Screening Indigenous Actinobacteria for Biocontrol of Ralstonia syzygii and Enhancing Chili Plant Growth

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Abstract

Bacterial wilt caused by *Ralstonia syzygii* subsp. *indonesiensis* is an important disease of chili plants that is difficult to control. Actinobacteria derived from the rhizosphere of chili plants have the potential as biological control agents of R. *syzygii* subsp. *indonesiensis*. The purpose of the study was to obtain actinobacteria isolates that have the potential to control bacterial wilt disease in planta and increase the growth and production of chili plants. The research consisted of four stages: 1.) Isolation and characterization of actinobacteria isolates, 2.) Inhibition test of actinobacteria isolates from the rhizosphere of chili plants against *R. syzygii* subsp. *indonesiensis* in vitro 3). The ability of selected actinobacteria to control *R. syzygii* subsp. *indonesiensis* and increase the growth and production of chili plants 4). Molecular identification of selected actinobacteria from the rhizosphere of chili plants. The results obtained 21 isolates of actinobacteria from the rhizosphere of chili plants. After the biosafety test, 14 isolates could be used in the next test. Actinobacterial isolates that can suppress *R. syzygii* subsp. *indonesiensis* and produce enzymes and growth-promoting hormones in as many as 12 isolates. A total of 10 best actinobacterial isolates suppressing the development of R. *syzygii* subsp. *indonesiensis* are RZATABY 7.1, RZATABY 8.11, RZATABY 8.3, RZATABY 8.5, RZATABY 8.6, RZATABY 7.4, RZATBY 8.4, RZATBY 8.8, RZATPY 8.5, and RZATPY 8.6. The best actinobacteria isolate in increasing the growth and yield of chili plants was RZATPY 8.5, with an increase in plant height of 15.97 cm, an increase in the number of leaves 32 strands, accelerating the emergence of flowers 24.4 days and an increase in fresh fruit weight 126.33 g compared to control plants. 16sRNA sequence homology test results from the ten best actinobacteria isolates belong to *Streptomyces* spp. and *Amicolatopsis* sp. GLM4.

Keywords Actinobacteria, bacterial wilt, *Ralstonia syzygii* subsp. *Indonesiensis*

Introduction

Bacterial wilt disease caused by *Ralstonia syzygii* subsp. *indonesiensis* is one of the important diseases in chili plants (Yanti *et al*., 2018). Bacterial wilt disease can cause plant death (Adriani *et al*., 2012; Ahmed *et al*., 2022) and cause losses of up to 90% (Palupi *et al*., 2015). Bacterial wilt disease quickly develops and spreads from infected plants to other plants, making it difficult to control. Efforts to control bacterial wilt disease have been made, namely crop rotation, resistant varieties, technical culture, and synthetic chemicals. Using synthetic chemicals from the bactericide group is a negative environmental risk because it can cause residues and bacterial resistance to bactericides. Thus, environmentally friendly control alternatives are needed, such as control using antagonistic microorganisms (Glare *et al*., 2012).

Antagonistic microorganisms that can be utilized as biological agents are actinobacteria (Subramaniam *et al*., 2016). Using biological agents such as actinobacteria is one solution to reduce the dangers of using synthetic bactericides that are intensively carried out by the community. The principle of using biological agents is not destructive and environmentally friendly (Aamir *et al*., 2019). Actinobacteria also indirectly function in the formation of humus and produce antibiotics that can destroy plant and animal residues, so using biological agents is safer for environmental conditions (Bhatti *et al*., 2017).

Actinobacteria are Gram-positive bacteria that can produce bioactive compounds and are antimicrobial in nature. (Anandan *et al*., 2016). Actinobacteria are also known to produce various types of secondary metabolite compounds and active compounds such as enzymes and antibiotics (Boukhatem *et al*., 2022). Actinobacteria include bacteria that are dominant in the soil, can overgrow, and have an important role in protecting plants from pathogen attacks. Actinobacteria can also produce plant growth hormones (Loqman *et al*., 2009). Actinobacteria can increase plant growth with two mechanisms, namely, directly and indirectly (Mingma *et al*., 2014). According to Goudjal *et al*. (2016), actinobacteria increase plant growth by the direct mechanism by producing chitinase enzymes and *b -1.3-glucanase* that can degrade microbial cell walls. Actinobacteria have the potential to dissolve phosphate, siderophore production, IAA (Indole Acetic Acid) production, ammonia production, and other lytic enzymes (Jog *et al*., 2012). Actinobacteria can act as biocontrol agents of pathogenic bacteria and fungi in plants (Mingma *et al*., 2014) and are known as *plant growth-promoting actinobacteria (*PGPA) (Kumar & Dubey, 2020). Actinobacteria can increase plant height, root length, dry weight, and photosynthetic pigments. Actinobacteria can produce phytohormones (Auxin, gibberellins, and cytokinins), siderophores, ammonia, dissolve phosphate and produce hydrogen cyanide (Chukwuneme *et al*., 2020). Actinobacteria from the streptomyces group are the most widely reported genus capable of controlling pathogens and increasing plant growth (Selim *et al*., 2019). The mechanism of Streptomyces in inhibiting plant pathogens is through competition that occurs in the rhizosphere, hyperparasitism, auxin expression, and produces indole-3-acetic acid (IAA) and antimicrobial metabolite compounds (Toumatia *et al*., 2015). Rubin *et al*. (2017) reported that *Streptomyces* sp. showed significant antibacterial activity against the development of Gram-negative bacteria such as *Xanthomonas* sp. *Streptomyces thermocarboxydus* isolates were shown to be able to inhibit the fungus *Fusarium oxysporum,* which causes late blight on aloe vera by 93.4% in vitro, while in vivo in the greenhouse, the isolate was able to suppress the pathogen by 70% (Jones & Elliot, 2017). The research aimed to obtain actinobacterial isolates that have the potential to control bacterial wilt disease and increase the growth and production of chili plants.

