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Isolation of *Pesudomonas fluorescens* from Infected and Uninfected Soil by *Ralstonia solanacearum* Capable of Producing Siderophore, HCN and Soluble Phosphate

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ABSTRACT

Ralstonia solanacearum, the causal agent of bacterial wilt disease, ranks among the most devastating pathogen in solanaceaus crops. In West Sumatra, the bacterial decreased tomato production up to 11% in 2014. This reduction could potentially occur annually due to the wide range of host and destructive levels of R. solanacearum. One of the efforts to suppress R. solanacearum is by utilizing the indigenous biological agents. In several cases, bacterial group of Pseudomonas fluorescens [Pf] has been reported suppress some soilborne pathogens regarding to its ability to produce secondary metabolite such as siderofor and HCN and to dissolve phosphate. This study aimed to select Pf bacterial thus produce secondary metabolite and dissolve phosphate. Pf was isolated from soil at the rhizosphere of tomato grown in severely attacked [>30%] by R. solanacearum in the District of Payakumbuh and a healthy site in the District of Tanah Datar. Both of the districts are in the region of The Province of West Sumatra Indonesia. The observation parameters were the production of siderophore, HCN and soluble phosphate. From 24 of collected Pf isolates, 16 were able to produce siderophore, HCN and to dissolve phosphate. The results showed that isolate Pf-Stj11 derived from the healthy tomato plant grown in infected soil by R. solanacearum produced the highest siderophore with absorbance was 1.776. While higher HCN was produced by isolates Pf-Stj3 and Pf-Stj8 of tomato diseased plant grown in severely infected soil by R. solanacearum as well as Pf-Stj9 and Pf-Stj1 from the healthy tomato plant grown in infected soil by R. solanacearum. The highest ability to dissolve phosphate was by the isolate Pf-Stj4 from tomato diseased plant grown in severely infected soil by R. solanacearum.

Keywords: *Pseuodomonas fluorescens, Siderophore, HCN, Phosphate, Ralstonia solanacearum*

INTRODUCTION

Ralstonia solanacearum, the causal agent of bacterial wilt disease, is a soilborne and waterborne pathogen [1-12]. This pathogen is one of the world's most destructive bacteria to horticulture crops due to its lethality, persistence, wide host range and broad geographic distribution. The visible symptoms of bacterial wilt are usually seen on the foliage of plants, including on tomato. Chemical pesticide is a common method to control *R. solanacearum* to date. However, negative impact of using chemical pesticides in terms of environment, health and economy have been reported everywhere in the world [13-24].

In the island of Sumatra, West Sumatra is one of the main producing provinces of tomatoes to neighboring provinces such as Jambi, Bengkulu, Riau and Riau Islands. But the attack of *R. solanacearum* potentially damaged tomato agribusiness in West Sumatra. In the period of one year, from 2013 to 2014, it was reported that tomato production in West Sumatra has decreased 11%. This decline has the potential to occur every year because R. *solanacearum* has about 450 other hosts [3,24], therefore it is very difficult to be controlled. One of the efforts that can be done to suppress the attacks of *R. solanacearum* is to utilize the group of Pf as biological control agent [25-35].

Based on field studies at the several centre of tomato planting areas in the province of West Sumatra since 2012, plants were found attacked by *R. solanacaearum* up to 30%. In contrast, several other tomato plants were still healthy even grown in a very close distance with tomato diseased plant. It seems there is a specific condition of these plants associated with their ability to remain healthy as it is assumed as the role of Pf. Pf protects plants from diseases caused by various soil-borne pathogens [2,25,32]. Factors that make Pf capable as a biocontrol agent due to its ability to produce siderophore and HCN and as well as to dissolve phosphate that suppress the activity of pathogenic bacteria *R. solanacearum* [22]. This study aimed to compare the ability of Pf isolates which were isolated from rhizosphere of tomato plants those were cultivated at heavy infected area by *R. solanacearum* and at un-infected site to produce siderophore, HCN and to dissolve phosphate.

