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Mass Propagation of Antagonistic Bacteria *Pseudomonas fluorescens* as an Environmental-Friendly Biocontrol Agent

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ABSTRACT

Biological control is a long-term and environmentally friendly method of pest management. The use of biological control agents has several advantages. These advantages include limiting the growth and development of plant-disrupting organisms over a relatively long period. Furthermore, biological agents have advantages in maintaining the equilibrium of the ecosystem that is present in agricultural environments. Due to their ability to create antimicrobials and stimulate plant development, as well as the fact that they are plant growth-promoting rhizobacteria (PGPR) and can survive in adverse environmental circumstances, antagonistic bacteria are one of the biological or biocontrol agents for managing illness. Antagonizing microorganisms can boost plant resilience to disease attack without polluting the environment or leaving toxic residues in the food chain Bacteria with good antagonistic agent properties can prevent the growth of pathogens and stimulate plant resistance responses. In addition, antagonistic bacteria can simultaneously boost the growth response of plants *(plant growth promoter*). One of the antagonistic bacteria used as PPGF is *Pseudomonas fluorescens*. *Pseudomonas fluorescens* has been widely recognized as having the potential as a biological agent to inhibit several plant pathogens. *P. fluorescens*is a group of root bacteria that effectively suppresses various plant diseases, including damping off of seedlings, soft rot, bacterial wilt, and others on many plant varieties. An antibiotic substance produced by *P. fluorescens*. (2,4-diacetylphloroglucinol/ 2,4-DAPG) can increase soil resistance to pathogens.

Keywords: Biological control, Antagonist bacteria, PGPR, Pseudomonas fluorescens.

Introduction

The agricultural sector plays a role in fulfilling and supporting the needs of human life, especially food, horticulture, and plantations. Plant pests and diseases are one of the barriers that can reduce plant productivity. The existence of attacks by the pest is dynamic and interrelated between plants (hosts), pests, and the environment (Dong & Lin, 2021). Pest and disease control usually uses chemicals because they are effective in suppressing Plant Pest Organisms populations. However, if used continuously and for an extended period, it will have a negative impact on plants, the environment, and even humans. The above problems can be overcome by biological control (Jatav & Dhar, 2014; Gisi & Leadbeater, 2010). Biological control is a long-term and environmentally friendly method of pest management. According to Muslim (2019), there are many Biological Control Agents that have been found in groups such as fungi (Plant Growth Promoting Fungi), bacteria (non-pathogenic bacteria: *Bacillus* sp. and *Pseudomonas* sp.), actinomycetes (*Streptomyces* sp.) (Suryaminarsih et al., 2022), and attenuated viruses. Novita et al. (2021) added that the use of biological control agents, apart from limiting the growth and development of plant-disturbing organisms over a relatively long time, the biological agent also has the advantage of protecting agricultural ecosystem balance.

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Antagonistic bacteria are one of the promising biocontrol agents for pest and disease because of their ability to produce antimicrobials and stimulate plant growth as well as their ability to suppress various types of plant pathogens such as plant growth-promoting rhizobacteria (PGPR) and can survive extreme environmental conditions (Wang et al., 2019; Raharjo et al., 2022). The use of antagonistic bacteria is also an environmental-friendly control because it does not contain toxic substances that can cause residues in the food chain, does not cause environmental pollution, and can increase plant resistance to pathogen attack. Antagonistic bacteria can suppress fungi or other bacteria through an antibiosis mechanism. These bacteria compete with nutrients or direct parasitism. Eris et al. (2017) added that good antagonistic agent bacteria are bacteria that can inhibit the growth of pathogens and stimulate plant resistance responses. In addition, antagonistic agent bacteria can simultaneously increase the growth response of plants (plant growth promoter).

Pseudomonas fluorescens potential as a biological agent to prevent numerous plant diseases has been extensively recognized. Several plant diseases, such as damping off of seedlings, soft rot, and bacterial wilt, can be prevented with the help of a species of rooting bacteria called *Pseudomonas* spp., according to research published by Istiqomah and Kusumawati (2018). Soil resistance to infections can be boosted with the use of *Pseudomonas* spp.-produced antibiotics (2,4-diacetylphloroglucinol/ 2,4-DAPG) (Weller et al., 2012). In light of the preceding context, it is vital to investigate, identify, and mass propagate that local-hostile microbes This research analyzed the rhizosphere layer of bamboo root soil in Made Village, Sambikerep District, Surabaya, for antagonistic microorganisms.

