Gram staining, which are typical of Gram-positive bacteria. These characteristics indicate that the isolate belongs to the actinomycetes group (Figure 3). The morphological characteristics of actinomycetes confirmed based on molecular analysis of 16S rRNA gene sequence to conduct their phylogenetic relationships. Phylogenetic analysis was based on methods including genomic DNA extraction, 16S rRNA gene amplification, sequencing, and phylogenetic tree reconstruction using the Neighbor-joining algorithm.



(a) (b)

Figure 3. (a) Macroscopic morphology of the RzAH-07 isolate colony and (b) microscopic morphology of filaments of cell isolate RzAH-07.

The extraction of actinomycetes DNA genomic using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research) yielded purity levels of 1.96 and 2.00 with a concentration of 175.9 ng/µl. The results indicated that genomic DNA extraction could proceed to 16S rRNA gene amplification using the universal primers 27F and 1492R. Visualization on agarose gel electrophoresis showed the presence of a DNA band at 1500 bp, similar to the 16S rRNA gene. The sequencing of 16S rRNA gene from the RzAH-07 actinomycetes isolate showed a DNA fragment length of 1,394 bp. The 16S rRNA gene sequence from the isolate (RzAH-07) was then compared with sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) program on the National Center for Biotechnology Information (NCBI) website, and showed that the RzAH-07 isolate is closely related to the genus *Streptomyces*, with a similarity percentage ranging from 98.35% to 99.14% based on the GeneBank database (Table 4). Phylogenetic reconstruction was performed in MEGA11 using the Neighbor-joining method with the Kimura 2-parameter model and 1,000 bootstrap analyses, indicating that isolate RzAH-07 is closely related to the genus *Streptomyces*, forming a clade together with the strains *Streptomyces carpaticus* NBRC 15390 & NRRL B-16359, *Streptomyces harbinensis* NEAU-Da3, and *Streptomyces ginkgonis* KM-1-2, with a bootstrap value of 98 (Figure 4).

Table 6. BLAST results for actinomycetes isolate RzAH-07 (PZ458432) with its closest match from the GenBank database.

Nearest phylogenetic neighbor	**Scientific name**	**Percent identity**	**E-value**	**Accession code**
Streptomyces ginkgonis strain KM-1-2 16S ribosomal RNA	*Streptomyces ginkgonis*	99.14%	0.0	NR_181625.1
Streptomyces ginkgonis strain KM-1-2 16S ribosomal RNA	*Streptomyces ginkgonis*	99.07%	0.0	NR_178933.1
Streptomyces harbinensis strain NEAU-Da3 16S ribosomal RNA	*Streptomyces harbinensis*	99.00%	0.0	NR_109622.1
Streptomyces carpaticus strain NRRL B-16359 16S ribosomal RNA	*Streptomyces carpaticus*	98.57%	0.0	NR_043814.1
Streptomyces carpaticus strain NBRC 15390 16S ribosomal RNA	*Streptomyces carpaticus*	98.35%	0.0	NR_112450.1