MATERIALS AND METHODS

Isolation of Actinobacteria from Chili Rhizosphere

Soil samples from the roots of healthy chili plants aged 6-10 weeks around chili plants with bacterial wilt symptoms. Sampling locations were taken using a purposive sampling method from chili production centers and endemic areas of bacterial wilt in West Sumatra, namely Jorong Koto Baruah, Nagari Aia Batumbuak, Gunung Talang District, Solok Regency; Jorong Koto Tuo, Nagari Panyalaian, X Koto District, Tanah Datar Regency and Jorong Simarasok, Nagari Simarasok, Baso District, Agam Regency Fig 1. A total of 10 g of soil sample was suspended in 100 ml of sterile distilled water and then homogenized using a *rotary shaker* for 15 minutes. Furthermore, serial dilutions were carried out with a dilution level of 10^{-1}-10^{-8}; 1 ml of dilution 10^{-7} and 10^{-8} were inserted into liquid water-yeast-agar (WYA) media as much as 1 ml each and then poured into a petri dish and then incubated for 5x24 hours. Single colonies of actinobacteria that grow then purified on the same medium.

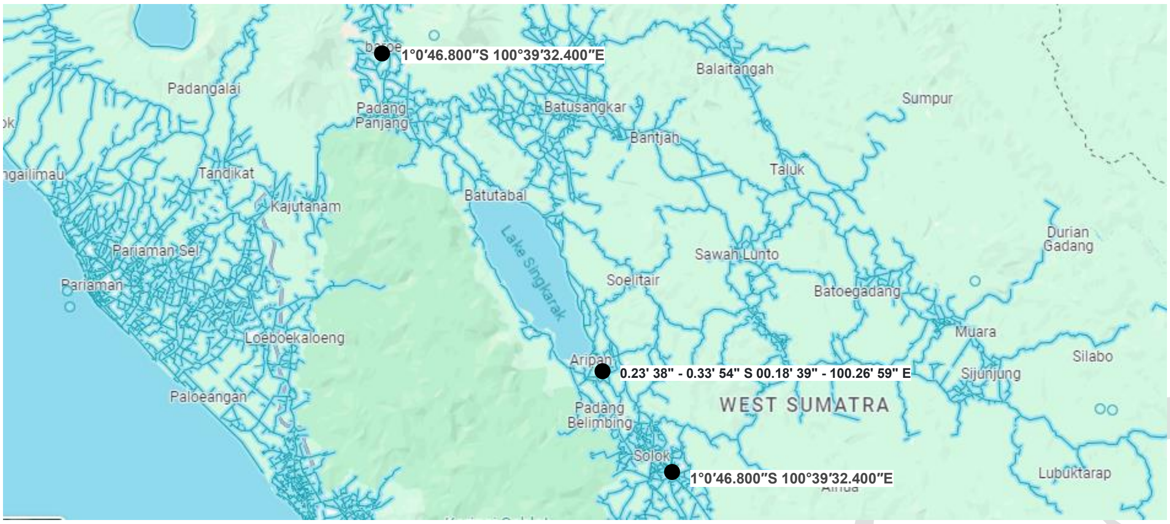

Fig 1. Geographic map of districts in West Sumatra ten locations surveyed for documentation of bitter rot disease and sample collection.

Biosafety test

a. Hypersensitivity Reaction Test

Hypersensitive reaction test aimed to determine whether the actinobacteria is classified as pathogenic or not. The density of spores in it reaches 10^6 spore cells/ml, calculated using a hemocytometer (Kawuri, 2012). The actinobacterial suspension was infiltrated on the lower surface of four o'clock flower plants (*Mirabilis jalapa*) leaves until saturated and incubated for 2x24 hours. Hypersensitive reactions are seen when no necrotic symptoms appear on the leaves infiltrated by actinobacteria (Yanti *et al*., 2017).

b. Pathogenicity Test

Pathogenicity tests were carried out on healthy chili fruits that were surface sterilized first by rinsing with sterile distilled water, soaking with 1% NaOCl for 2 minutes, and then rinsing with sterile distilled water. Chili fruit was dried and pierced with a sterile needle, and 10^8 spores/ml actinobacteria were applied in the center of the chili fruit and incubated for six days at room temperature. Isolates that cause necrotic in chili fruit are not used for further testing because they can potentially be pathogens in chili (Yanti *et al*., 2020).

c. Hemolysis Test

Actinobacterial isolates were cultured on a blood agar medium (sheep blood agar 5%) and incubated for five days. There are three types of hemolysis, namely, hemolysis a, b and g . Isolates that produce a or b hemolysis reactions were not used for further testing because they could be potentially pathogenic to humans and mammals (Soumare *et al*., 2021).

Inhibition Test of Actinobacterial Isolates from Chili Rhizosphere against *Ralstonia syzygii* subsp. *indonesiensis*

Inhibition test of actinobacterial isolates against chili plant pathogen causing bacterial wilt (*Ralstonia syzygii* subsp. *indonesiensis*) using *agar plug* method. Seed cultures of *Ralstonia syzygii* subsp. *indonesiensis* were grown on TZC media with an incubation time of 24 hours at 37°C on a rocking incubator at 150 rpm. Then, 1 mL of seed culture was mixed in 100 mL of TZC media and poured into Petri dishes. Agar plugs containing actinobacterial cultures on WYA solid media with an incubation period of 7 days were removed with a 6 mm diameter punch shortly after the seed media solidified. Then, the antagonist media was incubated for 48 hours to see the inhibition zone.