Material and Methods

Sampling and exploration

The rhizosphere layer was sampled on Kartono's bamboo plants with the Sendang Biru farmer group in Made Village, Sambikerep District, Surabaya City. Soil samples were taken at a soil depth of 0-5 cm, as much as 500 grams at 5 different points. Sampling was carried out in the rhizosphere layer of bamboo plants because it has been observed that the rhizosphere layer of bamboo has higher C-organic content than the non-rhizosphere layer of bamboo, according to Nurliana and Anggraini's (2018) research. High soil C-organic content can lead to high fungi, bacteria, and functional microbial populations because organic matter can be maximally fulfilled.

Sterilization of equipment and materials

Sterilization removes all living organisms, such as microorganisms (protozoa, fungi, bacteria, mycoplasma, and viruses) contained in an object. Sterilizing tools and materials are carried out with pressurized hot steam using an autoclave, according to Taufiq and Najmudin (2017). The working principle of the autoclave is sterilization at a temperature of 121ºC with a pressure of 1 atm for 30 minutes. Equipment sterilization is done for all tools, including Petri dishes, test tubes, media bottles, and Erlenmeyer. Tool sterilization activity begins with washing the equipment and then drying it. After drying, the petri dish is wrapped in unused paper with a side that has writing on the outside so that the writing ink does not stick to the petri dish. Furthermore, test tubes, media bottles, and Erlenmeyer are plugged using cotton, and the top surface is wrapped with aluminum foil.

Isolation of explored-bacteria

Bacterial isolation involves removing a bacterium from its native environment and cultivating it in an artificial medium. The soil samples were air-dried for 24 hours before isolation. Ten grams of soil from the bamboo rhizosphere were weighed and homogenized with 90 ml of sterile distilled water on the Erlenmeyer with a vortex (10^0). One ml of soil suspension was taken from Erlenmeyer with a pipette and dripped into the test tube 10^{-1}, then homogenized the solution in the test tube 10^{-1} by shaking for 30 times, then put the suspension into test tube 10^{-2}. Continue the same steps until test tubes 10^{-4}.

The streak plate and pour plate techniques are performed to isolate bacteria from the soil of the bamboo rhizosphere according to Sanders (2012). The streak plate method begins with taking the soil suspension in a 10^{-4} test tube, then dripping five drops of the soil suspension on the Kelman's media. After that, streaking and leveling the soil suspension over the media using an ose needle or glass slide.

After doing the streak plate, proceed with the pour plate method. Soil suspension in a 10^{-4} test tube were dripped five drops into Kelman's, then pour Kelman's media into the petri dish.

Selective media test

Bacterial isolates from exploration on the bamboo rhizosphere layer were streaked on Kelman's media. Isolation of bacteria using streak plate method. Initial streaks of isolates on Kelman's media were carried out by dividing the petri dish into 4 (four) parts and the bacterial isolates were streaked using an ose needle. Each isolate can only fill one side and not touch the other. Then the petri dish is wrapped and labeled. In one petri dish, there are 4 (four) bacterial colonies. Then the bacterial isolates were incubated for 24-48 hours. After that, the bacteria were observed and the bacterial culture was purified on new Kelman's media. Each bacterial colony is cultured in 1 (one) petri dish and there will be 4 (four) new bacterial isolates. The streak technique used is quadrant strokes, forming a continuous zig-zag in each quadrant, according to Prihanto et al., (2018).

Gram test (KOH 3%)

The gram test with 3% KOH determines whether the cultured bacteria are included in the gram-positive or gram-negative categories (Suyono & Farid, 2011). Three percent KOH was made by preparing 0.3 grams of KOH and 10 ml of sterile distilled water. KOH and sterile distilled water were put into a small Erlenmeyer and stirred until homogeneous. Sample bacteria on the preparation glass were mixed evenly with 1-2 drops of 3% KOH.

