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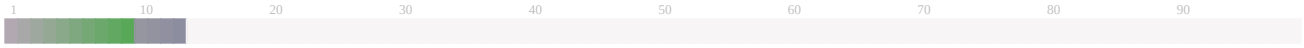

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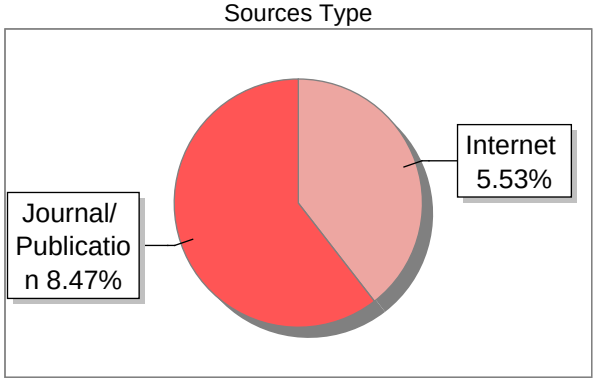

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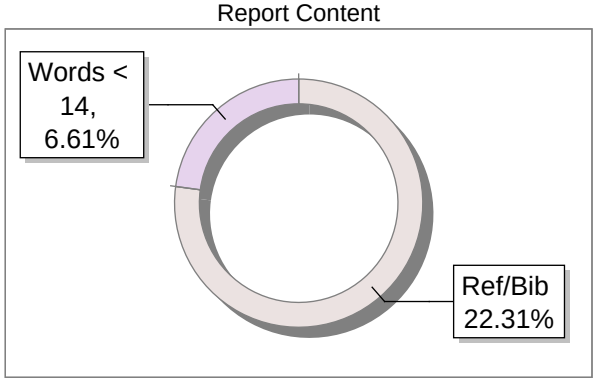

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Isolation and Antagonistic Activity of Actinomycetes Associated With Strawberry Plants (*Fragaria X Ananassa* Duch.) Against *Colletotrichum Acutatum* Simmonds

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ABSTRACT

Strawberries (*Fragaria x ananassa* Duch.) are a valuable horticultural crop in Indonesia, but their productivity is threatened by anthracnose disease caused by *Colletotrichum acutatum* Simmonds. Traditional control methods often involve synthetic fungicides, which pose risks of pesticide residues and environmental harm. This study investigates Actinomycetes isolated from various strawberry plant organs and their rhizosphere as potential biological control agents. Over 10 Gram-positive Actinomycetes strains were successfully isolated and characterized. Among them, isolates A3 and T2 exhibited the highest antagonistic activity against *Colletotrichum acutatum*, with inhibition percentages of 67.17% and 55.80%, respectively. These isolates demonstrated significant biocontrol potential through mechanisms such as competition, antibiosis, and hyperparasitism. The results suggest that Actinomycetes could serve as effective, eco-friendly alternatives to chemical pesticides in strawberry cultivation. Future research should focus on optimizing these strains for broader agricultural applications.

1. Introduction

Strawberries (*Fragaria x ananassa* Duch.) are high-value horticultural plants often grown for agrotourism in Indonesia (Karina et al., 2012; Oktaviana et al., 2022). They are popular due to their attractive appearance, sweet taste, and high content of antioxidants, vitamins, and nutrients (Karina et al., 2012). The demand for strawberries increases by up to 68% annually. However, strawberries are vulnerable to pathogen attacks, which can affect fruit quality and productivity (Istifadah et al., 2017; Sari et al., 2021). Anthracnose disease, caused by the fungus *Colletotrichum acutatum* Simmonds, poses a major threat to strawberry productivity, affecting fruits, stems, stolons, petioles, and leaves, and leading to significant yield losses (Lelana et al., 2015). The control of anthracnose typically involves the use of synthetic fungicides. However, the continuous use of synthetic fungicides can result in pesticide residues in fruit, environmental pollution, pathogen resistance, and harm to other microorganisms (Nurjasmi & Suryani, 2020; Lelana et al., 2015). As a result, there is a need for alternative control methods to mitigate the adverse effects of synthetic fungicides. Biological control, which involves using microorganisms with antagonistic properties against pathogens, presents a more environmentally friendly long-term solution (Syafriani et al., 2019). Microorganisms are advantageous as biocontrol agents due to their rapid growth on inexpensive substrates and their ability to produce potent enzymes (Oktaviana et al., 2022).