MATERIAL AND METHODS

Microscopic characterization of collected isolates

This study was conducted at the Laboratory of Microbiology, Biology Department, Faculty of Mathematics and Natural Sciences, Andalas University from October 2016 to July 2017. The isolates were collected from soil at the rhizosphere of tomato diseased plants which were severely attacked by *R. solanacearum* in Situjuah region in the District of Payakumbuh and at the rihozphere of tomato healthy plants in Tabek Patah region of the District of Tanah Datar. Isolates description as Pf was done based on Cappuccino [17]. The districts of Payakumbuh and Tanah Datar are in the Province of West Sumatra Indonesia.

Production of siderophores

Siderophore production by Pf was carried out according to Dirmawati [33]. Isolate was grown in the siderophore production medium [20 g sucrose, 2 g L-asparangin, 1 g K_2HPO_4 and 0.5 g $MgSO_4$] for 24 hours. Bacterial suspension was centrifuged at 11

000 rpm for 30 minutes. Detection of siderophore production was done by adding 1 mL of 0.01 M $FeCl_3$ into 3 mL of the supernatant. Then it was measured by using a spectrophotometer with a wavelength of 410 nm.

Production of HCN compounds

Isolates of collected Pf were grown on glycine media in 9 cm diameter petridish. The lid of petridish was affixed with a piece of filter paper that has been poured with HCN detection solution [2 g picric acid and 8 g of sodium carbonate in 200 mL of water]. Bacterial culture was incubated at a room temperature. Indicator of the production of HCN is based on discoloration on the filter paper of light yellow to dark brown [4].

Ability of dissolving phosphates

The ability of dissolving phosphate was carried based on Thakuria et al. [9] by using Pikovskaya agar medium with the addition of tri-calcium phosphate [TCP] as a source of phosphate. After sterilization with autoclave, the media poured into a petridish, allowed to harden and a 1 cm diameter of hole was prepared on the hardened media then 5 mL bacterial suspension was poured into this hole. Dissolve phosphate ability can be seen from hollow zone around the hole containing this 5 mL bacterial suspension.

RESULTS AND DISCUSSION

This study was conducted on tomato plantations at the infected and un-infected soil by *R. solanacearum*. Soil samples were collected from the rhizosphere of tomato plants severely infected [up to 30%] with *R. solanacearum* in a plantation site of 7000 tomato plants at 2 ha farmer property [540 masl] in Situjuah-the District of Payakumbuh. While soil samples derived from the rhizosphere of healthy tomato plants were selected from 11 000 tomatoes plants those grown at 3 ha farmer property [780 masl] free of *R. solanacearum* disease symptoms in Tabek Patah- the District of Tanah Datar. Distance between these two location sites is 50 km [36,37].

Macroscopic characterization of collected isolates

Macroscopic identification of the collected isolates based on Cappuccino [17] resulted in 24 isolates with characters similar to Pf (Table 1).

Based on of mcroscopic observation, all of observed isolates have different characters (Table 1). Macroscopic colonies consist of circular, irregular, filamentous and rhizoid types and were dominated by 16 isolates of circular forms. While the entire and undulates derived from 10 isolates. At the elevation observation of the colony, the most common type was umbonate which 15 isolates were while the color of the colony was dominated by creamy color within 17 isolates. One of the important characters of Pf is described by circular form, the dominated form type which was found in this study. Identified circular shaped of *Pf 's* colony was also found in the rhizosphre of banana, as well as the flat shape [26]. Rhodes [21a] insisted that circular shape of the colony is one of the distinct Pf character. From microscopic observation in this study, all of isolates have basil cell shape and gram negative results. Two important characteristics possessed by pseudomonad group bacteria are bacillus cell shape and gram negative [19]. Bacteria classified as a gram negative when it is treated with gram staining will shows the red color and when is dyed with KOH 3% there will be mucus, as obtained in this study. This is associated with a gram-negative cell wall arrangement

that has a thin peptidoglycan layer and a thick layer of phospholipids. During gram staining process, this phospholipid layer will be degraded and will absorb the colors of safranin that is red [11].