Pigmentation test

The pigmentation test is the observation of Pseudomonas bacteria under UV light to see the presence of fluorescent pigments. The presence of fluorescent pigments is characterized by bacteria that fluoresce under UV light. This test was carried out on King's B media. The pigmentation test is almost the same as the selective media test, where streak plates are carried out with quadrant streaks that form a continuous zig-zag in each quadrant. The difference is only in the media where the pigmentation test uses King's B media. After carrying out the streak plate, the bacteria are incubated for 24-48 hours. Then the bacteria were observed under UV light.

Virulence test

A virulence test was performed to distinguish bacteria that have low virulence, medium virulence, and high virulence. Testing for bacterial pathogenicity begins with the production of 1% Tetra Zolium Chloride (TZC). Initially, 0.5 grams of 1% TZC was weighed and added to 49 milliliters of sterile water solution. The solution was then homogenized and sterilized in an autoclave at 121 degrees Celsius and 1 atmosphere of pressure for seven minutes. The 100 cc Kelman's media in Erlenmeyer was then thawed and waited until it was neither too hot nor frozen. Next 1% of Kelman's media was dripped and homogenized, then it was placed into a petri dish and allowed to freeze. After cooling and freezing the TZC media, streak plate bacterial isolates using the quadrant scratch method. The bacteria were then cultured for 24 to 48 hours. High-virulence bacteria are white with a pink border, low-virulence bacteria are pink to dark red, and non-virulent bacteria are blue-red.

Soft rot test

The soft rot test is used to evaluate whether or not the bacteria being tested are pathogenic. If the potato is pathogenic, it will develop rotten spots within 24 to 48 hours. To conduct the soft rot test, healthy potatoes, Whatman paper, sterile distilled water, and Petri dishes are required. First, the potatoes are peeled and properly rinsed under running water, and then they are cut into approximately 5x3x1 cm squares. In the center of the potato, dig a 1-centimeter-deep hole. The potatoes were then gently cleaned with alcohol and sterile distillate water. After placing the Whatman paper on the bottom of the petri dish, sterile distilled water was dripped onto it until it was saturated. The potatoes are then laid on butcher paper. Using a loop needle, the bacterial isolates were then streaked into the potato trench. The bacteria were then cultured for 24-48 hours. The test result is affirmative if the potato exhibits soft rot signs.

Hypersensitive test

The purpose of the hypersensitivity test is to detect the pathogenicity of bacteria. Tobacco plants are used in the hypersensitivity test because they are susceptible to infections and have thick leaf bones, making testing more accessible. If the tobacco leaves were necrotic, positive findings were achieved, however negative results were obtained if the leaves were not necrotic. The following are the steps for hypersensitivity testing. Prepare the tobacco plant, the *P. fluorescens* isolate, a syringe, 70% alcohol, and sterile distilled water first. Finally, pour sterile distillate water into the Erlenmeyer and the petri dish. The bacteria are then dissolved in sterile distillated water using an ose needle. Moreover, the syringe is cleansed with alcohol once and sterile distilled water three times. Then, 1 ml of the liquid bacterial isolate was injected into the bones of the tobacco leaf using a syringe. After being labeled, the tobacco leaves were cultured for 24-48 hours.

Results and Discussion

The penalty for paying compensation is a consequence of deceit or corruption that endangers the country's finances or the country's economy. A Juridical means is needed to recover the losses, namely in the remittance of replacement money. Replacement money is an additional form of punishment (criminal) in corruption cases. In essence, both legally and doctrinally, judges are not required to always impose additional penalties.

Results of exploration of antagonistic bacteria in the Rhizosphere Layer of bamboo plants

Exploration of the rhizosphere layer of bamboo plants found bacterial isolates with antagonistic properties (*Bambusoideae* sp.). The rhizosphere layer of bamboo plants (*Bambusoideae* sp.) was selected because there have been numerous reports of potential antagonistic microorganisms from the bamboo rhizosphere that have antagonistic ability against soil-borne pathogens (soil-borne disease) via antagonistic mechanisms such as competition for life, parasitism, antibiotics, and induced systemic resistance (Fuke et al., 2021; Xing et al., 2021). According to Susanti et al. (2015), apart from suppressing the development of pathogens, rhizosphere microbes can also increase plant growth through various mechanisms, including the production of growth-stimulating compounds such as phytohormones. Safuf et al., (2019), added that bamboo rhizobacteria are a group of beneficial bacteria that can actively colonize the rhizosphere, which is essential in increasing plant growth, soil fertility, and crop fertility yields. The sampling location for the rhizosphere layer was carried out on bamboo plants in Made Village, Sambikerep District, Surabaya City. Bamboo rhizosphere soil samples were planted on Kelman's media in 2 Petri dishes where each petri dish was divided into 4 so there were 8 isolates. Moreover, the results of the exploration and isolation produced 4 bacterial isolates that grew on Kelman's media (Figure 1.). Then the bacteria were purified and tested for selective media, gram test, pigmentation test, virulence test, soft rot test, and hypersensitivity test for each isolate.

