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Selection of Antagonistic Rhizobacteria Potential for Biological Control of *Fusarium oxysporum*

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Abstract

Fusarium oxysporum is a pathogen that causes wilt disease in many plants and can couse losses of up to fifty percent. Standard control is to use synthetic fungicides. However, continuous use of fungicides has a high environmental risk, so environmentally friendly and safe control efforts are needed. One way is by using rhizosphere bacteria or rhizobacteria. This research aims to obtain rhizobacteria isolates that can control the pathogen F. oxysporum , which causes fusarium wilt in vitro. This research was conducted from June to July 2023. The methods in this research included isolating rhizobacteria, observing the morphology of bacterial colonies, gram staining, gram reaction test, catalyst test, and in vitro antagonist test. Research data shows that of the thirty rhizobacteria isolates observed, all isolates had the potential to inhibit the growth of F. oxysporum . The KMTK2 showed the highest inhibitory ability isolate with an inhibitory ability of 73,99%, and the lowest inhibitory ability was shown by the TBA1 isolate with an inhibitory ability of 51,56%. Potential rhizo-bacteria isolates can suppress the growth of the pathogen F. oxysporum and can be used as a biological agent to reduce the use of pesticides in treating plant diseases.

Keywords : Wilt Disease; Inhibition test; fungicide; rhizosphere bacteria

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1. Introduction

Fusarium oxysporum is a pathogen that can attack many types of plants [1]. *F. oxysporum* can cause plants to wilt and end in death [2]. *F. oxysporum* is a soil borne pathogen that can survive in soil and plant debris for quite a long time [3]. *F. oxysporum* causes significant losses to its host plants, both planted in open fields and greenhouses and is a major limiting factor in the plant cultivation process. Attacks due to this pathogen can cause losses of up to 50% [4].

Chemicals are commonly used to control the *F. oxysporum* pathogen. However, intensive use of synthetic fungicides can lead to the accumulation of toxic compounds that can harm the environment and humans and cause resistance to pests and diseases [5]. Based on this, environmentally friendly control alternatives are needed. One of them is biological control using microorganisms as biological agents. The pathogen *F. oxysporum* can be controlled with soil microbes that have antagonistic properties [6].

The control technique for controlling pathogens is rhizosphere bacteria (Rhizobacteria). Rhizobacteria are a collection of bacteria that live and colonies on plant roots [7]. Rhizobacteria can act as antagonistic bacteria by producing siderophores and extracellular metabolites [8]. Inhibition of pathogen growth by rhizobacteria can occur due to mechanisms of competition for nutrients and space [9]. Apart from that, rhizobacteria can also trigger Induced Systemic Resistance (ISR) to increase host plants' response to pathogen attacks [10]. Using rhizobacteria to suppress *F. oxysporum* attacks is a highly recommended control method. Several studies report that the antagonistic activity of rhizobacteria is produced through different mechanisms, rhizobacteria can reduce pathogen populations through

competition and the produc-tion of antimicrobial compounds.

This research aims to obtain rhizo-bacteria isolates that can be used to control the pathogen *F. oxysporum* , which causes fusarium wilt so that an environmentally friendly control alternative can be obtained.

2. Materials and Methods

2.1 Time and Place of Research

This research was carried out from June to July 2023. The research was carried out at the Phytopathology Laboratory, Department Plant Protection, Faculty of Agriculture, Sriwijaya University.

2.2 Tools and materials

The tools used in this research were autoclave, cover glass, Erlenmeyer, vernier caliper, ose needle, laminar air flow, micropipette, microscope, petridish, tweezers, preparation, sprayer, and triangle. The materials used are Potato Dextrose Agar (PDA), Nutrient Agar (NA), alcohol 96%, alcohol 70%, KOH 3%, *F. oxysporum* isolates from the collection of Phytopathology Laboratory, and the root soil of alang-alang (*Imperata cylindrica*) , bamboo (*Bambusa* sp.), banana (*Musa balbisiana*), lemongrass (*Cymbopogon citratus*), Jati (*Tectona grandis*), hevea (*Hevea brasiliensis*), gaharu (*Aquilaria malaccensis*), snake fruit (*Salacca zalacca*), sugar cane (*Saccharum officinsrum* L.), and rumput gajah (*Pennisetum purpureum*).