Actinomycetes, which are a group of Gram-positive bacteria found in plant tissues and soil, are known for producing bioactive compounds with antibiotic, anticancer, and phytohormone properties (Fatmawati et al., 2014; Elsie et al., 2018). They also produce antifungal compounds that inhibit pathogen growth through mechanisms such as competition for space and nutrients, antibiosis, and mycoparasitism (Abidin et al., 2015; Marsaoli et al., 2019). Previous studies have shown that Actinomycetes can inhibit *Colletotrichum acutatum* in chili plants (Hartanto & Eti, 2016). This study aims to explore the potential of Actinomycetes isolated from strawberry plant organs and rhizosphere as natural antifungal agents against anthracnose disease in strawberries caused by *Colletotrichum acutatum* Simmonds.

2. Methods

2.1. Equipment and Materials

The equipment used includes Erlenmeyer flasks, Petri dishes, measuring cylinders, pipettes, autoclaves, Bunsen burners, scalpels, slides, microscopes, mortars, pestles, analytical balance, incubators, ovens, microwaves, vortexes, rulers, test tubes, and clamps. Materials used include strawberry plants (*Fragaria x ananassa*), *Colletotrichum acutatum* fungus, soil samples, Himedia Starch Casein Agar (SCA) medium, Potato Dextrose Agar (PDA) medium, and various chemicals such as sterile distilled water, 70% alcohol, 0.5% NaClO solution, nystatin, chloramphenicol, rifampicin, crystal violet, iodine, and safranin.

2.2. Research Procedure

2.2.1. Isolation of Actinomycetes from Strawberry Plant Organs (Fragaria x ananassa Duch.)

Healthy strawberry plants (*Fragaria x ananassa* Duch.) were collected from Magelang, Central Java. Roots, stems, and leaves were washed with running water. The organ samples were surface-sterilized with 70% (v/v) alcohol for 1 minute, 0.5% (v/v) NaClO solution for 3 minutes, 70% (v/v) alcohol for 30 seconds, then rinsed twice with sterile distilled water and dried in sterile Petri dishes. The organ samples were then cut and ground. SCA medium was prepared and supplemented with 50 g/mL nystatin and 25 mg/mL rifampicin. The organ samples were placed on the medium and incubated at room temperature (25-28°C) for 2-3

weeks. Each Actinomycetes isolate that grew was subcultured using the 4-quadrant streak method on SCA medium (Passari et al., 2015).

2.2.2. Isolation of Actinomycetes from Strawberry Rhizosphere (Fragaria x ananassa Duch.)

Soil samples from strawberry plants (*Fragaria x ananassa* Duch.) were randomly taken from four points at a distance of 0-10 cm from the plant stand and at a depth of 3-20 cm. The surface soil layer of 0-3 cm was discarded. The soil samples were placed in clean zip lock bags and brought to the laboratory. The samples were mixed and placed in Petri dishes, then left in the open air for 5 days (Susilowati dkk, 2007; Nurrochman, 2015). One gram of soil sample was placed in a test tube containing 9 mL of sterile 0.85% NaCl solution and shaken for 5 minutes for a 10^{-1} dilution. The sample suspension was placed in a water bath at 50°C for 10 minutes, then serially diluted. One milliliter was transferred to another test tube containing sterile 0.85% NaCl solution for a 10^{-2} dilution. This step was repeated until a 10^-4 dilution was obtained. Suspensions at 10^{-3} and 10^{-4} dilutions were inoculated using the pour plate method. SCA medium and 1% nystatin were added to Petri dishes containing the 10^{-3} and 10^{-4} dilution suspensions. The plates were gently shaken to homogenize and incubated at 25°C for 4 days to 2 weeks (Sembiring dkk, 2000).