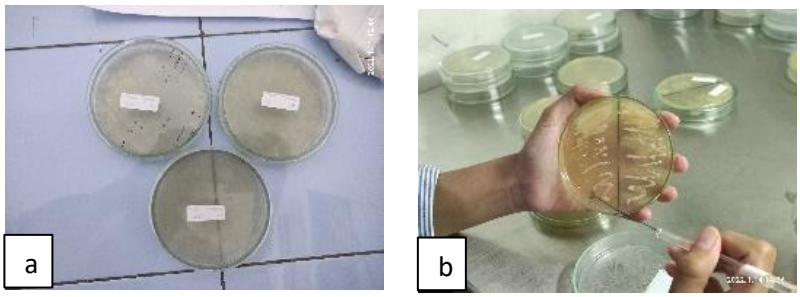

Figure 1. Exploration result. a) Bacteria PF on Kelman’s media, b) The results of bacterial isolates that have grown

Results of identification characterization of colony morphology of antagonist bacterial isolates

Antagonistic bacterial isolation in the rhizosphere layer of bamboo plants produced 8 isolates, but only 4 isolates were taken to be tested (Figure 2.). This is because the other 4 isolates did not grow well.

According to Putri et al., (2017), growth media can also be used for micro-organism isolation, identification, and making pure cultures. Growth media must meet the nutritional requirements needed by bacteria such as carbon (CO_2 and CH_4), nitrogen (NO_2 and NO_3), as well as the most crucial mineral elements such as Ca, Zn, Na, K, Cu, Mn, Mg, and Fe, Vitamins, Water, and Gases. The four bacterial isolates were then purified in Kelman's medium using the streak plate method using the quadrant streak technique. Using the quadrant scratch technique, a single colony of bacterial isolates will be obtained so that the morphology of the colony can be observed. According to Wulandari et al. (2019), bacterial growth media based on their properties and functions are classified into enriched media, exclusive media, cell-active media, culture media, and media used to study the biochemical properties of a particular bacterium. Observation of the morphological characteristics of the colonies and bacterial cells aims to determine the characters and characteristics of the bacteria obtained for bacterial identification. According to Wulansari et al. (2019), macroscopic morphological characterization was carried out by observing bacterial colonies growing on the surface of the agar medium, such as the shape, color, size, and edges of the colonies.

The results of the observation of the colony morphology of the antagonistic bacterial isolates were that the four bacterial isolates were round, milky white, and had a convex surface. However, the edges of isolate PF 1 are wavy, and the edges of isolates PF 2, PF 3, and PF 4 (table 1). From these results, it can be assumed that PF 2, PF 3, and PF 4 are isolates of Pseudomonas bacteria. This is following the research of Adiathy et al. (2017), that one of the morphological characteristics of the genus Pseudomonas is that the colonies are round and white-cream yellow.

Table 1. Description of colony morphology of antagonist bacterial isolate in the rhizosphere layer of bamboo plants (*Bambusoideae* sp.)

No	Isolate Code	Colony Morphological Characteristics			
		Shape	**Colour**	**Elevation**	**Margin**
1.	PF 1	Circular	White	Convex	Endulate
2.	PF 2	Circular	White	Cembung	Entire
3.	PF 3	Circular	White	Cembung	Entire
4.	PF 4	Circular	White	Cembung	Entire

