ANTAGONISTIC POTENTIAL OF FLUORESCENT *Pseudomonas* AGAINST *Xanthomonas oryzae* pv. *oryzae*, THE BACTERIAL LEAF BLIGHT PATHOGEN IN RICE AND RELATED MECHANISMS

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ABSTRACT

A total of 158 bacterial strains of fluorescent Pseudomonas (FPs) were isolated from the rhizosphere soil of 20 rice fields in 3 districts Long My, Vi Thuy, and Vi Thanh in Hau Giang province. Through in vitro antagonistic potential evaluation against Xanthomonas oryzae pv. oryzae caused bacterial leaf blight disease in rice, five strains Ps.HG-11, Ps.HG-82, Ps.HG-44, Ps.HG-35, and Ps.HG-17 showed high inhibition activity against Xoo. bacteria (strain Xoo.HG.08) under in vitro conditions. In addition, these FPs bacterial strains exhibited the ability to secrete proteolytic protease enzymes on skim milk medium. Especially, three strains (Ps.HG-11, Ps.HG-82, and Ps.HG-44) showed good colonization ability on the rice leaves of the Jasmine 85 variety under greenhouse conditions. These FPs bacterial strains should be further investigated as potential biological control agents for bacterial leaf blight disease in rice under greenhouse conditions.

Keywords: Fluorescent Pseudomonas, biocontrol, *Xanthomonas oryzae* pv. *oryzae*.

INTRODUCTION

Bacterial leaf blight disease, caused by *Xanthomonas oryzae* pv. *oryzae*, is a serious disease in tropical rice-growing countries. The disease causes damage and reduces rice yield by 20-30% (Mew 1992). Under heavy rain conditions, the damage caused by the disease is up to 74-81.3% of the total output, but the extent of this damage depends on rice varieties and seasons (Ahmed and Singh 1975). In Vietnam, bacterial leaf blight disease was recorded in 1970 with an infection area of 18% on the NN8 variety and a yield reduction of 30-60% (Le Luong Te and Vu Trieu Man 1999).

Currently, many management measures are proposed, in which resistant varieties are mainly used. On the other hand, disease management using rhizosphere microorganisms in controlling plant diseases is a promising research direction around the world. *The Rhizosphere* microorganisms such as *Azotobacter*, *Bacillus*, *Pseudomonas*, *Burkholderia*, etc. are microorganisms that have the ability to widely distribute in the environment and give good preventive effects. In published studies, fluorescent *Pseudomonas* is one of the leading antagonistic bacteria to the pathogens in agriculture and suppresses plant diseases by protecting seeds and roots from infection (Ganeshan and Kumar 2005). Fluorescent *Pseudomonas* is a gram-negative, rod-shaped bacterium that is commonly found in soil and in decaying organic compounds (IRRI 1994). Research results by Tran Thi Cuc Hoa (1997) confirmed that fluorescent *Pseudomonas* bacteria has the ability to control wilt disease in soybean caused by *Rhizoctonia solani*. In addition, some bacterial strains belonging to the genus *Pseudomonas* spp. have the ability to reduce the diseased leaf area on rice caused by *Xanthomonas oryzae* pv. *oryzae* by up to 60%

(Yasmin et al. 2016). Based on the achievements of previous biocontrol studies of fluorescent *Pseudomonas* in controlling plant pathogen diseases, in this study, we evaluated the antagonistic activity as well as the biocontrol potential of 158 bacterial strains of fluorescent *Pseudomonas* against *Xoo.*, causing bacterial leaf blight in rice under laboratory and greenhouse conditions.

MATERIALS AND METHODS

Materials

The rice leaf blight bacteria (*Xoo*.HG-08 strain) was isolated in Vi Thanh district, Hau Giang province. Of the 158 bacterial strains from the rhizosphere soil of the rice fields in the 3 districts of Long My, Vi Thuy, and Vi Thanh of Hau Giang province were tested. The Jasmine 85 rice variety was provided by the Department of Plant Protection, CuuLong Delta Rice Research Institute.