2.3 Research Implementation

Research activities include the isolation and selection of rhizobacteria growth inhibition test of the pathogenic fungus *F. oxysporum* using rhizobacteria in vitro.

Isolation and Selection of Rhizobacteria

Bacteria were obtained from several soil samples from plant roots. 75 g of each sample was used and soaked in 200 ml of distilled water. Then, incubate for one day in a closed container. After incubation, the samples were homogenized using a rotary shaker at a speed of 200 ppm for 48 hours. After that, bacteria were isolated using the serial dilution method. Bacterial isolation was carried out at a dilution of 10-8 in NA media, then incubated for 48 hours at 30^{o} C. To obtain pure bacterial cultures, bacterial colonies that grow are purified on NA media and incubated for 48 hours at 30^{o} C [11].

Morphology of Bacterial Colony

The morphology of the bacterial isolates was observed in the culture of isolates that had been purified on NA media to obtain a single isolate. Observation were made by observing bacterial colonies shape, elevation, margin, and color [12].

Gram Stain

Bacterial cultures are taken with a loop needle and streaked onto a glass slide. The bacterial streaks were covered with crystal violet dye solution for 1 minute, after which the excess dye solution was removed using distilled water. Next, the bacterial scratches are treated with iodine for 2 minutes, and the excess iodine is removed using distilled water. After that, the bleaching process is done by adding alcohol 96% to the bacterial scratches and then rinsing with distilled water. The bacterial smear is then soaked in safranin for 30 seconds; the excess dye is then rinsed with distilled water. Observe the bacterial streaks using a microscope. Gram-positive bacteria are indicated by the bacteria staining purple, if the color produced is red, then the bacteria are gram-negative bacteria.

Gram Reaction

This test is carried out by taking a bacterial culture using a loop needle and streaking it on a glass slide dripped with KOH 3%. Bacteria and KOH 3%are mixed by stirring using a loop needle. If the bacteria are not slimy, they are gram positive bacteria, whereas if the bacteria produce mucus, they are gram negative.

Catalyst Test

The catalyst test is carried out by stirring the bacterial culture on a glass slide treated with H_2O_2 5%. Gram positive bacteria will produce gas bubbles, while gram negative bacteria cannot produce gas bubbles.

Antagonism Test

The rhizobacteria inhibition test against *F. oxysporum* was carried out using the dual culture method. The *F. oxysporum* isolate was cut to a diameter of 0.5 cm and then transferred to NA media at 3 cm from the rhizobacteria. Rhizobacteria isolates were streaked lengthwise in the opposite direction to the pathogen. The percentage of rhizobacteria inhibitory power is calculated using the following formula:

$$I\% = \frac{R1-R2}{R1} \times 100\%$$

I %; Percentage of inhibition zone, R1; Average length of radius of *F. oxysporum* fungus colonies in controls, R2; Average length of *F. oxysporum* fungus colonies in the treatments.

3. Results and Discussion

Isolation of Rhizobacteria

The results of bacterial isolation from plant root-soil obtained 30 different isolates. These isolates were obtained from the root soil of banana (KMP) as many as 2 isolates, 4 isolates from the soil of the roots of sedge grass (KMTK), 4 isolates from the soil of the roots of sugar cane (KMT), 3

isolates from the soil of the roots of agarwood (MJG), 3 isolates from the soil of salak roots (MJSL), 5 isolates from rumput gajah root soil (MJRG), 4 isolates from alang-alang root soil (TBA), 3 isolates from lemongrass root soil (TBS) and 2 isolates from bamboo plant root soil (TBB).

Tabel 1: Identification of Morphological Characteristics of Rhizobacteria Isolates