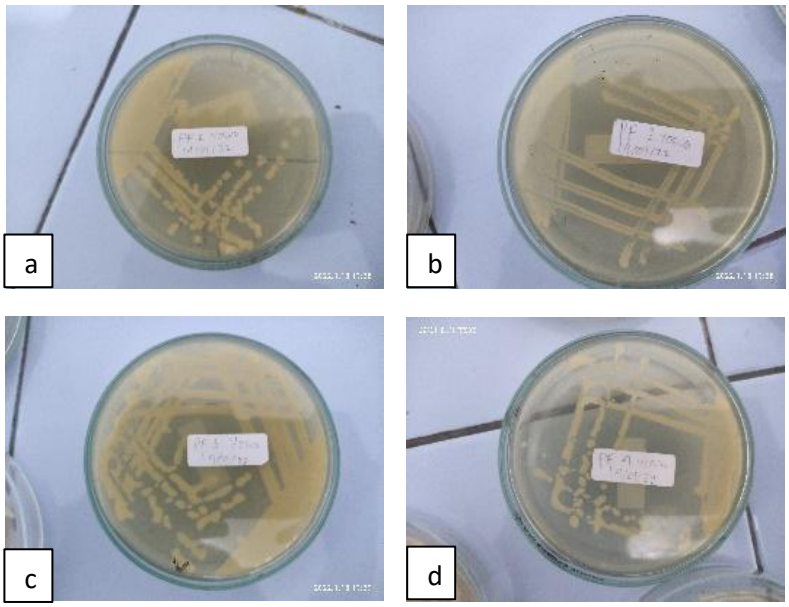

Figure 2. Results of observation of bacterial morphology PF. a) Isolate PF 1, b) Isolate PF 2, c) Isolate PF 3, d) Isolate PF 4

Identification results of physiological and biochemical characterization of antagonist bacterial isolates

Physiological and biochemical characterization is the basis for identifying bacteria down to the genus level. Bacteria were identified at the biological agent's laboratory center UPT Protection of Food Crops and Horticulture, including the Gram test (3% KOH), Pigmentation test, Virulence test, Soft Rot test, and Hypersensitivity test (table 2). Physiological identification is carried out to determine bacteria based on their cell activity (Doberenz et al., 2017). In Contrast, biochemical identification studies the compounds present in living systems, the arrangement of these compounds into cells, and the chemical interactions that occur (Amrulloh et al., 2021). Physiological and biochemical identification is also essential in characterizing unknown bacterial species because morphologically different cultures or bacterial cells will look similar if physiological and biochemical observations are not carried out (Liu et al., 2014).

Table 2. Results of physiological and biochemical characteristics of endophytic bacteria isolates

Num-ber	Isolat Code	Colony Morphological Characteristics				
		Gram Test	Pigmentasi Test	Virulensi Test	*Soft Rot* Test	Hypersensi-tive Test
1.	PF 1	-	+	Has no virulence	-	-
2.	PF 2	+	+	Has no virulence	-	-
3.	PF 3	-	+	Has no virulence	-	-
4.	PF 4	+	+	Has no virulence	-	-

Explanation:

Gram Test: The sign negative (-) means the result is negative and the bacteria has a gram-positive character. The sign (+) indicates that the test result is positive and the bacteria has a gram-negative character.

Pigmentasi Test: The (+) sign indicates that the bacteria has a fluorescent power under ultraviolet light and the (-) sign indicates that the bacteria does not have a fluorescent.

Virulence Test: Bacteria with high virulence will be white with pink edges, bacteria with low virulence will be pink to dark red, and bacteria that do not have virulence will have a bluish-red color.

Soft rot test: The (+) sign indicates that the bacteria can cause potatoes to rot, and the (-) sign indicates that the bacteria do not cause the rotting of potatoes.

Hypersensitive: The (+) sign indicates that the bacteria are pathogenic and the (-) sign indicates that the bacteria are non-pathogenic.

Gram test result (KOH 3%)

The gram test using 3% KOH is carried out to determine whether the cultured bacteria are included in the gram-positive or gram-negative category. This test is a suitable method of identifying bacteria in determining the dominant type of active bacteria, which is indicated by the presence of mucus (Eliopoulus & Bush, 2001). According to Oviana et al. (2015), if unbroken threads of mucus are formed, the bacteria being cultured are gram-negative bacteria. However, if they are not formed, the bacteria are gram-positive.