Methods

Experiment 1: Evaluation of the antagonistic activity of fluorescent *Pseudomonas* against *Xoo.* under *in vitro* conditions

A method of antagonistic activity of fluorescent *Pseudomonas* against pathogenic bacteria (strain *Xoo*.HG-08) proposed by Khoa et al. (2016) was used. In this assay, 1 mL of *Xoo.* bacterial suspension (10^8 cfu/ml) was spread uniformly on 20 mL of Wakimoto's medium in the petri plate. A hole of 5 mm diameter was punched in the center of the petri dish containing the Wakimoto's medium, in which the *Xoo.* were inoculated with the help of a cork borer. A fresh culture of 48h-old fluorescent *Pseudomonas* on King's B medium was taken out as a disc with a diameter of 5 mm by a cork borer and then placed on *Xoo*. pathogen dish. The plate was then allowed to incubate at 30^{o}C for seven days before observing and measuring the formation of a clear zone surrounding the disc. King'B medium disc without pathogen was also placed on BLB pathogen to serve as controls.

Experiment 2: Evaluation of protease enzyme-producing ability of promising strains of fluorescent *Pseudomonas* under *in vitro* conditions

Active antagonistic fluorescent *Pseudomonas* were selected from the previous experiment and evaluated for hydrolytic enzyme production. The activity of protease was detected on agar plates containing King' B medium (Shyamala and Sivakumaar 2012). A fresh culture of 48 h-old fluorescent *Pseudomonas* on King's B medium was placed in the center of a petri plate containing skimmed milk agar. The plate was incubated at 28^{o}C. The experiment was arranged in a completely random with 4 replicates, each independent variable was a bacterial strain. The ability of antagonistic bacterium to produce protease enzyme was judged by observing the growth of bacterial colonies and the radius of the transparent area appearing around the bacterial colony on skimmed milk agar medium at 24, 48, and 72 hours after the experiment.

Experiment 3: Evaluation of the rice leaf colonization by active antagonistic fluorescent *Pseudomonas*

Active antagonistic fluorescent *Pseudomonas* were selected from the previous experiment and evaluated for colonizing on rice plant based on a method of Namasivayam and Sahayaraj (2008). A loop of 2 old-days fluorescent *Pseudomonas* on King's B medium was inoculated on 100 mL NB liquid medium and placed on a shaker at 1,500 rpm for 48 hours at 28^{o}C. About 10 mL of fluorescent *Pseudomonas* suspension from the initial culture was suspended into a falcon tube. The tube was allowed to centrifuge at 1000 rpm for 20 minutes in order to separate bacterial cells from the liquid solution. A loop of bacterial culture rod containing fluorescent *Pseudomonas* cells deposited at the bottom of the falcon tube was diluted in an eppendorf tube containing 1 mL of sterile distilled water, then bacterial suspension was obtained to a density of 10^8 cfu/mL. The bacterial suspension was sprayed on the leaf of 38-day-old rice plant at the concentration of 10^8 cfu/mL. The survival of

fluorescent *Pseudomonas* on the rice leaf was recorded at 1, 3, 5, and 7 days after inoculation. The rice leaves were washed under running tap water and cut into small segments of 1-2 mm before its surface was sterilized with 1% of sodium hypochlorite for 10 mins, then followed by rinsing with sterile distilled water. The rice leaf segments were weighed to about 1g and then dipped into 10 mL steriled distilled water. The suspension was thoroughly mixed and diluted into 4-fold serial dilutions before 100 µL was spread onto King's B medium. The King's B plates were incubated at 24°C for 48 hours, and three replicates were used for each treatment. Bacterial colony formed on the plate was counted and expressed as colony-forming unit (CFU) per fresh weight of rice leaf (g) acording to the formula of (Tran Linh Thuoc 2006).

Data analyses

Raw data was processed by Excel software. Statistical analysis was performed with the software of SPSS version 2.0.

RESULTS AND DISCUSSION

Antagonistic potential of fluorescent *Pseudomonas* against *Xoo. in vitro* conditions

▪ Initial screening of isolated strains of fluorescent *Pseudomonas*

From rapid assessment of the antagonistic ability of 158 strains of fluorescent *Pseudomonas* against *Xoo.* bacteria causing bacterial leaf blight disease on rice which were collected in Hau Giang province by co-culture method on Wakimoto medium indicated that, 12 strains of fluorescent *Pseudomonas* showed antagonistic expression against *Xoo*. These promissing strains will be selected for the next experiment of antagonistic potential evaluation against *Xoo.* (strain *Xoo.*HG-08) under *in vitro* conditions.