Characterization of Chili Rhizosphere Actinobacteria In Vitro

a. IAA Production Test

Agar pieces (0.5 cm in diameter) from 5-day-old actinobacterial cultures grown on ISP2 agar were transferred into 18 × 180 mm^2 test tubes containing 5 mL of ISP2 broth. A 2% solution of L-tryptophan (Sigma-Aldrich, Beijing, China) was sterilized and added to the ISP2 broth. The culture was then inoculated, incubated in the dark at 30^0C, and homogenized using an orbital shaker at 150 rpm for one week. The supernatant was harvested by centrifugation at 11,000 rpm for 15 min. The reaction was evaluated for IAA production using a colorimetric assay by mixing the supernatant with Salkowski's reagent (1 mL of 0.5 M FeCl3 in 49 mL of 35% (w/v) HClO4). A pink-to-red color indicates IAA production.

b. Siderophore Production

Siderophore production was determined by placing a dotted line in the center of Chrome Azurol S agar (60.5 mg). Chrome azurol S was dissolved in 50 mL of distilled water and mixed with 10 mL of $FeCl_3.6H_2O$ solution (1 mM $FeCl_3.6H_2O$+10 mM HCl). 72.9 mg HDTMA was dissolved in 40 mL aqua dest; the solution was then added to 900 mL King's B agar (Hymedia®, per liter containing 20 g protease peptone, 1.5 g K2HPO4, 1.5 g MgSO4, 15 g agar, and glycerol pH 7.2)) and incubated at 28°C for five days. The medium's color change from blue to orange indicates siderophore activity (Mirza *et al*., 2001).

c. Ammonia Test

Each selected actinobacteria was put into a medium containing 10 mL of peptone water (1%) and incubated for 48-72 hours at 28±2 C. Then, 0.5 mL of Nessler reagent (SigmaAldrich® - HgI4K2 0.09m/L) was added, which changed the color to yellow. The color change to brown was ammonia production (Chen *et al*., 2015).

d. Protease activity

Proteases were tested on Luria Bertani Broth medium (composition per liter containing 10 g Casein enzyme hydrolyzate, 5 g yeast extract, 10 g NaCl, pH 7.5 (HiMedia®)), and modified with 2% skim milk powder (Nestle) and 15 g agar. A clear zone appeared around the actinobacterial colonies, indicating protease activity (McDonald and Chen, 1965).

e. Chitinolytic Activity Test

Actinobacterial isolates were streaked on solid chitin media (3 g colloidal chitin, 1 g K2HPO4, 0.2 g MgSO4.7H2O, 1 g yeast extract, 20 g agar and 1 L distilled water). Incubation was carried out for six days at 37°C. Observations were made by looking at the clear zone around the colony, which indicates the solubility of chitin by the isolate (Gherbawy *et al*., 2012).

Ability of Selected Actinobacteria to Control *R. syzygii* supsp. *indonesiensis* and Increase Chili Plant Production in planta

Actinobacteria selected from in vitro selection were then used to see their ability to suppress the development of R. syzygii subsp. Indonesiensis, increase the growth and yield of chili plants using a completely randomized design (CRD). A total of 12 isolates were used, with six replicates. The treatments were ten isolates of actinobacteria, positive control (without inoculation treatment of R. *syzygii* subsp. *indonesiensis*), negative control (with inoculation treatment of *R. syzygii* subsp. *indonesiensis*). The placement of experimental units was randomized.

Actinobacteria Propagation

Pure isolates of actinobacteria from microtubes were rejuvenated by scratch method in WYA medium and incubated 5x24 hours. One single colony of actinobacteria was inserted into 25 ml of WY Broth medium in a culture bottle and then incubated for 7x24 hours on a rotary shaker at 70 rpm (Kawuri *et al.,* 2012). Population density was determined with a suspension of actinobacteria diluted until the spore density reached 10^6 spore cells/ml. A population with a density of 10^6 spore cells/ml was introduced.

Chili Seedling Planting and Actinobacteria Introduction

Chili seedlings were transplanted to the field 21 days after seedling (das). The selected seedlings were the seedlings selected in the previous stage. The roots of chili seedlings were immersed in a suspension of actinobacteria with a density of 10^6 spore cells/ml for 15 minutes. The seedlings were then planted into polybags containing sterile soil.

Propagation of *R. syzygii* subsp. *indonesiensis*

R. syzygii subsp. *indonesiensis* isolates were propagated using the scraping technique on TZC media and then incubated for 2x24 hours.

Pathogenicity Test

The pathogenicity test of *R. syzygii* subsp. *indonesiensis* used the Yanti *et al*. (2017) method. Bacterial inoculation was carried out 35 days after planting (dap) chili plants by loosening the chili root soil first, cutting the roots on two sides of the chili plant, and then pouring bacterial suspension with a bacterial population density of 10^7 cells/ml.

Inoculation of *R. syzygii* subsp. *indonesiensis*

The soil around the roots of chili plants was loosened first to facilitate inoculation of the pathogen *R. syzygii* subsp. *indonesiensis*. Root openings of chili seedlings aged 35 dap were done by cutting the roots on both sides of the plant. Next, 30 ml of *R. syzygii* subsp. *indonesiensis* suspension was watered to the plants at a density of 10^7 cells/ml (Klement *et al*., 1990).