Table 1: Mcroscopic characterizations of colony of *Pseudomonas fluorescens* [Pf] collected from rhizosphere of tomato plants at an infected plantation site by *Ralstonia solanacearum* in Situjuah [Stj] District of Payakumbuh and at a healthy plantation site in Tabek Patah [TbP] District of Tanah Datar.

No	Code of isolate	Source of isolates	Shape of colony	Margin of colony	Type of colony elevation	Color of colony
1.	Pf-Stj1	Diseased tomato plant in infected site by *R. solanacearum*	Circular	Entire	Flat	Yellowish white
2.	Pf-Stj2	Diseased tomato plant in infected site by *R. solanacearum*	Irregular	Undulate	Umbonate	Cream
3.	Pf-Stj3	Diseased tomato plant in infected site by *R. solanacearum*	Circular	Entire	Flat	Yellow
4.	Pf-Stj4	Diseased tomato plant in infected site by *R. solanacearum*	Circular	Undulate	Convex	Cream
5.	Pf-Stj5	Diseased tomato plant in infected site by *R. solanacearum*	Irregular	Undulate	Umbonate	Whitish green
6.	Pf-Stj6	Diseased tomato plant in infected site by *R. solanacearum*	Irregular	Undulate	Umbonate	Cream
7.	Pf-Stj7	Diseased tomato plant in infected site by *R. solanacearum*	Circular	Entire	Flat	Cream
8.	Pf-Stj8	Diseased tomato plant in infected site by *R. solanacearum*	Circular	Undulate	Umbonate	Whiteish green
9.	Pf-Stj9	Healthy tomato plant in infected site by *R. solanacearum*	Filamentous	Undulate	Umbonate	Cream
10.	Pf-Stj10	Healthy tomato plant in infected site by *R. solanacearum*	Filamentous	Undulate	Umbonate	Yellowish white
11.	Pf-Stj11	Healthy tomato plant in infected site by *R. solanacearum*	Circular	Entire	Umbonate	Cream
12.	Pf-Stj12	Healthy tomato plant in infected site by *R. solanacearum*	Filamentous	Undulate	Umbonate	Cream
		Healthy tomato plant in				

13.	Pf-Stj13	infected site by *R. solanacearum*	Circular	Undulate	Umbonate	Cream
14.	Pf-Stj14	Healthy tomato plant in infected site by *R. solanacearum*	Rhizoid	Filiform	Umbonate	Cream
15.	Pf-Stj15	Healthy tomato plant in infected site by *R. solanacearum*	Circular	Entire	Umbonate	Yellow
16.	Pf-Stj16	Healthy tomato plant in infected site by *R. solanacearum*	Circular	Entire	Umbonate	Cream
17.	Pf-TbP1	Healthy tomato plant in uninfected site by *R. solanacearum*	Circular	Entire	Flat	Yelowish white
18.	Pf-TbP2	Healthy tomato plant in uninfected site by *R. solanacearum*	Circular	Entire	Flat	Cream
19	Pf-TbP3	Healthy tomato plant in uninfected site by *R. solanacearum*	Circular	Lobate	Umbonate	Cream
20.	Pf-TbP4	Healthy tomato plant in uninfected site by *R. solanacearum*	Circular	Lobate	Flat	Cream
21.	Pf-TbP5	Healthy tomato plant in uninfected site by *R. solanacearum*	Irregular	Undulate	Umbonate	Cream
22.	Pf-TbP6	Healthy tomato plant in uninfected site by *R. solanacearum*	Circular	Entire	Umbonate	Cream
23.	Pf-TbP7	Healthy tomato plant in uninfected site by *R. solanacearum*	Filamentous	Lobate	Flat	Cream
24.	Pf-TbP8	Healthy tomato plant in uninfected site by *R. solanacearum*	Circular	Entire	Flat	Cream

Siderophores production of collected isolates

The production of siderophores, HCN and soluble phosphate is presented in Table 2. The capability of Pf to produce siderophores was determined by spectrophotometers using a wavelength of 410 nm. Duffy and Defago [6] stated that the production rate of greenish-green or greenish pigment of filter paper used is positively correlated with the production of siderophores. When detected siderophores was associated with a wavelength of 410 nm as it was used in this study, it is certain that the kind of siderophore obtained is pseuodobactin [14]. The absorbance value data using the wavelength of 410 nm in the bacterial isolate can be seen in Table 2.