▪ Antagonistic activity of antagonistic strains of fluorescent *Pseudomonas* against *Xoo*

Results of evaluation on the antagonistic potential of fluorescent *Pseudomonas* strains against *Xoo.* under *in vitro* conditions confirmed that the evaluated bacterial strains were effective in inhibiting *Xoo.* (strain *Xoo*.HG-08) in a stable manner and remaining antagonistic efficacy up to 72 hours after the experiment (HAE). At 24 hours after the experiment (HAE), the highest antagonistic strain was observed on Ps.HG-44 with an average inhibition zone radius of 7.4 mm, followed by Ps.HG-82 and Ps.HG-11 with inhibition zone radius (6.3 mm and 4.8 mm). The remaining treatments had a radius of inhibition zone in the range of (2.1-3.0 mm), in which the strain of Ps.HG-76 had the lowest inhibition level (2.1 mm). At 48 HAE, Ps.HG-82 expressed the highest effectiveness in inhibiting the growth of *Xoo.*, reaching 9.3 mm. Two strains of Ps.HG-44 and Ps.HG-11 occupied the second and third rank in terms of antagonistic efficacy compared to Ps.HG-82, with radius of inhibition zones equivalent to 8.3 mm and 7.8 mm. The lowest antagonistic efficacy was found on Ps.HG-76. At 72 HAE, three strains (Ps.HG-11, Ps.HG-44, and Ps.HG-82) showed the highest inhibition activity against *Xoo.*, with inhibition zone radius (12.3 mm), whereas the lowest inhibition efficacy strain belonged to Ps.HG-01 (4.1 mm). Thus, three strains (Ps.HG-11, Ps.HG-44, Ps.HG-82) were selected to investigate the mechanism related to antagonistic activity under *in vitro* conditions.

Table 1. Inhibition zone radius of fluorescent *Pseudomonas* against *Xoo.* (strain *Xoo.*HG-08) at different times of observation.

No.	Treatments	Inhibition zone radius (mm)		
		24 HAE	48 HAE	72 HAE
1	Ps.HG-01	2.0^{i}	4.5^{h}	4.1^{i}
2	Ps.HG-11	4.8^{c}	7.8^{c}	12.3^{a}

3	Ps.HG-17	3.8^{e}	8.2^{b}	8.1^{c}
4	Ps.HG-18	3.0^{f}	6.0^{f}	6.1^{f}
5	Ps.HG-25	2.3^{h}	4.5^{h}	3.7^{j}
6	Ps.HG-32	4.0^{d}	6.1^{f}	6.3^{e}
7	Ps.HG-35	3.0^{f}	7.3^{d}	8.3^{b}
8	Ps.HG-44	7.4^{a}	8.3^{b}	12.3^{a}
9	Ps.HG-59	2.5^{g}	6.8^{e}	7.3^{d}
10	Ps.HG-60	3.0^{f}	5.5^{g}	5.9^{g}
11	Ps.HG-76	2.1^{i}	4.4^{h}	4.4^{h}
12	Ps.HG-82	6.3^{b}	9.3^{a}	12.3^{a}
	F	**	**	**
	CV (%)	3.3	1.3	1.2