Molecular identification of selected Actinobacteria

The genomic DNA of selected Actinobacteria was extracted using a Genomic DNA Mini Kit (Blood/Cultured Cell) extraction kit. The 16S rRNA gene sequence was amplified using universal primers of the 16S rRNA gene for bacterial domain 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 387r (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al*. 1998) with a target length of ~1300 pb amplicons. The PCR reaction mix for a total volume of 50 µL consisted of Go Taq Green 25 µL, reverse and forward primers 4 µL each, DNA 8 µL and nuclease-free water 9 µL. PCR conditions began with initial denaturation for 4 minutes at 94°C followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, elongation for 1 minute at 72°C and final elongation for 7 minutes at 72°C.

Genomic DNA and PCR products were electrophoresed to determine the product size and quality. Electrophoresis was performed using 1% agarose gel and migration at 80 V for 45 minutes. Furthermore, the gel was soaked in Ethidium Bromide (EtBr) for 15 minutes and then visualized on a UV transilluminator. PCR products were sequenced through the First Base sequencing service. Sequencing results were aligned with GenBank data using the Basic Local Alignment Search Tool- Nucleotida (BlastN) program from the National Center for Biotechnology Information (NCBI) website. Phylogenetic analysis was performed using the MEGA 6.0 program with the Neighbor Joining (NJ) method with a 1000X bootstrap**.**

Observation

a. Morphology and Biosafety of Actinobacteria

Parameters observed were substrate mycelium, aerial mycelium, elevation, edge, size, hypersensitivity reaction, pathogenicity, and hemolysin.

b. Inhibition of Actinobacteria of chili plant rhizosphere against *R. syzygii* subsp. *indonesiensis*

The parameter observed was the diameter of the clear zone around the growing actinobacterial colonies. Observations were made after the culture was incubated for 48 hours. Calculation of inhibitory power to calculate the inhibitory index using the formula

$$\text{Inhibition index} = \frac{\text{clear zone diameter - disk diameter}}{\text{disk diameter}}$$

c. Characterization of rhizosphere actinobacteria of chili plants in vitro

The ability test to produce enzyme chitinase, dissolve phosphate, produce IAA, and tether nitrogen. Actinobacteria selected further tested its ability to produce chitinase enzyme using the method of Hsu and Locwood (1975), produce IAA with the method of Gordon and Weber (1951), siderophore production, ammonia, and protease activity tested on Luria Bertani Broth media.

d. Disease progression

i. Incubation Period (Days after Inoculation)

The incubation period was observed every day after inoculation of *R. syzygii* subsp. *indonesiensis* until the plants showed the first wilting symptoms of chili.

ii. Disease incidence (%)

Disease incidence was the proportion of pathogen-affected plants in a plant population. Disease incidence was observed every day after inoculation until the first symptoms appeared. Disease incidence was calculated using the formula:

$$KP = \frac{n}{N} \times 100\%$$

Description: KP : disease incidence (%)
n : number of affected plants
N : number of plants observed

iii. Disease Severity (%)

Observations of disease severity were carried out simultaneously with observations of disease incidence by counting wilted leaves every week until the chili plants died. The percentage of disease severity can be calculated using the formula:

$$S = \frac{\sum(n \times v)}{N \times V} \times 100\%$$

Description: S : Disease Severity
n : Number of plant leaves in each scoring
v : Scale value of disease attack per individual plant
V : Highest value of damage category
N : Number of plant leaves observed

The bacterial wilt disease severity scale used the modified Fegan *et al*. (1998) scale to calculate the severity of bacterial wilt disease in chili plants (Table 1).

Table 1. Scale and criteria for measuring disease severity:

Scale	**Attack Rate (%)**	**Criteria**	**Documentation**
0	no wilting symptoms	Healthy	

1	1-25% leaf wilting	Lightweight	
2	26-75% leaf wilting	Medium	
3	76-100% leaf wilting	Weight	

e. Plant Growth (Generative Phase)

i. First Flower Appearance (Days)

The first flower appearance was observed on the first day of flower appearance on each chili plant.

ii. Fruit Weight (g)

The harvested chili fruits were weighed and totaled each harvest.

Data Analysis

Data were analyzed using variance using Statistix 8 software, if significantly different, followed by Duncan's Multiple Range Test (DMRT) at the 5% level.

RESULTS AND DISCUSSION

Actinobacteria isolates that have been isolated from the roots of chili plants obtained as many as 21 isolates. The substrate mycelium color is generally white and gray, while the aerial mycelium is generally green (Table 2).

Table 2. Morphological diversity of Actinobacteria isolates and biosafety test

No.	Isolate	Substrate mycelium	Aerial mycelium	Elevation	Edge	Size	HR Test	Pathogenicity Test	Hemolysis Test
1	RZATABY	Gray	Green	Undulate	Convex	2 mm	-	-	-

	8.12								
2	RZATABY 8.8	Gray	Green	Undulate	Convex	2 mm	-	-	-
3	RZATABY 7.4	White	Green	Undulate	Convex	2 mm	-	-	-
4	RZATABY 8.11	Gray	Green	Undulate	Convex	2 mm	-	-	-
5	RZATABY 8.2	White	Green	Entire	Convex	2 mm	+	+	+
6	RZATABY 8.10	Yellow	Greenish yellow	Undulate	Convex	2 mm	+	+	+
7	RZATABY 8.6	Gray	Green	Undulate	Flat	2 mm	-	-	-
8	RZATABY 8.5	Gray	Green	Undulate	Flat	2 mm	-	-	-
9	RZATABY 8.3	Gray	Green	Entire	Flat	2 mm	-	-	-
10	RZATABY 7.3	White	Gray	Undulate	Convex	2 mm	+	+	+
11	RZATABY 8.9	White	Greenish yellow	Entire	Convex	2 mm	+	+	+
12	RZATABY 7.1	Gray	Green	Undulate	Convex	2 mm	-	-	-
13	RZATABY 8.1	Gray	Green	Undulate	Convex	2 mm	+	+	+
14	RZATBY 8.7	White	Green	Entire	Convex	2 mm	+	-	-
15	RZATBY 8.8	White	White	Undulate	Convex	2 mm	-	-	-
16	RZATBY 8.1	White	White	Undulate	Convex	2 mm	-	-	-
17	RZATBY 8.6	Red	White	Undulate	Convex	2 mm	-	-	-
18	RZATPY 8.6	Red	White	Undulate	Convex	2 mm	-	-	-
19	RZATBY 8.4	White	White	Undulate	Convex	2 mm	-	-	-
20	RZATBY 8.5	Yellowish white	Gray	Undulate	Convex	2 mm	+	+	+
21	RZATPY 8.5	Yellow	White	Undulate	Convex	2 mm	-	-	-