Isolate code	Margin	Elevansi	Color	Gram stain	Gram reaction	Katalis
KMT1	Entire	Umbonate	Yellowish	-	+	+
KMT2	Endulate	Umbonate	White	+	+	+
KMT3	Endulate	Pulvinate	Yellowish	-	-	-
KMT4	Entire	Flat	Yellowish	-	+	+
KMP1	Erose	Raised	White	+	+	+
KMP2	Entire	Raised	White	+	+	-
KMTK1	Endulate	Umbonate	White	-	+	+
KMTK2	Entire	Convex	Yellowish	+	+	-
KMTK3	Filamentous	Raised	Yellowish	-	+	+
KMTK4	Lobate	Convex	White	-	+	+
MJG1	Entire	Convex	White	-	-	+
MJG2	Lobate	Convex	Yellowish	-	-	+
MJG3	Entire	Convex	Yellowish	+	+	-
MJRG1	Filamentous	Raised	Yellowish	+	+	-
MJRG2	Erose	Flat	Yellowish	-	-	+
MJRG3	Erose	Flat	Yellowish	+	+	+
MJRG4	Entire	Convex	White	-	-	+
MJRG5	Lobate	Convex	Yellowish	+	+	+
MJSL1	Lobate	Convex	Yellowish	-	-	+
MJSL2	Entire	Convex	Clear	-	-	+
MJSL3	Erose	Flat	Clear	+	+	+
TBS1	Filamentous	Raised	Yellowish	+	+	+
TBS2	Endulate	Raised	White	+	+	-
TBS3	Entire	Convex	White	+	+	+
TBA1	Endulate	Flat	Purple	+	+	+
TBA2	Entire	Pulvinate	Clear	+	+	+
TBA3	Entire	Convex	White	-	-	+
TBA4	Endulate	Raised	Clear	-	-	+
TBB1	Endulate	Raised	Yellowish	-	+	+
TBB2	Endulate	Raised	White	-	-	+

(+) indicates a positive reaction, (-) indicates a negative reaction

The research showed that each isolated rhizobacteria isolate had different morphological characteristics regarding color, elevation, and margins of the observed bacterial colonies. The bacterial gram test aims to determine the grams of a bacteria. Based on the results of the tests that have been carried out, there are bacterial isolates that have gram positive and negative cell walls. According to Lailatus et al., gram positive bacteria are indicated by the absence of mucus formed after the bacterial isolate reacts with KOH 3%. Gram negative bacteria show a reaction to KOH 3% by producing mucus [13]. This is because gram positive bacteria have thick peptidoglycan walls [14].

From the results of the catalyst tests that were carried out, many isolates produced bubbles after being given H_2O_2 (+), indicating that these isolates were capable of producing the catalase enzyme. The catalase enzyme is needed to break down H_2O_2, which is toxic to cells because it can activate enzymes in cells. This compound is formed during aerobic metabolic conditions [15]. Meanwhile, the gram staining results showed positive results (+) with the formation of a purple color in the bacterial cells. This is because bacteria have a low lipid content, so the cell walls are easily dehydrated by alcohol [16].

Antagonism Test

The antagonist test of rhizobacteria isolates against *F. oxysporum* was carried out by calculating

the ability of rhizobacteria to inhibit the growth of *F. oxysporum* .

Tabel 2: Percentage of inhibition of antagonistic bacteria against F.oxysporum pathogens in vitro