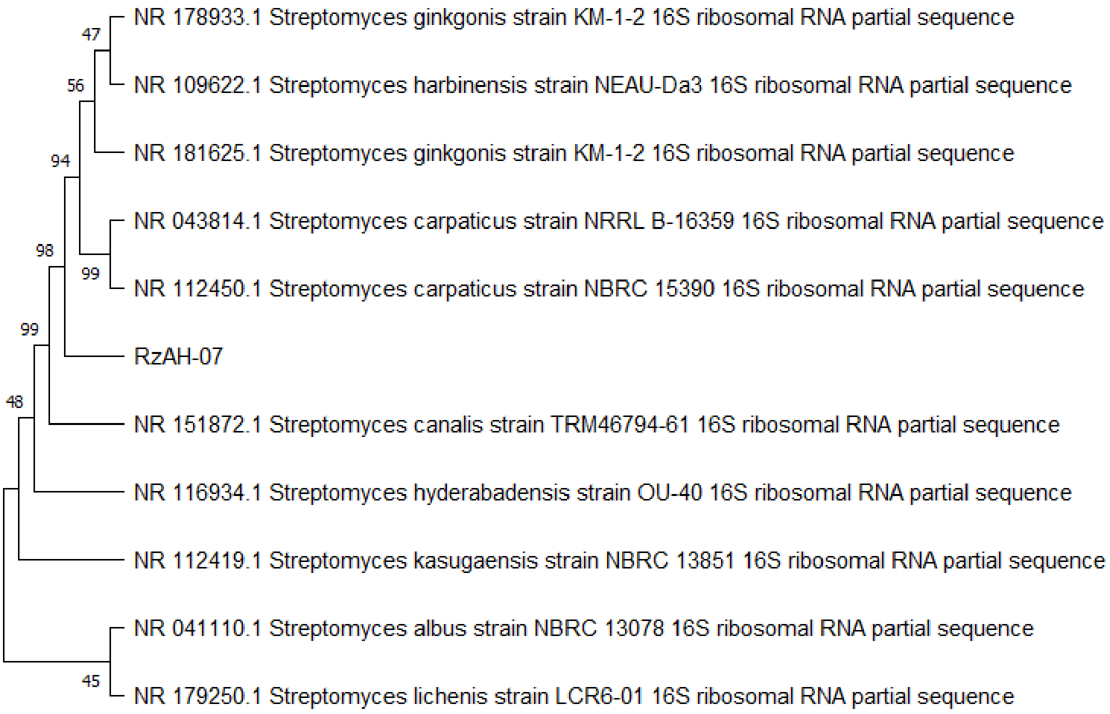


Figure 4. Phylogenetic tree of the RzAH-07 (PZ458432) isolate reconstructed based on the neighbor-joining algorithm using bootstrap 1000x.

Discussion

The Hungayono karst ecosystem has unique soil physicochemical characteristics and varying slopes, thereby providing a variety of ecological niches for actinomycetes. In this study, nine types of actinomycetes isolates were successfully isolated from the rhizosphere soil of *Alocasia macrorrhizos, Ficus microcarpa,* and *Acrostichum aureum* L. in the Hungayono karst ecosystem. Previous research has shown that actinomycetes are a group of bacteria capable of growing under extreme environmental conditions (Retnowati *et al.,* 2024). However, these findings suggest that the presence of actinomycetes in this karst region is likely influenced by ecological adaptation. This is due to the measured physicochemical parameters, with a fairly neutral pH range (6.8–7), temperature (29–37 °C), and humidity (10–20%). These ranges fall within the optimal range for actinomycete growth, as reported by Ngamcharungchit *et al.* (2023); actinomycetes can survive under pH conditions within the optimal pH range (6.5–8.0) and at temperatures of 20–37 °C (Dewi *et al.,* 2024).

The presence of actinomycetes in the rhizosphere is also likely influenced by interactions with the host plant. This is supported by the findings of Retnowati *et al.* (2024) that the association of actinomycetes in the plant rhizosphere is influenced by root exudates produced by plant roots. Root exudates have a varied composition that can modify pH, thereby increasing nutrient availability even under extreme conditions (Hugoni *et al.,* 2018). According to Lamichhane *et al.* (2024), root exudates are rich in sugars, amino acids, organic acids, vitamins, nucleosides, phytociderophores, phenolic compounds, fatty acids, and others, including unidentified compounds. This is further emphasized by Yadav *et al.* (2018), who note that actinomycetes colonize the rhizosphere, which possesses high nutritional value due to the exudation, secretion, and deposition of important compounds from the roots, such as amino acids, fatty acids, organic acids, phenols, sterols, sugars, and vitamins.

Actinomycetes in rhizospheric soil have a high potential in producing bioactive compounds (Praptiwi *et al.,* 2023). Consistent with this statement, the antagonistic testing of actinomycetes in this study showed that 33% of the isolates were antifungal-producing actinomycetes. Among the 9 isolates tested, 3 isolates (33%) exhibited antagonistic activity against the test pathogenic fungi. This percentage is relatively low and consistent with the findings of Meliani *et al.* (2022), who successfully isolated 45 actinomycetes isolates from soil in the rhizosphere zone of colocynth (*Citrullus colocynthis*) in the Sahara, Morocco; of these isolates, only 12 were capable of inhibiting the growth of the pathogens Aspergillus flavus and *Fusarium oxysporum* f. sp. *albedinis*. Additionally, Shirokikh's (2019) study identified 48 actinomycetes isolates from three locations: the Nurgush State Nature Reserve (Russia), Belovezhskaya Pushcha National Park (Belarus), and the Novyi Svet Nature Reserve (Crimea); only 40% of the isolates were capable of inhibiting pathogen growth, including *Fusarium oxysporum*. This indicates that antagonistic ability is likely a trait specific to certain isolates, rather than a general trait of karst actinomycetes.