**: statistically significant in probability 1%; HAE: hours after the experiment.

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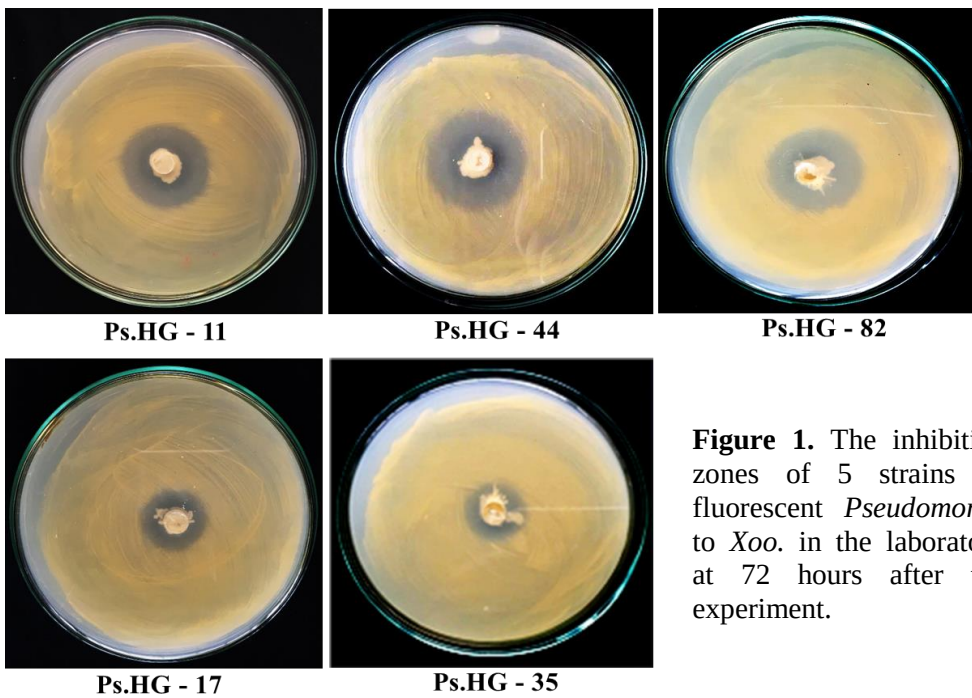

Figure 1. The inhibition zones of 5 strains of fluorescent *Pseudomonas* to *Xoo.* in the laboratory at 72 hours after the experiment.

Protease enzyme production of promising strains of fluorescent *Pseudomonas*

To study the antagonistic mechanism of fluorescent *Pseudomonas*, we examined the ability of antagonistic activity strains to produce antagonist-related lytic enzymes. Translucent hydrolysis circles were formed around all colonies of fluorescent *Pseudomonas* on the medium for protease, indicating that fluorescent *Pseudomonas* could produce protease enzyme. Among five experimental strains, Ps.HG-82 showed the highest ability to produce protease enzyme with a radius of the proteolytic circle (13, 26, and 41 mm) at (24, 48, and 72 HAE),

followed by strains of *(Ps.HG-11, Ps.HG-44* and *Ps.HG-35).* The strain of *Ps.HG-17* exhibited the lowest protease enzyme production for all observed times.

Table 2. Protease enzyme secretion ability of promising strains of fluorescent *Pseudomonas* at different times of observation.

No.	Treatments	Radius of proteolytic circle (mm) at different times of observation		
		24 HAE	48 HAE	72 HAE
1	Ps.Hg – 82	13.0^{a}	26.0^{a}	41.0^{a}
2	Ps.Hg – 11	10.0^{b}	18.0^{b}	37.0^{b}
3	Ps.Hg – 35	8.0^{c}	13.0^{d}	25.0^{d}
4	Ps.Hg – 44	8.3^{c}	15.0^{c}	27.0^{c}
5	Ps.Hg – 17	3.0^{d}	4.0^{e}	8.0^{e}
F		**	**	**
CV (%)		7.9	4.8	3.0