2.2.3. Regeneration of Colletotrichum acutatum Simmonds

The strain of *Colletotrichum acutatum* Simmonds was obtained from the Laboratory of Biology at Universitas Ahmad Dahlan in Yogyakarta. The isolate was cultured on PDA medium supplemented with chloramphenicol. The mycelia were then transferred to Petri dishes containing PDA medium and incubated for 7 days at a room temperature of 25-28°C (Nurjasmi & Suryani, 2020).

2.2.4. Observation of Morphological Characteristics of Actinomycetes

Actinomycetes isolates were grown on SCA media for 10 days at room temperature (25-28oC), then observed the morphological characteristics of colonies including macroscopic and microscopic observations. Macroscopic observations consisted of edges, elevations, colony shape, presence or absence of aerial mycelium, aerial mycelium color, and substrate mycelium color. Microscopic observations are carried out using the gram staining stage first, then the cell shape of the Actinomycetes is observed (Li et al., 2016).

2.2.5. Screening of Antagonism Activity of Actinomycetes against Fungus Colletotrichum acutatum Simmonds

Colletotrichum acutatum Simmonds fungus was revived, and Actinomycetes isolates were cultivated on SCA medium as stock for 3 days. Pieces of pure culture of *Colletotrichum acutatum* Simmonds fungus (± 5 mm) were inoculated in the center of petri dishes containing PDA media. Actinomycetes isolates were streaked (± 2 cm) on all four sides of the petri dish at a distance of 2.5 cm from the center. In the negative control, pieces of *Colletotrichum acutatum* Simmonds fungus (± 5 mm) were placed in the center of the Petri dish containing PDA media without adding Actinomycetes isolates. Both treatment dishes were then incubated at room temperature (25-28oC) for 5-7 days until the fungal colonies of *Colletotrichum acutatum* Simmonds in the negative control treatment grew to fill the petri dish. The radius of the fungal colonies formed was measured every day for 5 days (Khucharoenphaisan et al., 2016; Audinah and Ilmi, 2019). The percentage of inhibition of Actinomycetes against *Colletotrichum acutatum* Simmonds was calculated using the formula from Rahman et al. (2009):

$$\text{PH} = \left(\frac{R1 - R2}{R1}\right) x100\%$$

In this formula, PH represents the percentage of inhibition, R1 is the growth radius of *Colletotrichum acutatum* Simmonds in the control, and R2 is the growth radius in the treatment. This calculation quantifies the effectiveness of Actinomycetes in inhibiting fungal growth.

2.3 Analisis Data

The experiment was repeated three times to ensure reliability. Data obtained from the percentage of inhibition results were analyzed to determine which Actinomycetes isolates had the highest efficacy in inhibiting the fungus *Colletotrichum acutatum* Simmonds during the antagonism activity screening. A One-Way ANOVA test was used to analyze the data. If the One-Way ANOVA test indicated significant differences among the mean values, the Duncan test was subsequently performed to identify which isolates significantly differed from others.

3. Results and Discussion

3.1. Results

Actinomycetes isolates were successfully obtained from various plant organs and the rhizosphere of strawberry plants (*Fragaria x ananassa* Duch.). While some isolates exhibited similar characteristics, 10 distinct isolates were selected based on their unique traits. The isolation process yielded 10 Actinomycetes isolates: 3 from the roots, 2 from the stems, 2 from the leaves, and 3 from the rhizosphere (soil). The highest number of isolates were obtained from the roots and rhizosphere of the strawberry plants. The appearance of these isolates is displayed in Figure 1. Macroscopically and microscopically, all isolates have distinct colony forms. These observations suggest that the isolates in groups A, B, D, and T represent different species or strains of Actinomycetes, indicating varied potential functions or activities.

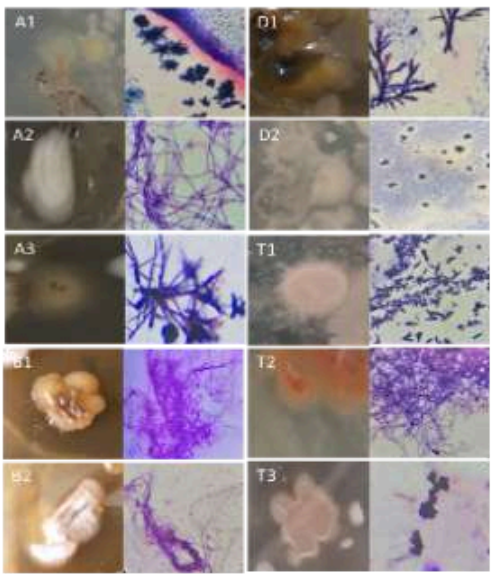

Figure 1. Isolates from strawberry plant organs and rhizosphere (*Fragaria x ananassa* Duch.) on SCA medium. Note: (A) root; (B) stem; (D) leaf; and (T) soil.