Antagonism testing using the cross-streak method showed that isolate RzAH-07 was capable of inhibiting the growth of *Fusarium oxysporum* and *Neocosmospora solani* relatively well, although the level of inhibition was classified as moderate to weak. Evidence of this isolate's antagonism was demonstrated by the presence of clear inhibition zones around the colonies. The presence of these inhibition zones indicates that this isolate produces active antifungal compounds. Several previous studies have shown that actinomycetes inhibit fungal growth. For example, *Streptomyces cellulosae* strain JKP5 was reported in the study by Fatmawati et al. (2018) to be capable of inhibiting the growth of *Fusarium oxysporum* by 42.8%, but was unable to inhibit Sclerotium rolfsii. Meanwhile, the study by Al-Dhabi et al. (2019) identified a potential strain of *Streptomyces* sp., Al-Dhabi30, which exhibited inhibition zones of 16–22 mm against plant pathogens (*F. oxysporum, A. niger, A. flavus, B. orizae,* and *F. solani*).

Variations in antagonistic ability may also be influenced by the resistance of pathogenic fungi to secondary metabolites produced by actinomycetes. As reported by Al-Hatmi *et al.* (2016), pathogenic fungi can reduce the effects of antifungal metabolites via efflux pumps (AMB/MFS transporters) that can expel foreign compounds (xenobiotics) from fungal cells, such as *Fusarium* (Bai *et al.,* 2024). However, this mechanism has not been tested on the fungal isolates used in this study. The results of the antagonistic assay also showed high inter-replicate variability, indicating that the interactions observed were not entirely stable under in vitro conditions. Furthermore, this is a limitation of the cross-streak method that leads to the formation of inconsistent inhibition zones.

Actinomycetes isolated from the rhizosphere soil in the Hungayono karst ecosystem, Gorontalo, exhibited varying levels of tolerance to two types of fungicides (thiram and benomyl) at concentrations of 100–1000 ppm. Interestingly, the results showed a negative RIR value of up to -35%, indicating higher growth on the treated media compared to the control. This phenomenon may be due to differences in colony growth rates or media composition. Another possibility is that the isolates utilize the fungicides as a carbon source and/or activate detoxification mechanisms such as efflux pumps (Yin *et al.,* 2023). However, this is merely a hypothesis and requires further testing. Among the tested isolates, RzAH-07 exhibited the most promising combined profile, although its antagonistic activity remained moderate to weak under in vitro conditions. This dual capability is crucial for biological control strategies, as RzAH-07 has the potential to remain functional even when applied concurrently with synthetic fungicides. Although Benomyl (*single-site fungicide*) and thiram (*multi-site inhibitor*) have different mechanisms of action (Campanale *et al.,* 2023), RzAH-07's ability to persist suggests a superior defense mechanism, likely through drug inactivation and target modification (De Simeis & Serra, 2021). However, these tolerance results can only serve as an initial screening and require field-scale efficacy testing to validate the potential of the isolates.

Phylogenetic analysis based on the 16S rRNA gene indicates that isolate RzAH-07 forms a clade with the strains *Streptomyces carpaticus* NBRC 15390 & NRRL B-16359, *Streptomyces harbinensis* NEAU-Da3, and *Streptomyces ginkgonis* KM-1-2. *Streptomyces carpaticus* is known to produce secondary metabolites exhibiting antimicrobial properties (ohmyungsamycin, pellasoren, naringenin, and ansamycin), which have been identified in its genome (Bataeva et al., 2024). *Streptomyces harbinensis* exhibits metabolites important for agriculture, including terpenes (geosmin, lycopene, albaflavenone, and carotenoids), alkylresorcinols, lanthipeptides, butyrolactones, polyoxypeptides, ectins, as well as desferrioxamine B and E, and naringenin (Babalola et al., 2025). Additionally, *Streptomyces ginkgonis* is a newly described species (Yin et al., 2018) for which metabolite data remain limited. However, the study by Yin et al. (2018) revealed its closeness to *S. carpaticus* JCM 6915T (99.3%), *S. harbinensis* (98.9%), and *S. cheonanensis* (98.5%). Thus, this reference supports the capabilities of isolating RzAH-07 in this study.

CONCLUSIONS

In conclusion, this study successfully isolated nine actinomycete isolates from the rhizosphere of the Hungayono karst in Gorontalo. Approximately 3 isolates (33%) exhibited inhibitory activity against *Fusarium oxysporum* and *Neocosmospora solani*, with the best isolate, RzAH-07, showing inhibition zones of 5.22 mm and 4.18 mm. Isolate RzAH-07 also demonstrated tolerance to thiram up to 500 ppm and benomyl up to 1000 ppm. Molecular analysis based on the 16S rRNA gene indicated that isolate RzAH-07 belongs to the genus *Streptomyces*, specifically closely related to *Streptomyces carpaticus* NBRC 15390 & NRRL B-16359, *Streptomyces harbinensis* NEAU-Da3, and *Streptomyces ginkgonis* KM-1-2. This research is still limited to in vitro testing. Therefore, further validation will be needed, such as characterization of bioactive compounds and field testing, before these isolates can be evaluated for their potential as biological control agents.

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