**: statistically significant in probability 1%; HAE: hours after the experiment.

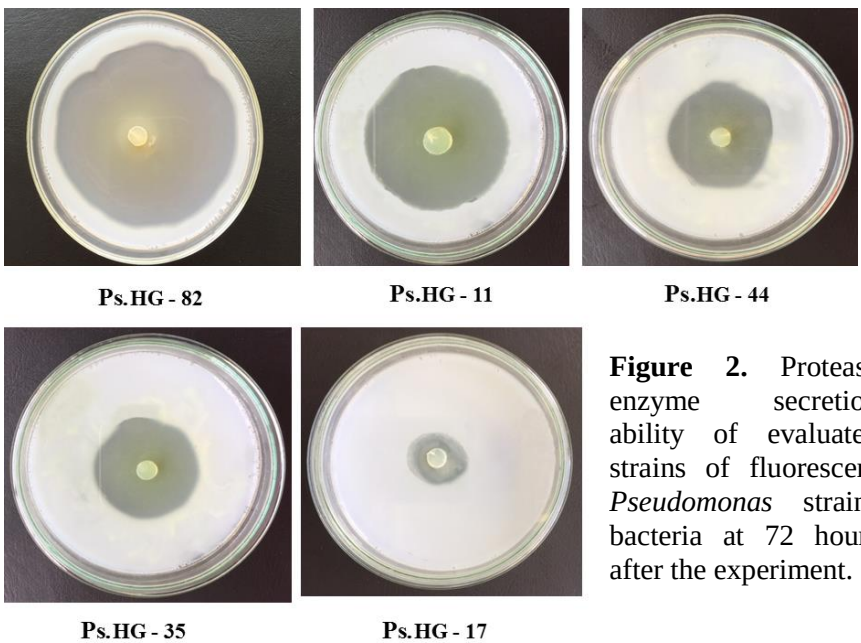

Figure 2. Protease enzyme secretion ability of evaluated strains of fluorescent *Pseudomonas* strains bacteria at 72 hours after the experiment.

Colonization ability of promising strains of fluorescent *pseudomonas* on rice leaves

Colonization of inoculated bacteria on Jasmine 85 rice variety was detected at 1, 3, 5, and 7 days after the experiment (DAE) and the finding proved that the maximum population density of fluorescent *Pseudomonas* was recorded at 3 DAE. Among the tested microorganisms, three strains of Ps.HG-11, Ps.HG-82, and Ps.HG-44 showed the highest ability to colonize on rice leaves of the Jasmine 85 variety with the highest density of viable population at 3, 5, and 7 days after inoculation, whereas two strains of Ps.HG-35 and Ps.HG-17 had a low ability to colonize on rice leaves in which its survival ability was maintained up to 3 days after the experiment.

Table 3. The density of fluorescent *Pseudomonas* on rice leaves (x10^4 cfu/g) at different times of observation, Jasmine 85 rice variety, CLRRI.

No.	Treatments	Density of fluorescent *Pseudomonas* on rice leaves (x10^4 cfu/g)			
		1 DAE	3 DAE	5 DAE	7 DAE
1	Ps.HG – 11	4.3^{a}	56.8^{a}	23.8^{a}	12.5^{a}
2	Ps.HG – 82	3.5^{ab}	42.5^{b}	17.5^{b}	8.0^{b}
3	Ps.HG – 44	3.5^{ab}	25.5^{c}	17.0^{b}	7.3^{b}
4	Ps.HG – 35	3.5^{ab}	8.0^{d}	0.0^{c}	0.0^{c}
5	Ps.HG – 17	2.5^{b}	7.0^{e}	0.0^{c}	0.0^{c}
6	Control	2.5^{b}	0.0^{f}	0.0^{c}	0.0^{c}
	F	*	**	**	**
	CV (%)	18.5	3.0	3.6	6.6