Actinobacteria tested on *Mirabilis jalapa* plants obtained seven isolates that showed positive hypersensitive reaction tests, and seven isolates showed positive results in pathogenicity and hemolysis tests. The negative hypersensitive reaction test indicates that the isolate can be used for the next stage, namely introducing chili plants in pathogen control. While isolates with a positive hypersensitive reaction test indicate that the isolate is pathogenic to plants, it cannot be used for the next stage. Isolates that show a clear zone on the blood medium are not used because they are pathogenic to mammals with no clear zone are isolates that are not pathogenic (Figure 1d).

Actinobacteria isolates showed varied characteristics both morphologically and physiologically. The substrate mycelium color is generally white and gray, while the aerial mycelium color is generally green Figure 1a and 1b. Actinobacteria isolate RZATABY 7.1 showed negative results of hemolysin test characterized by the absence of a clear zone Figure 1c, and Actinobacteria isolate RZATABY 8.12 showed positive hemolysin test with the formation of a clear zone and not used for further tests Figure 1d.

Inhibitory Activity of Chili Rhizosphere Actinobacteria Isolates against *R. syzygii* subsp. *indonesiensis*

Twelve isolates from 14 actinobacteria isolates obtained from the first stage of the test were known to inhibit *R. syzygii* subsp. *indonesiensis* using the plug agar method on YWA solid media. The inhibitory activity of chili rhizosphere actinobacteria against *R. syzygii* subsp. *indonesiensis* using the plug agar method was indicated by the formation of inhibition zones that vary between 8-28.5 mm (Table 3). All isolates were able to inhibit *R. syzygii* subsp. *indonesiensis*. Based on these data, some isolates have diverse antimicrobial activity depending on the origin of the isolate.

Table 3. Inhibitory activity of chili rhizosphere actinobacteria against *R. syzygii* subsp. *indonesiensis* using the agar plug method on WYA media

Isolate	Diameter of clear zone (mm)
RZATABY 7.1	9.3 ± 0.6
RZATABY 8.11	9.0 ± 1.0
RZATABY 8.3	9.3 ± 0.6
RZATABY 7.3	11.0 ± 1.7
RZATABY 8.6	9.7 ± 1.1
RZATBY 8.8	15.7 ± 4.2
RZATPY 8.5	13.0 ± 1.0
RZATPY 8.6	12.0 ± 2.6
RZATABY 7.4	9.2 ± 0.4
RZATBY 8.4	9.7 ± 1.1
RZATBY 8.1	6.3 ± 1.0
RZATBY 8.6	6.1 ± 0.7
RZATABY 8.8	1.2 ± 0.6
RZATABY 8.12	0.9 ± 0.4

***In Vitro* Characterization of Chili Rhizosphere Actinobacteria**

Actinobacteria rhizosphere chili tested in vitro include siderophore production, IAA concentration, ammonia production, protease, and chitinolytic activity of actinobacteria isolates (Table 4). It has obtained ten actinobacteria isolates that produce siderophores, ammonia, and protease enzymes and can produce chitinolytic.

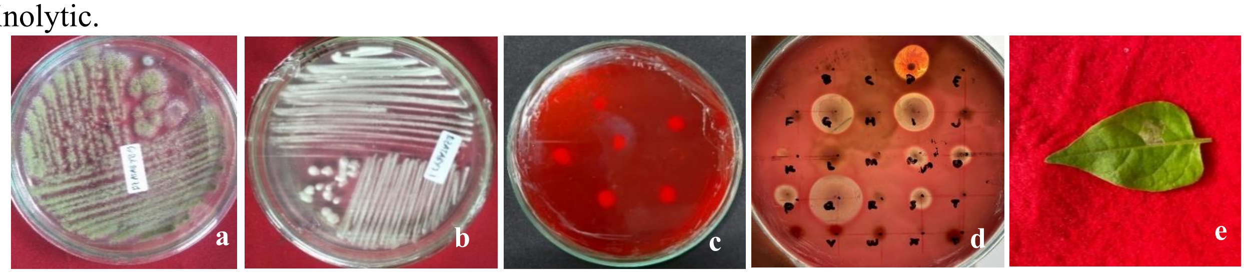

Figure 2. Actinobacteria colonies, (a) Isolate RZATABY 8.6 (b) Isolate RZATBY 8.1 (c) Actinobacteria isolate hemolysis test RZATABY 7.1 showed negative results with no clear zone (d) Hemolysis of actinobacterial isolate RZATABY 8.12 showed positive results with the presence of a clear zone (e) Actinobacterial hypersensitivity test RZATPY 8.5 showed negative results with necrosis on *M. jalapa.*