Based on Figure 1. and Table 1., the 10 isolates observed are suspected to belong to the Actinomycetes group due to their colony shapes (circular) and various other characteristics such as colony edge, elevation, cell shape, aerial mycelium color, and substrate mycelium color. The Gram staining results indicated that all isolates produced a purple color, indicating that they are gram-positive bacteria, like Actinomycetes.

Table 1. Macroscopic and microscopic characterization of microbial isolates obtained from strawberry plant organs and rhizosphere (*Fragaria x ananassa* Duch.)

Source	No	Isolate Code	Colony Shape	Edge	Elevation	Cell Shape	Aerial Mycelium Color	Substrate Mycelium Color
Plant organs	1.	A1	Circular	Undulate	Umbunate	Monoverticillate no spirals	Yellowish white	Yellowish brown
	2.	A2	Circular	Entire	Convex	Primitive spirals	Yellowish white	Yellowish white
	3.	A3	Circular	Undulate	Raised	Straight	Yellowish white	Yellowish white
	4.	B1	Circular	Entire	Convex	Primitive spirals	Brownish white	Brownish white
	5.	B2	Circular	Entire	Convex	Primitive spirals	Yellowish white	Kuning kecoklatan
	6.	D1	Circular	Undulate	Umbunate	Flexous	Oranye	Oranye
	7.	D2	Circular	Undulate	Convex	Fascicled	Yellowish white	Yellowish white
Soil	8.	T1	Circular	Felamen-tous	Flat	Bivarticillate no spirals	Pink-white	White
	9.	T2	Circular	Filamen-tous	Flat	Monoverticillate no spirals	Pink	Pink
	10.	T3	Circular	Filamen-tous	Flat	Bivarticillate no spirals	Pink-white	Pink-white

The results of the antagonistic activity screening test of Actinomycetes isolates from the plant organs and rhizosphere of strawberry plants (*Fragaria x ananassa* Duch.) against the fungus *Colletotrichum acutatum* Simmonds after 5 days of incubation are presented in Figure 2. All isolates show varying degrees of inhibition. Isolate A3, exhibit significant inhibition zones, indicating strong antagonistic activity. Isolate T2 also shows considerable inhibition. The percentage of inhibition for each isolate was calculated and analyzed, as shown in Figure 3.

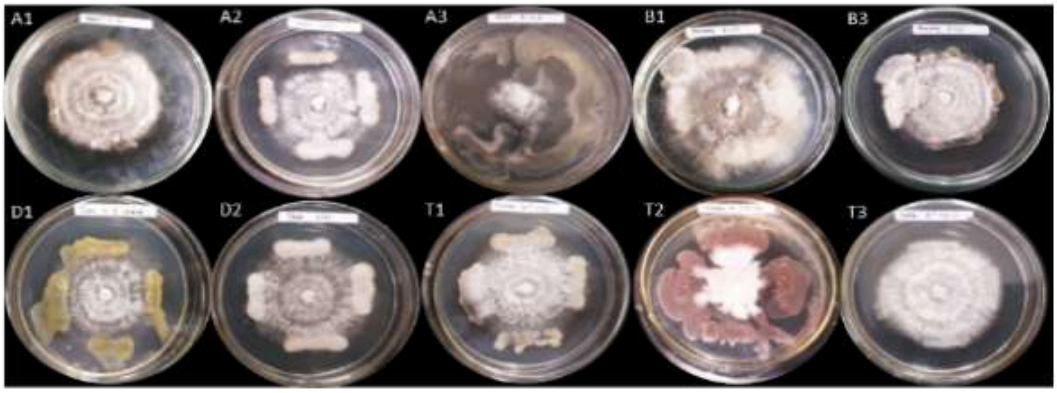

Figure 2. Antagonistic activity screening results of Actinomycetes isolates from strawberry plant organs and rhizosphere (*Fragaria x ananassa* Duch.) against *Colletotrichum acutatum* Simmonds.