Table 2: The ability of pseudomonads fluorescent bacterial collected from the rhizosphere of diseased and healthy tomato plants those were grown in the infected and uninfected sites by *R. solanacearum* in producing siderophore, [A_{410}], HCN and soluble phosphate[Ø cm].

No	Code of isolate	Production of		
		Siderophore	HCN	Soluble phosphate
1	Pf-Stj2	1.147	++	-
2	Pf-Stj3	1.166	+++	-
3	Pf-Stj4	0.808	++	2.45
4	Pf-Stj5	0.370	+	-
5	Pf-Stj6	0.581	+	1.63
6	Pf-Stj8	0.279	+++	1.30
7	Pf-Stj9	1.720	+++	1.48
8	Pf-Stj10	0.831	++	1.83
9	Pf-Stj11	1.776	+++	1.73
10	Pf-Stj13	1.567	++	1.48
11	Pf-Stj15	0.450	+	1.68
12	Pf-Stj16	0.786	++	1.80
13	Pf-TbP1	1.677	++	1.73
14	Pf-TbP2	0.246	+	1.58
15	Pf-TbP3	0.390	++	1.60
16	Pf-TbP4	1.362	++	2.13

Note: = -No soluble phosphate detected. +, ++ and +++ = low, medium and high HCN production respectively.

In this study, the highest absorbance value was 1.776 produced by Pf-Stj11 isolate. However, when compared with others Pf isolates derived from the rhizosphere of tomato diseased plant, the highest absorbance values were found in Pf-Stj9 isolate with an absorbance value of 1.720. While the highest absorbance value of Pf isolate from rhizosphere of tomato healthy plant grown in un-infected site by *R. solanacearum* was 1.677 derived from Pf-TbP1 isolate. This explains that Pf-Stj11 isolate was capable of producing more siderophore than other isolates. The high ability of Pf-Stj11 to produce siderophore showed its potential to be a biological agent. This is associated with the function of siderophores to chelate Fe that causes Fe becomes unavailable for pathogens. The bacterial capable of producing siderophore will monopolize the rhizosphere and suppress the growth of pathogens, because siderophore has a high affinity for Fe_3^+ and facilitates cellular iron transport [22].

The antagonistic mechanism of Pf to produce siderophore against *R. solanacearum* is in competing with Fe element and inhibiting the growth of pathogen by excreting pseudobactin [14]. Besides, siderophore is also responsible to dissolve insoluble phase minerals such as phosphate to be utilized by plants [22]. Phosphate plays an important role in improving the quality of plants, among others, for cell growth, formation of fine roots and root hair, formation of flowers, fruits and seeds and strengthens plant resistance to disease [9].

HCN production of collected isolates

HCN compounds produced by the Pf group act as antimicrobials because they inhibit cytochrome C at the time of respiration and affect the release of metal ions [5]. *Fluorescens* bacteria group is also very effective and aggressive as root colonizer than non-*fluorescens* [2]. From the results of this study, it was found that HCN producing ability of the Pf isolates derived from the rhizosphere of tomato diseased plant in the infected area by *R. solanacearum* higher than in uninfected area (Table 2). While the production of HCN compounds from Pf isolates in rhizosphere of tomato healthy plant was lower. In fact, the production of HCN compounds was influenced by the activity of pathogens present in the plant rhizosphere [2]. The existence of the pathogens stimulates Pf to compete in absorbing minerals such as P bound to Fe and Al. However, in this study, the high production of HCN compounds did not relate to the ability of the isolates to produce siderophores and dissolve phosphate (Table 2). The indicator of the production of HCN by Pf bacteria is the change of color of filter paper which is dropped with picric acid and sodium carbonate [4]. The picric acid is a strong acid that acts as a proton donor for release H^+, the carbonate ions of Na_2CO_3 react with H^+ from the OH group of picric acid and form sodium picrate [picrate alkaline]. The nitro group presents in the picrate alkaline carries a partial positive charge and draws the electrons from the benzene ring, allowing for the reaction of the cyanide ion to the picrate compound. While the Pf bacteria produce HCN, it is captured by a picrate alkaline paper originally yellow to brownish. The higher the production of HCN, the indicator color which has originally colored yellow will turn brown proportional to the concentration of HCN produced by Pf [4,8].