**: statistically significant in probability 1%; DAE: Days after the experiment.

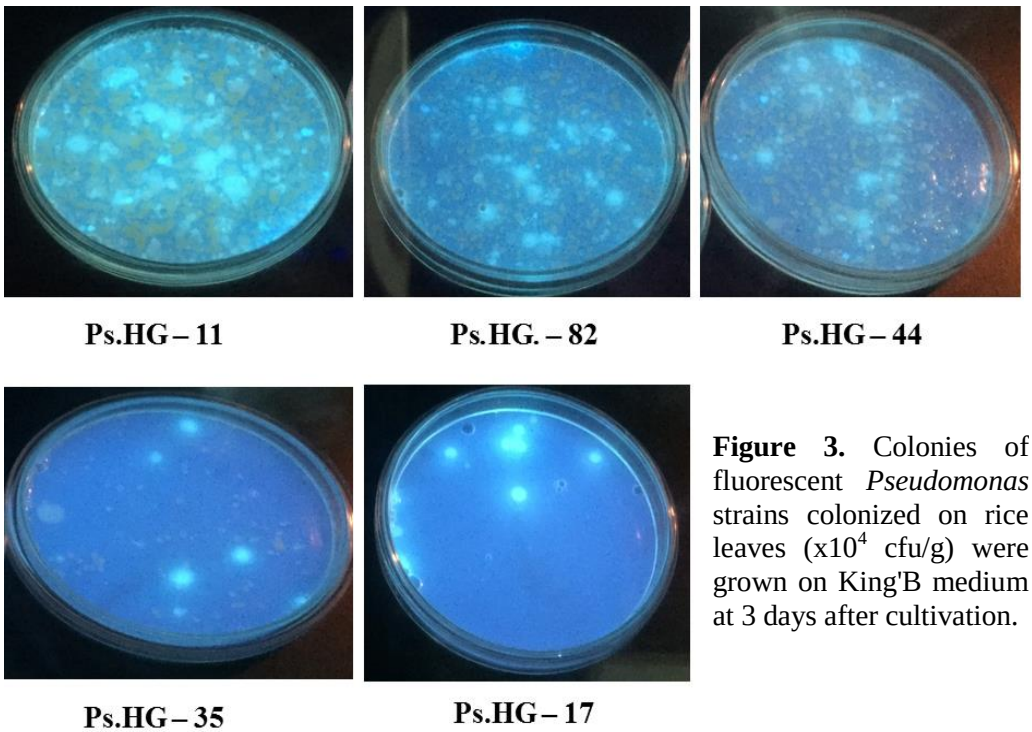

Figure 3. Colonies of fluorescent *Pseudomonas* strains colonized on rice leaves (x10^4 cfu/g) were grown on King'B medium at 3 days after cultivation.

CONCLUSIONS

Five strains of fluorescent *Pseudomonas* (Ps.HG-11, Ps.HG-44, Ps.HG-82, Ps.HG-35, and Ps.HG-17) had high antagonsictic activity against *Xoo*. (strain *Xoo*.HG.05) causing bacteria leaf blight disease of rice *in vitro* conditions. In addition, three strains of Ps.HG-11, Ps.HG-82, and Ps.HG-44 exhibited the ability to colonize on rice leaves of the Jasmine 85 variety under greenhouse conditions. Investigation result of protease enzyme production by promising strains of fluorescent *Pseudomonas* indicated that, these five fluorescent *Pseudomonas* strains showed the

ability to produce protease enzyme on skim milk medium.

COMPETING INTERESTS

The authors declare they have no conflict of interest, financial or otherwise.

AUTHOR'S INFORMATION AND CONTRIBUTIONS

Tran Thi Kieu provided data and wrote the draft version of manuscript in English. Nguyen Duc Cuong edited and approved the final draft.

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ĐÁNH GIÁ KHẢ NĂNG ĐỐI KHÁNG CỦA VI KHUẨN *PSEUDOMONAS* PHÁT HUỲNH QUANG VỚI VI KHUẨN *XANTHOMONAS ORYZAE PV. ORYZAE* GÂY BỆNH BẠC LÁ LÚA VÀ CÁC CƠ CHẾ LIÊN QUAN

Tổng số có 158 chủng vi khuẩn Pseudomonas phát huỳnh quang (PHQ) được phân lập từ đất vùng rễ lúa tại 20 ruộng của 3 huyện Long Mỹ, Vị Thủy, và Vị Thanh thuộc tỉnh Hậu Giang. Qua kết quả đánh giá khả năng đối kháng của vi khuẩn Pseudomonas PHQ đối với vi khuẩn Xanthomonas oryzae pv. oryzae gây bệnh bạc lá lúa trong điều kiện phòng thí nghiệm đã xác định 5 chủng (Ps.HG-11, Ps.HG-82, Ps.HG-44, Ps.HG-35 và Ps.HG-17) thể hiện khả năng đối kháng cao. Ngoài ra, năm chủng vi khuẩn nêu trên còn thể hiện khả năng phân giải enzyme protease trên môi trường skim milk. Đặc biệt, ba chủng (Ps.HG-11, Ps.HG-82 và Ps.HG-44) có khả năng định cư tốt trên lá lúa của giống Jasmine 85 trong điều kiện nhà lưới.

Từ khóa: *fluorescent Pseudomonas, kiểm soát sinh học, Xanthomonas oryzae pv. oryzae.*