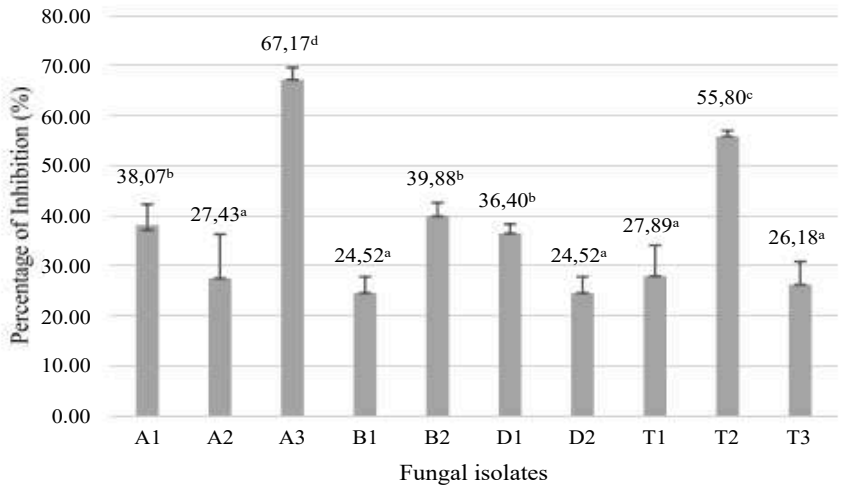

Figure 3. Percentage of inhibition of fungal isolates against *Colletotrichum acutatum* Simmonds. Different letters denote statistically significant differences based on Duncan's test at $p < 0.05$.

Based on Figure 3, the screening results of the antagonistic activity of Actinomycetes isolates, which were isolated from strawberry plant organs and rhizosphere (*Fragaria x ananassa* Duch.), against the fungus *Colletotrichum acutatum* Simmonds, showed that all 10 Actinomycetes isolates had the ability to inhibit the fungus. The isolate A3 stands out with the highest inhibition percentage of 67.17% and is significantly different from all other isolates. The isolate T2 also shows a high inhibition percentage of 55.80%, significantly different from many other isolates except A3. Isolate A1, B1, and B2 have moderate inhibition percentages ranging from 36.40% to 39.88%, and they are not significantly different from each other. Isolates A2, D1, D2, T1, and T3 show lower inhibition percentages, ranging from 24.52% to 27.89%, and are not significantly different from each other.

3.2. Discussion

The samples used as sources of Actinomycetes isolates were taken from the organs (roots, stems, and leaves) and the rhizosphere of strawberry plants (*Fragaria x ananassa* Duch.), isolated using the planting method. The choice of vegetative organs and rhizosphere as sources of Actinomycetes is based on their presence in the healthy vegetative parts of plants (Elsie et al., 2018). Additionally, Sastrahidayat and Rochdjatun (2014) stated that at soil depths of 3-20 cm, there is an abundant and diverse population of Actinomycetes. The isolation process resulted in more than 10 bacterial isolates from plant organs and the rhizosphere. The root organs and rhizosphere were the most abundant sources of isolates due to their ability to produce and release root exudates, which serve as energy sources for Actinomycetes, influencing microbial diversity and population in the roots and rhizosphere. Soil properties, such as pH conditions, also affect microbial populations (Zunairoh et al., 2019). Based on the morphological characteristics observed both microscopically and macroscopically, it is known that the 10 isolates from strawberry plant organs and the rhizosphere are Gram-positive, producing purple color in Gram staining, with circular colonies and varying other characteristics. These findings align with Fatmawati et al. (2014), indicating Actinomycetes are Gram-positive bacteria. Sulistiyani and Akbar (2014) also noted Actinomycetes colonies are round or circular with varied edges and surfaces. The observed morphological characteristics align with those reported by Fatmawati et al. (2014) and Sulistiyani and Akbar (2014), suggesting the 10 isolates from strawberry plants and the rhizosphere belong to the Actinomycetes group and can be used for further testing. Morphological characterization of Actinomycetes serves as a reference for classification but cannot determine specific types of Actinomycetes (Barka et al., 2016).