Table 4. Characterization of Actinobacteria in vitro

No.	Isolate	IAA concentration (ppm)	Siderofor	Ammonia	Protease	Chitinolytic Activity
1	RZATABY 7.4	31.78	+	+	+	+
2	RZATABY 8.11	27.57	+	+	+	+
3	RZATABY 8.6	12.44	+	+	+	+
4	RZATABY 8.5	33.75	+	+	+	+
5	RZATABY 8.3	16.74	+	+	+	+
6	RZATABY 7.1	10.41	+	+	+	+
7	RZATBY 8.8	25.95	+	+	+	+
8	RZATBY 8.1	5.31	+	+	+	-
9	RZATBY 8.6	4.13	+	+	+	+
10	RZATBY 8.4	57.85	+	+	+	+
11	RZATPY 8.6	2.67	+	+	+	-
12	RZATPY 8.5	80.56	+	+	+	+

- No reaction

+Made reaction

A total of 10 actinobacterial isolates have chitinolytic activity (Figure 2). Chitinolytic activity is seen in clear zones on colloidal chitin media.

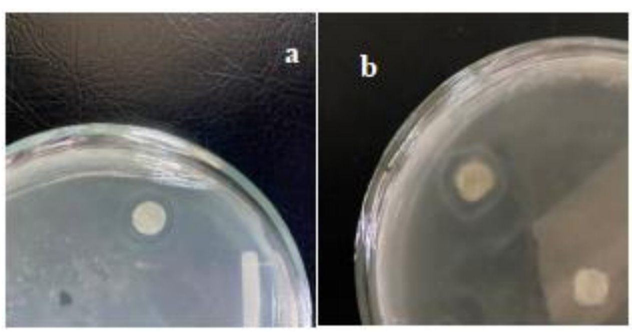

Figure 2. The chitinolytic activity of rhizosphere actinobacteria isolates (a) Isolate RZATABY 8.6 (b) Isolate RZATBY 8.8

Selection of Actinobacteria to Control *R. syzygii* subsp. *Indonesiensis* and Increase Chili Crop

Production

a. Disease progression

Compared to the negative control, chili plants introduced with actinobacteria showed that all isolates can slow the incubation period. Ten isolates of actinobacteria can slow the incubation period at 45.00 Hsi. A total of 10 actinobacteria isolates could suppress the incidence of disease until the end of observation showed no symptoms of attack by *R. syzygii* subsp. *indonesiensis* to 0%.

Chili plants introduced actinobacteria showed that all treatments of actinobacterial isolates showed lower disease severity than the negative control (Table 5). A total of 10 actinobacterial isolates were able to reduce the percentage of disease severity to 0%.

Table 5. Incubation period, disease incidence, and severity of bacterial wilt disease in chili plants that have been introduced with Actinobacteria

Treatment	**Incubation period**	**Disease incidence (%)**	**Disease severity (%)**
RZATABY 7.1	42.00 a	0.00** b	0.00** b
RZATABY 8.11	42.00 a	0.00** b	0.00** b
RZATABY 8.3	42.00 a	0.00** b	0.00** b
RZATABY 8.5	42.00 a	0.00** b	0.00** b
RZATABY 8.6	42.00 a	0.00** b	0.00** b
RZATBY 8.8	42.00 a	0.00** b	0.00** b
RZATPY 8.5	42.00 a	0.00** b	0.00 **b
RZATPY 8.6	42.00 a	0.00** b	0.00** b
RZATABY 7.4	42.00 a	0.00** b	0.00** b
RZATBY 8.4	42.00 a	0.00** b	0.00** b
RZATBY 8.1	28.00 b	33.33 ab	8,33 b
RZATBY 8.6	28.00 b	33.33 ab	6,66 b
Control -	21.67 c	100.00 a	100,00 a
Coeficient of Varians (CV)	5,19	7,46	8.67

*Numbers followed by the same lowercase letter in the same column are not significantly different according to DNMRT at the 5% level.

*The incubation period of 42 days after inoculation indicates that the plants did not show symptoms until the last observation day.

The initial symptoms caused by *R. syzygii* subsp. *indonesiensis* are leaves wilting of chili plants starting from the top leaves. Then, further symptoms will be followed by wilting from all plant leaves and dead chili plants. A comparison of wilted plants and healthy plants can be seen in Figure 3.

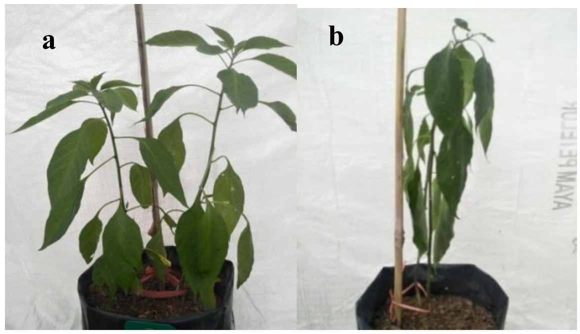

Figure 3. Comparison of healthy and diseased plants (14 hsi). (a) chili plants introduced with actinobacteria RZATPY 8.5 (b) diseased plants (only inoculated with *R. syzygii* subsp. *indonesiensis*).

b. Plant Growth (Generative Phase)

Table 6. Plant height, leaf number, first flower appearance, and fruit weight of chili peppers introduced with actinobacterial isolates