The 3% KOH test result on pseudomonas bacteria showed that the bacteria PF 1 and 3 were gram-positive and PF 2 and 4 were gram-negative. Hardiansyah et al. (2020) added that 3% KOH testing on bacteria indicated gram (+) bacteria had thick cell walls and thin fat, while gram (-) had thick fat and thin cell walls located in the periplasmic space. KOH will attack fat (lipid bilayer) and make gram (-) cells break. The ruptured cell releases genetic material (DNA), an abundant substance in bacterial cells. Very long DNA molecules are sticky strings that appear as slime when removed with an inoculum needle.

Pigmentation test result

The pigmentation test is carried out on King's B media to determine whether a bacteria is classified as a fluorescent bacterium (Figure 4.). According to Soesanto (2008) who stated that *P. fluorescens* secretes green, red green, pink, and yellow pigments, especially in a medium lacking iron. *P. fluorescens* forms a fluorescent pigment known as fluorescein. Bacterial isolates that produce glow are PF 2, PF 3, and PF 4 with yellow pigments. According to Rahayu (2008), this pigment is an extracellular organic compound with a low molecular weight and a very strong affinity for Fe, soluble in water, and fluoresces under ultraviolet. It acts as an antibiotic, so it functions as a biocontrol. Hasanuddin (2011) added that fluorescein-fluorescent pigments would become raw materials for a broad spectrum of bioactive (for example, antibiotics, siderophores, volatile compounds, and phytohormones).

Virulence test result

A virulence test is performed to determine bacteria that have high virulence (malignancy), low virulence, and no virulence. Virulence test using TZC media, a selective media that can distinguish between a-virulent and virulent bacteria (Cosson et al., 2002). Based on the results of the virulence test, the four bacteria namely, PF 1, PF 2, PF 3, and PF 4 were not virulent because they were red in TZC media. According to James et al. (2003), when growth on semi-selective media (tetrazolium chloride), it will form slimy white colonies with a pink color in the middle (virulence) (Figure 5.), while a-virulent colonies are dark red.

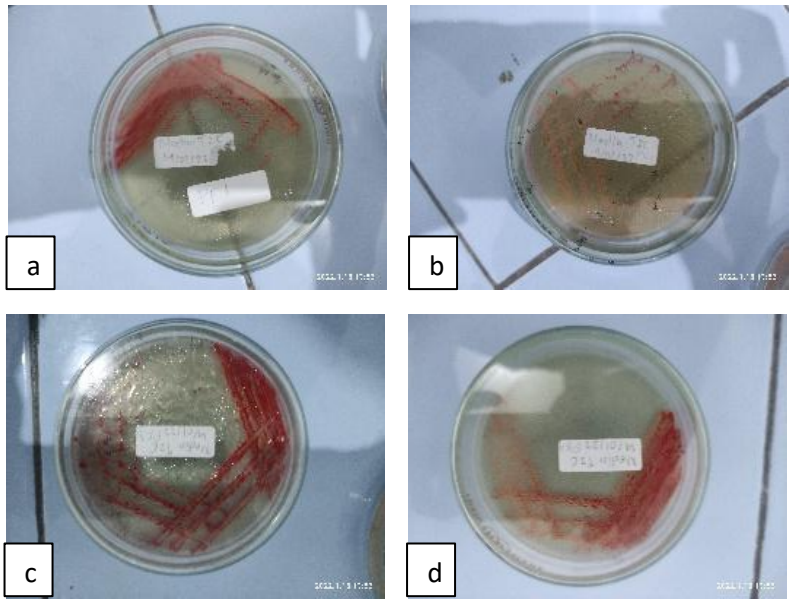

Figure 5. Virulence Test. a) Isolate PF 1 on TZC media, b) Isolate PF 2 on TZC media, c) Isolate PF 3 on TZC media, dan d) Isolate PF 4 on TZC media.

Soft rot test result

The soft rot test is a test that aims to distinguish pathogenic and non-pathogenic bacteria. Pathogenic bacteria will rot potatoes within 12-24 hours (Li et al., 2007). Based on the potato decay test (soft rot) carried out, the four isolates, namely PF 1, PF 2, PF 3, and PF 4 were negative and did not spoil the potato media. It can be concluded that the four bacterial isolates tested were non-pathogenic. Bacteria that are positive in the potato rot test (soft rot) are indicated by the rotting of the potato's middle part, which is scratched by the bacteria. According to Oviana et al. (2015), soft rot bacteria are a group of bacteria that cause soft rot in plants and are pathogenic.