The antagonist test assesses the bacteria's ability to inhibit fungal growth (Lestari, 2017). The 5-day incubation was chosen because the petri dish containing only *Colletotrichum acutatum* Simmonds (negative control) was fully occupied. According to Khucharoenphaisan et al. (2016), inhibition percentages of 61%-100% are categorized as highly efficient in inhibiting growth, while percentages of 41%-60% indicate the ability to inhibit fungal mycelium growth. Referring to Khucharoenphaisan et al. (2016), only two isolates had average inhibition percentages above 61% with A3 (highly efficient) and T2 above 41% (able to inhibit growth). The mechanism of inhibition by Actinomycetes against *Colletotrichum acutatum* Simmonds, known as antagonistic activity in biological control, includes competition for space and nutrients, antibiosis, and microparasitism (Marsaoli et al., 2019). Actinomycetes also produce antifungal compounds that inhibit pathogenic fungal growth and are antagonistic (Abidin et al., 2015). The antagonistic activity of isolate A3 against *Colletotrichum acutatum* Simmonds, shown in Figure 2, indicates rapid growth and spread compared to the fungus, suggesting space and nutrient competition between A3 and *Colletotrichum acutatum* Simmonds (Marsaoli et al., 2019; Abidin et al., 2015). Competition for space and nutrients allows biocontrol agents to grow faster than pathogens (Sriyanti et al., 2015). When multiple microorganisms occupy the same space simultaneously, they compete for nutrients to grow and reproduce, leading to suppressed growth of some microorganisms (Rifai et al., 2020). The inhibition process by isolate T2 against *Colletotrichum acutatum* Simmonds, shown in Figure 2, indicates direct contact between the isolate and the fungus. According to Edy et al. (2017), direct contact between a pathogen and bacterial isolate can further inhibit pathogen growth. Direct contact also enables bacteria to perform hyperparasitism (microparasitism) (Chen et al., 2016). Hyperparasitism involves antagonistic activity where one organism harms another by producing antibiotics or inhibitory substances to suppress pathogen growth (Rifai et al., 2020).

Isolates other than A3 and T1 also exhibited inhibitory effects, with inhibition percentages below 41%. The inhibition percentages of the other eight isolates varied,

reflecting the different capabilities of each isolate in inhibiting the growth of *Colletotrichum acutatum* Simmonds. This variability is attributed to differences in the types of Actinomycetes bacteria and the amounts of inhibitory secondary metabolites produced by each isolate (Pitasari and Ali, 2018). The mechanism by which pathogens' growth is inhibited through the production of metabolic compounds is known as antibiosis (Fiko and Widiantini, 2018). The findings indicate that Actinomycetes isolated from strawberry plants and their rhizosphere have significant potential as biocontrol agents against fungal pathogens. The effective isolates, particularly A3 and T2, demonstrate strong antagonistic activity, making them promising candidates for developing biocontrol strategies in agriculture. Utilizing such natural biocontrol agents can reduce the reliance on chemical pesticides, promoting sustainable and eco-friendly farming practices.

4. Conclusion

The research on Actinomycetes isolated from strawberry plants and their rhizosphere reveals their significant potential for agricultural biocontrol. Over 10 Gram-positive Actinomycetes strains were successfully isolated, with A3 and T2 showing the highest antagonistic activity against *Colletotrichum acutatum*, indicating their potential efficacy as biocontrol agents. These strains use mechanisms such as competition, antibiosis, and hyperparasitism to inhibit fungal growth, offering a sustainable alternative to chemical pesticides. The variability in inhibitory effects suggests these Actinomycetes can be tailored for specific pathogens or conditions, enhancing their practical use in agriculture. This research highlights the promising role of Actinomycetes in managing fungal pathogens and supports their application in sustainable farming practices. Future studies should focus on optimizing these strains for broader biocontrol use.

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