Soluble phosphate production of collected isolates

In this study, all isolates of Pf which were isolated from rhizosphere of healthy or tomato diseased plants, either grown in infected or uninfected site by R. *solanacearum*, have potentiality to dissolve phosphate (Table 1). This indication can be seen in the presence of a hollow zone around the isolates grown on the medium of Glisin (Figure 1).

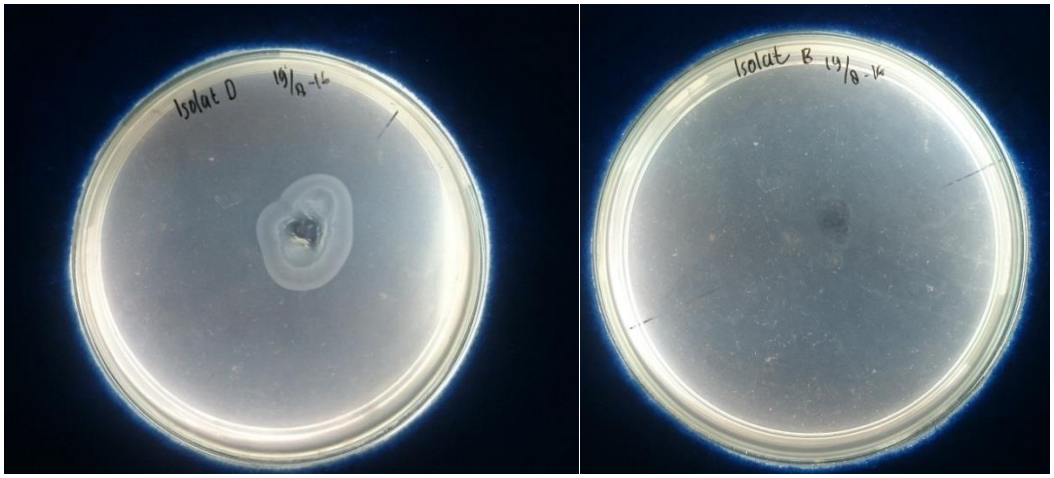

Figure 1: Hollow zone formed on isolate Pf-Stj4 [left], and no hollow zone formed on isolate Pf-Stj2 [right]

From this study, the largest hollow zone was 2.40 cm which was caused by Pf-Stj4 isolate (Table 2). This isolate derived from the rhizosphere of diseased tomato plant on the infected site by *R. solanacearum*. According to Alexander and Zuberer [7], hollow zone around the colony is qualitatively characterized by the ability of bacteria in dissolving phosphate, which in this study

was by isolate Pf-Stj4, not by Pf-Stj11. Isolate Pf-Stj11 has the highest siderophore and higher HCN production capability, but did not follow by its high posphate dissolving ability. The ability of Pf in dissolving phosphate varies depending on the type of its strain [15].

CONCLUSION

From 24 isolates of the Pf collected in this study, 16 produced siderophore, HCN and soluble phosphate. The highest siderophore production was by isolate Pf-Stj11, however did not follow by its ability to dissolve phosphate. The highest ability to dissolve phosphate was from isolate Pf-Stj4. The isolate of Pf-Stj11- together with isolates Pf-Stj3, Pf-Stj8, Pf-Stj9 - also produced similar higher amount of HCN compare with others.

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