Treatment	Plant Height (cm)	Number of Leaves (blade)	First Flower Appears	Fruit weight (gram)
Control +	39.33 f	99.667 c	55.00 a	*0.000 i
RZATABY 7.4	43.76 ef	115.33 bc	49.67 abcd	89.667 bcde
RZATBY 8.4	44.16 ef	118.33 bc	48.00 abcde	96.333 bc
RZATABY 8.5	45.00 def	120.33 bc	48.00 abcde	102.67 b
RZATABY 8.6	45.33 def	125.33 abc	47.67 abcde	93,000 bcd
RZATABY 7.1	47.06 cdef	126.33 abc	47.67 abcde	64.333 g
RZATBY 8.8	52.40 bcde	127.33 abc	46.00 bcde	78.667 defg
RZATPY 8.6	52.93 bcde	128.33 abc	45.33 bcde	77,000 efg
RZATABY 8.11	53.50 bcde	130.00 abc	41.67 cdef	87.333 cdef
RZATABY 8.3	54.43 bcde	131.00 abc	38.00 ef	79.333 def
RZATPY 8.5	55.30 bcd	131.67 abc	34.67 f	126.33 a
KK	12.59	18.07	19.56	1.52

*Numbers followed by the same lowercase letter in the same column are not significantly different according to DNMRT at the 5% level.

In general, all isolates can increase plant height and leaf number, accelerate the first flower's appearance, and increase the chili fruit's weight. A total of 10 isolates showed significantly different results on plant height, number of leaves, first flower appearance, and fruit weight of chili compared to the control (Table 6).

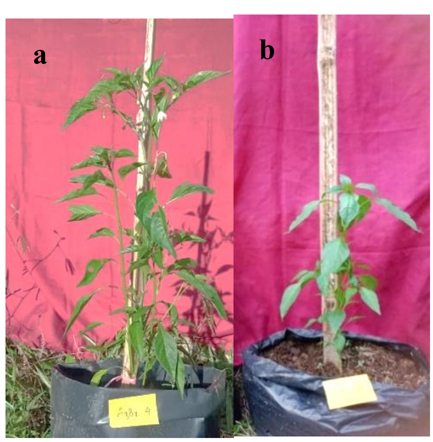

Figure 4. Comparison of chili plants appeared the first flower, (a) the introduction of actinobacteria isolates with code RZATPY 8.5, (b) control.

Figure 5. Comparison of the amount of chili production introduced actinobacteria from the rhizosphere of chili plants with the control in the first harvest, (a) control, (b) introduction of actinobacteria isolates with code RZATPY 8.5

Molecular identification of selected Actinobacteria

The 16S rRNA gene amplification results of 10 selected isolates showed a query cover percentage of 100%. Eight isolates were identified in the streptomyces group.

Table 5. BlastN results of 16S rRNA gene sequences of rhizosphere actinobacteria isolates of chili plants

Isolate Code	GenBank isolate	Query Cover (%)	Homolo gy (%)	Number of accessions
RZATABY 8.11	*Streptomyces pharvulus* NBRC 13193	100	99	AB184326
RZATABY 8.5	*Streptomyces pharvulus* NBRC 13193	100	99	AB184326
RZATBY 8.4	*Streptomyces pharvulus* NBRC 13193	100	99	AB184326
RZATABY 7.1	*Streptomyces roseofulfus* NBRC 13194	100	99	AB184327
RZATPY 8.6	*Streptomyces roseulus* NBRC 12816	100	99	AB189337
RZATABY 8.6	*Streptomyces deserti* C63	100	99	MK929476.1
RZATABY 8.3	*Streptomyces* sp GETU1	100	99	MW897733
RZATBY 8.8	*Amicolatopsis* sp GLM4	100	99	MW897838
RZATPY 8.5	*Amicolatopsis* sp GLM4	100	99	MW897838
RZATABY 7.4	*Streptomyces* sp GIG2	100	99	MW897844

Discussion

Isolation of actinobacteria from the root soil of chili plants obtained as many as 21 isolates from 3 districts. Overall, the isolates obtained have different morphological and physiological characteristics. Isolates from Solok Regency have more diverse morphological characters (61.90%) compared to Agam Regency (28.57%) and Tanah Datar Regency (9.53%). The diversity of isolates and population numbers of actinobacteria is thought to be due to different cropping patterns and locations in sampling host plants. Chili plants in Solok District and Agam District are planting patterns done by farmers with polyculture, while in Tanah Datar District with monoculture. According to Mitra *et al*. (2022), the diversity of actinobacteria was influenced by the diversity of plant species that grow. Actinobacteria can thrive in humus litter and leaf litter layers.

Morphological characterization of actinobacteria isolates carried out observation of the color of the substrate mycelium, aerial mycelium, Gram test, and hypersensitive reaction test. On mycelium substrate, diverse colors are gray, white, yellow, and red. While the areal mycelium is green, gray, and white). Abdelgawad *et al*. (2020) Abdelgawad *et al*. (2020) state that the color diversity of actinobacteria is due to the presence of spore chain pigments owned by actinobacteria; hyphae will turn into a certain color when spore formation occurs so that different colors are obtained. Pigments produced have biological capabilities such as antibiotics, antitumors, vitamins, and others. (Lasudee *et al*., 2018). In the Gram test, all actinobacterial isolates showed no sticky mucus formation from a mixture of bacterial colonies. This condition is known as a negative reaction, and the isolates tested are classified as gram-positive (Sousa & Olivares, 2018). Hypersensitivity reaction and pathogenicity tests were conducted to determine the potential of actinobacterial isolates as plant pathogens, especially in chili peppers. Hypersensitivity reaction is programmed cell death at the site of pathogen entry. Chloroplasts play an important role in HR and defense reactions in plants. Hypersensitivity starts from the most important sources in defense signals, such as *reactive oxygen species* (ROS), reactive *nitrogen oxide intermediates* (NOI), defense hormones salicylic acid, and jasmonic acid. These HR reactions can inhibit the growth of plant pathogens. Hypersensitivity is also a plant mechanism in preventing pathogen growth in *incompatible plant-pathogen interactions*, causing disease resistance (Coll *et al*. 2011). Hypersensitive reaction testing on *Mirabilis jalapa* leaves infiltrated with a suspension of actinobacteria at 106 spore cells/ml density. Seven isolates show a positive reaction hypersensitive. Pathogenicity reaction testing on chili plants infiltrated with actinobacteria suspension showed seven isolates with a positive reaction, so it can not be used in the next test.