Isolate	Average percentage of inhibitory				
	Day 2	**Day 4**	**Day 6**	**Day 8**	**Day 10**
Kontrol	0.00^{b}	0.00^{e}	15.00^{b}	0.00^{h}	0.00^{k}
KMT 1	43.08^{a}	34.40^{abc}	47.69^{a}	$61.39^{abcdefg}$	$62.06^{bcdefgh}$
KMT 2	19.84^{a}	30.76^{bc}	47.11^{a}	63.57^{abcdef}	62.72^{bcdefg}
KMT 3	13.08^{a}	28.70^{d}	48.01^{a}	$62.11^{abcdefg}$	63.21^{bcdef}
KMT 4	18.28^{a}	33.89^{abc}	51.34^{a}	60.91^{bcdefg}	$60.85^{cdefghi}$
KMP 1	20.68^{a}	42.78^{abc}	57.14^{a}	68.99^{abc}	68.66^{abc}
KMP 2	16.79^{a}	33.37^{abc}	52.27^{a}	67.82^{abcd}	68.30^{abc}
KMTK 1	43.61^{a}	44.81^{abc}	58.61^{a}	64.92^{abcde}	65.30^{abcde}
KMTK 2	22.40^{a}	47.60^{ab}	55.77^{a}	73.83^{a}	73.99^{a}
KMTK 3	32.55^{a}	43.34^{abc}	52.68^{a}	$61.57^{abcdefg}$	61.72^{cdefgh}
KMTK 4	25.27^{a}	38.85^{abc}	46.24^{a}	66.99^{abcd}	67.53^{abcd}
MJG 1	28.30^{a}	31.89^{bc}	41.39^{a}	52.35^{fg}	53.87^{hij}
MJG 2	29.94^{a}	39.08^{abc}	45.73^{a}	57.86^{bcdefg}	58.68^{efghij}
MJG 3	21.75^{a}	14.76^{d}	50.98^{a}	70.31^{ab}	73.15^{a}
MJRG 1	27.86^{a}	34.83^{abc}	51.79^{a}	$62.27^{abcdefg}$	$62.31^{bcdefgh}$
MJRG 2	37.98^{a}	40.94^{abc}	48.64^{a}	62.97^{abcdef}	62.90^{bcdef}
MJRG 3	20.43^{a}	27.38^{cd}	45.03^{a}	55.97^{defg}	56.55^{fghij}
MJRG 4	16.54^{a}	36.60^{abc}	45.01^{a}	63.61^{abcdef}	64.72^{abcdef}
MJRG 5	21.77^{a}	34.81^{abc}	44.97^{a}	60.33^{bcdefg}	58.30^{efghij}
MJSL 1	39.81^{a}	31.92^{bc}	44.40^{a}	57.19^{cdefg}	58.06^{efghij}
MJSL 2	24.33^{a}	30.37^{bc}	44.35^{a}	56.76^{cdefg}	56.20^{fghij}
MJSL 3	28.07^{a}	41.57^{abc}	48.04^{a}	58.85^{bcdefg}	$60.02^{cdefghi}$
TBS 1	40.37^{a}	40.44^{abc}	56.07^{a}	60.59^{bcdefg}	$61.00^{cdefghi}$
TBS 2	30.95^{a}	54.02^{a}	53.59^{a}	$61.34^{abcdefg}$	$61.86^{bcdefgh}$
TBS 3	25.18^{a}	29.48^{bc}	46.31^{a}	59.88^{bcdefg}	$59.29^{defghij}$
TBA 1	23.51^{a}	34.82^{abc}	39.31^{a}	50.75^{g}	51.56^{j}
TBA 2	23.55^{a}	39.53^{abc}	49.82^{a}	59.06^{bcdefg}	58.34^{efghij}
TBA 3	30.14^{a}	33.28^{bc}	42.21^{a}	53.02^{efg}	54.28^{ghij}
TBA 4	33.79^{a}	30.64^{bc}	44.08^{a}	52.13^{fg}	52.72^{ij}
TBB 1	28.27^{a}	44.09^{abc}	57.06^{a}	70.42^{ab}	70.83^{ab}
TBB 2	23.18^{a}	37.38^{abc}	52.65^{a}	67.39^{abcd}	67.91^{abcd}
F-Hitung	4.53^{*}	15.97^{*}	3.88^{*}	90.13^{*}	181.40^{*}
P-Value	2.55×10^{-7}	2.0×10^{-16}	3.21×10^{-6}	2.0×10^{-16}	2.0×10^{-16}
BNJ 5%	3.02	1.60	2.41	0.78	0.56

Description: *significantly different; values in columns with the same letter are not significantly different at $P < 0.05$ according to Tukey's HSD test; Original data were square root transformed before statistical analysis.

The antagonist test aims to determine the ability of Rhizobacteria to inhibit *F. oxysporum* . Several bacterial strains are known to have the ability to suppress the growth of phytopathogens and can be applied as biocontrol agents [17]. The research results show that rhizobacteria treatment can inhibit pathogenic fungus *F. oxysporum* development in vitro. The percentage of inhibitory power for each rhizobacteria isolate showed different results. This was because the rhizobacteria isolates used were other, so the metabolite components activity was different. Different bacterial strains will produce different metabolite compounds so that their effects are also different [18].