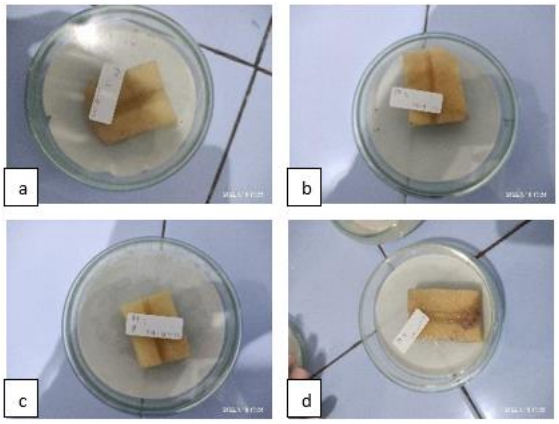

Figure 6. Soft rot test. a) Isolat PF 1 on potato, b) Isolat PF 2 on potato, c) Isolat PF 3 on potato, d) Isolat PF 4 on potato

Hypersensitive test result

Hypersensitivity test is a form of defense from the host plant that develops rapidly in the face of incompatible pathogens with the host plant resulting in cell death. Hypersensitivity reactions occur only in incompatible combinations between the host plant and the pathogen (Klement & Goodman, 1967). Based on hypersensitivity testing, the four isolates namely PF 1, PF 2, PF 3, and PF 4 were negative and did not cause necrosis on tobacco leaves. From these results, the four PF bacterial isolates can be used as biological control agents. According to Kurniawati et al. (2020), the hypersensitivity test is a method that can be used to quickly detect the potential for the pathogenicity of a bacterium in plants. Hypersensitivity test reactions occur within 24-48 hours and are localized. Cellular membranes on tobacco plant leave that come in contact with pathogenic bacteria will be destroyed and necrosis. This response indicates that bacteria in contact with tobacco plant leaves have the potential to become plant pathogens.

Figure 7. Hypersensitive test on tobacco plants

Mass propagation of P. fluorescens bacteria

Mass propagation of *P. fluorescens* bacteria is the last step before the bacteria can be used as PGPR. Material for mass propagation utilizes soybeans (*Glycine max* L.) as a liquid growth medium. According to Fauziah et al. (2016), soybeans are the best source of protein, fat, vitamins, minerals, and fiber. This mass propagation also utilizes a simple fermenter because *P. fluorescens* bacteria in liquid media needs to be fermented first. Bacterial propagation has several requirements that must be met, including 1) the new product must contain 2.5x 10^8 cfu/g, 2) 3 months after storage at room temperature, the population must be 8-9 x10^7 cfu/ g, 3) the storage period of 3-4 months, and 4) finished product moisture not more than 20%. However, the mass propagation that was carried out was not based on the literature because it was carried out with a simple fermenter and did not make it difficult for farmers to carry out mass propagation on their own.

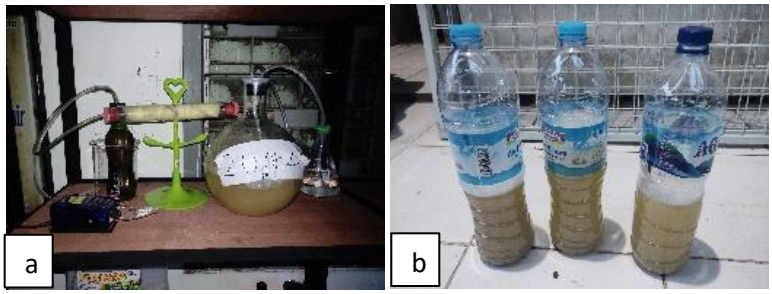

Figure 8. Mass Propagation of *P. fluorescens* bacteria. a) Mass propagation result with fermentor, b) bacteri *P. fluorescens* PGPR product

Conclusion

The antagonistic bacteria were obtained from the rhizosphere layer of bamboo plants (*Bambusoideae* sp.) located in Made Village, Sambikerep, City of Surabaya. Bacterial identification activities went through several stages, namely the Gram test KOH (3%), Pigmentation test, Virulence test, Soft Rot test, and hypersensitivity test. Furthermore, this test showed that the bacterial isolates found were *Pseudomonas fluorescens* and can be mass propagated as a biological control agent.

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