After testing Actinobacteria from the roots of chili plants in vitro, we obtained as many as ten isolates, which can produce chitinase enzymes, siderophore production, and IAA growth enzymes. Actinobacteria can produce siderophores used in iron chelators in a limited environment that affects plant growth and nutrition. It follows the statement of Sathya *et al*. (2017) that actinobacteria produce siderophores that play an important role in plant protection capabilities that display activity against phytopathogens by producing siderophores in a competitive environment. Based on the IAA production test, all actinobacteria isolates produced IAA at concentrations between 0.12 to 80.56 μg/mL. However, there are ten isolates of actinobacteria that produce IAA at high concentrations. These results follow the concentration of IAA reported in the study of Sameera *et al*. (2018), which found that the production of IAA from actinobacteria is less than 140 μg/mL.

Introducing actinobacteria isolates to chili seeds can increase field emergence, seedling height, and number of leaves of chili seedlings. The best isolate in increasing seedling growth is RZATABY 8.11, with a recapitulation of 36.62% effectiveness. It is because actinobacteria secrete growth hormones that affect the roots and can spur the growth of chili plants during the seedling phase. The results of this study follow the research of Romano-Armada *et al*. (2020), which showed that the Streptomyces genus, which is a species of actinobacteria, is known to play an important role in nutrient cycling, nitrogen fixation, production of secondary metabolites, and spur plant growth. In addition, research by Myo *et al*. (2019) reported that inoculation of IAA-producing actinobacteria can effectively increase seed germination, along with overall growth and root elongation in various plants.

Actinobacterial isolates extended the incubation period, reducing the incidence and severity of bacterial wilt disease compared to the negative control (inoculated with *R. syzygii subsp. indonesiensis*). There are ten isolates of actinobacteria from 12 isolates introduced in chili plants that are best in suppressing the incidence of disease and disease severity until the end of the observation day (35 days after inoculation), namely isolates RZATABY 7.1, RZATABY 8.11, RZATABY 8.3, RZATABY 8.5, RZATABY 8.6, RZATABY 7.4, RZATBY 8.4, RZATBY 8.8, RZATPY 8.5, and RZATPY 8.6. The ability of actinobacteria to suppress the growth of *R. syzygii* subsp. *indonesiensis* because actinobacteria produce antibiotics from each isolate of actinobacteria in the inhibition of pathogen growth. As one of the microorganisms inducing plant resistance, actinobacteria can produce several secondary metabolites. The production of various extracellular enzymes assists the ability to degrade macromolecules. Some actinobacteria from the genus Streptomyces produce *xylanase* enzymes that degrade complex lignocellulose (Akilandeswari & Pradeep, 2017). Actinobacteria has the potential to dissolve phosphate and produce siderophores in the mechanism of nutrient competition carried out by biological agents with pathogens (Setiawati *et al*., 2021).

Introducing actinobacteria isolates in chili plants can increase plant height, number of leaves, flower appearance, fruit weight, and yield of chili plants. The best isolate in increasing the growth and yield of chili plants is RZATPY 8.5, with an increase in plant height of 15.97 cm, the number of leaves with a value of 32.003 strands, accelerating the emergence of flowers 20.4 and an increase in fresh fruit weight of 126.33 g compared to positive control plants. It is suspected that actinobacteria can spur the growth of chili plants. Igarashi *et al.* (2004) reported that *S. hygroscopius* S-17, a member of actinomycetes, was able to spur plant growth twice as high and eight times greater than the control treatment because actinobacteria are known to produce toyocamycin. This cytokinin-like hormone can spur callus growth, and pteridic acid, an auxin-like hormone, spurs root development.

From the results of this study, we can see that using actinobacteria as a biological agent can suppress the development of bacterial wilt disease caused by *R. syzygii* subsp. *indonesiensis.* The research of Rehan *et al*. (2021) reported that in the planta test, *Streptomyces* sp*.,* which is a type of actinobacteria, was able to suppress *Ralstonia. solanacearum* wilt disease in chili plants reaching 100%. It follows the opinion of Prudence *et al*. (2020) that actinobacteria in spurring plant growth can occur directly or indirectly. Directly includes producing phytohormones, soluble phosphate, nitrogen fixation, and increased nutrient uptake. Indirectly, it can control pathogens through secondary metabolic production, competition, parasitism, and induce resistance. The ten best actinobacterial isolates are suppressing the development of *R. syzygii* subsp. *indonesiensis* are RZATABY 7.1, RZATABY 8.11, RZATABY 8.3, RZATABY 8.5, RZATABY 8.6, RZATABY 7.4, RZATBY 8.4, RZATBY 8.8, RZATPY 8.5, and RZATPY 8.6. Actinobacteria isolates are best in increasing the growth and yield of chili plants is RZATPY 8.5 with an increase in plant height of 15.97 cm, the number of leaves of 32.003 strands, accelerated the emergence of flowers of 20.4 days and an increase in fresh fruit weight as much as 126.33 g compare with positive control. After the 16sRNA sequence Homology test, the ten best actinobacteria isolates belonged to *Streptomyces* spp. and *Amicolatopsis* sp. GLM4.

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