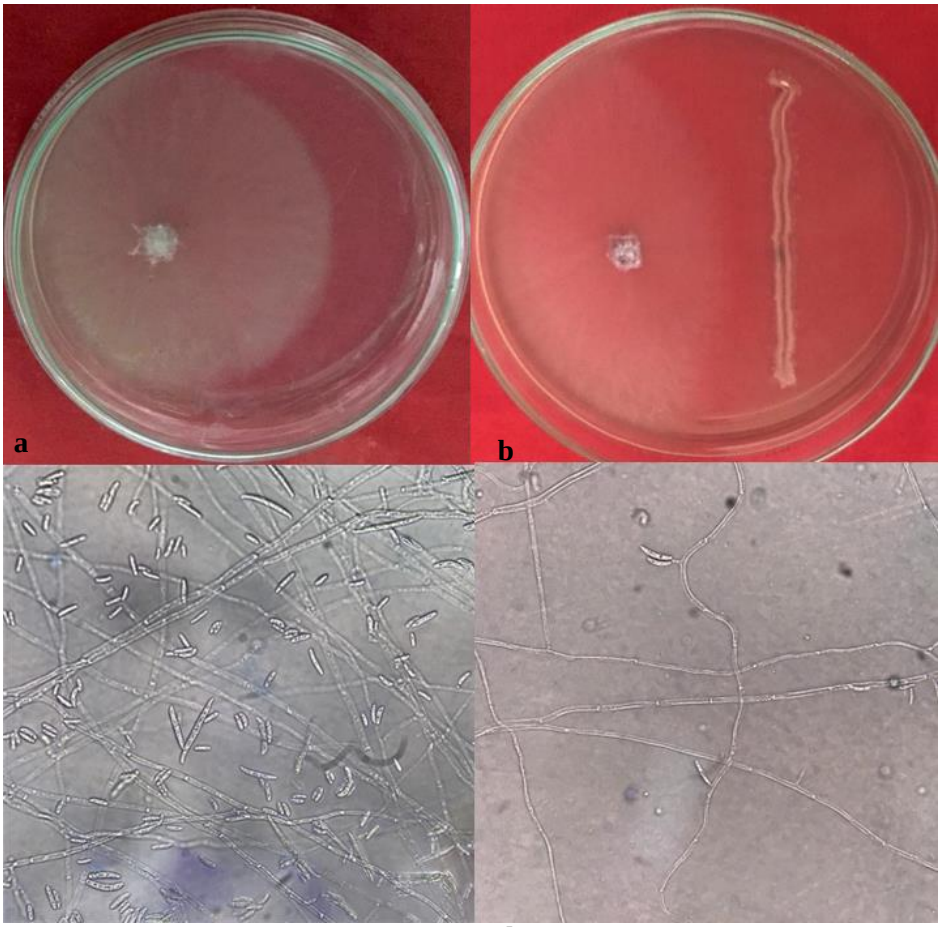

Figure 1 : Growth inhibition of *F. oxysporum* (a) control ; (b) effect of rhizobacteria isolat. Microscopic observation impact of rhizobacteria on hyphal morphology (c) control ; (d) effect of rhizobacteria.

Rhizobacteria can have an antagonistic mechanism in the form of antibiosis against pathogenic fungi. This can be shown by the *F. oxysporum* fungus colony being shortened, and there is a distance separating the pathogenic fungus colony and the rhizobacteria (Figure 1b). Forming a separation distance or inhibition zone indicates that the bacteria produce antifungal compounds [19]. In addition, antifungal activity can produce biochemical substances such as surfactin, chitinase, and β-1,3-glucanase, which can inhibit the growth of pathogen mycelium and can damage and inhibit the formation of pathogen cell walls [20]. Apart from that, the shape of inhibition zones can occur due to mechanisms of competition for nutrients and space. According to Bubici et al., microbes living in the same environment will compete for nutrients and other resources [21].

Microscopic observation showed that *F. oxysporum* fungal hyphae experienced abnormal growth due to antifungals produced by rhizobacteria. Abnormal growth in hyphae is indicated by the size of the hyphae being larger compared to normal hyphae of the *F. oxysporum* fungus, as well as the tip of the hyphae being smaller. Paisal et al., stated that antifungal compounds produced by bacteria generally result in abnormal growth of hyphae (malformations), which is indicated by shortening, swelling, shrinking, lysis, twisting and bending of the hyphae which results in the hyphae not being able to develop develop properly [16].

4. Conclusion

From this research, the results showed that all isolates had the potential to inhibit the growth of *F. oxysporum* . The highest inhibitory ability was shown by the KMTK2 isolate with an inhibitory ability of 73.99% and the lowest inhibitory ability was shown by the TBA1 isolate with an in-hibitory ability of 51.56%.

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