Isolation and characteristics of *Pseudomonas fluorescens* to inhibit *Phytophthora palmivora* causing rot disease in durian

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ABSTRACT

Plant growth promoting Rhizobacteria (PGPR) have long been selected as biocontrol agents for plants. The PGPR are beneficial bacteria that live in plant roots and enhance plant growth by various of mechanisms. The PGPR have many species of the bacterial genus Pseudomonas, in which the *Pseudomonas fluorescens* strains is suitable for application as a biocontrol agent due to its abundance in natural soil and plant root systems. *Pseudomonas fluorescens* strains have important characteristics such as the ability to adhere to soil particles and to rhizomes, the ability to synthesize antibiotics, and to produce hydrolytic enzymes. Furthermore, *P. fluorescens* strains also possess plant growth promoting characteristics such as proteolysis, phosphate solubilization, iron chelation, and phytohormone production. Durian is a crop that has been expanded in Southeast Asian countries but the rate of durians infected with root, stem and fruit rot caused by *P. palmivora* is quite large. In this study, five native *P. fluorescens* isolates were isolated from 70 soil and rhizome samples collected from 10 durian growing provinces and evaluated for their ability to antagonize *P. palmivora* in petri plates. These isolates were gram negative, small, single isolated rods without sporulation when observed under microscope. All of them tested positive for catalase test, oxidase test, starch hydorolysis, gelatine liquefaction, H_2S, citrate utilization and negative with indole, Voges-proskauers, methyl red. All of 5 native *P. fluorescens* isolates were capable of phosphate solubilizing activity, N_2 fixation, siderophore and IAA production. The study showed that *P. fluorescens* P. fDN strain was able to inhibit *P. palmivora* causing rot disease in durian with 51.85% inhibition of radial growth.

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1. Introduction

Pseudomonas sp. are considered biological control agents because they are widely present in agricultural soils and have many suitable characteristics in the PGPR group. Around the world, many studies have been established to exploit the potential of pseudoluminescent bacteria, in which the most concentrated aspects are phosphate solubilizing activity, N_2 fixation, siderophore and IAA production. Reported by Glick et al. (1999) showed that IAA-producing rhizobacteria enhanced root length, which is one of the rhizobacteria promoting plant growth activity. Gupta et al. (2002) isolated fluorescent *Pseudomonas* strains capable of producing IAA from potato rhizosphere. Report by Pandey et al. (2013) also showed that, *Pseudomonas* strains are indeed rhizosphere endophytes promoting good growth of sunflower (*Helianthus annus*). In Vietnam, Tran et al. (2010) studied the effect of *P. fluorescens SS01* on black mold wilt (*Aspergillus niger*) on peanuts, showed that this strain has the ability to survive 30 days after inoculation with high

density and decrease in density at later times. Ngo et al. (2011) determined the level of nitrogen fertilizer replacement of *Pseudomonas* sp. BT1 and BT2 with high yielding rice plants grown in pots. In the case of replacing 50% N with *Pseudomonas*, the rice yield in potting could be increased by 23.9% compared to the control. Chu et al. (2020) isolated and selected *Pseudomonas* three strains capable of stimulating the growth of peanut under saline conditions both in vitro and in nursery. *P. fluorescens* had been reported to manage several diseases caused by soil borne pathogens (Hoffland et al., 1996; Wei et al., 1996). The purpose of this study is to isolate and characterrize *P. fluorescens* antagonists against rot durian disease caused by *Phytophthora palmivora*.

2. Materials and Methods

2.1. Isolation and morphological description

2.1.1. Soil sample collection

Samples were collected from the rhizosphere soils of durian farms at Tay Ninh, Binh Phuoc, Binh Duong, Dong Nai, Daklak, Dac Nong, Lam Dong, Vinh Long, Ben Tre, Dong Thap provinces. Samples were taken as follows: first, the topsoil of about 10 cm was removed and a metal spoon was used to collect soil at a depth of 10 - 20 cm. Then, 2 - 3 tablespoons of soil was taken at each point and the soil samples around the roots of many points were pooled to have a soil sample of 500 g. Thus, fives samples taken from a garden (or farm) included 4 diagonal points and 1 center point of the gardens. Seventy soil samples were collected from the rhizosphere of durian farms. The soil intimately adhering to the roots was collected and mixed to provide a composite soil sample.

2.1.2. Isolation of *P. fluorescens* isolates

Methods of Vlassak et al. (1992) was used to isolate *P. fluorescens*. Ten g of soil sample was put into a 90 mL conical flask of salt water and shaken for 15 min on a shaker. Then, it was aspirated and serially diluted the suspension to concentrations from 10^{-2} to 10^{-4}. About 0.1 mL of the corresponding diluent was added onto a sterilized petri dish containing King's B medium (KB). The petri dishes were incubated at room temperature (30°C ± 2°C) for 24 - 72 h. Each dilution was repeated 3 times. The growth of *Pseudomonas* colonies on the KB was observed during 24 - 72 h.

2.1.3. Morphological characterization

Pseudomonas strains were purified and studied for colony morphology and pigmentation by gram staining, described on specialized media. Cultural characterization of isolates was observed by different colony characteristics such as shape, size, surface, margin, color, odor, pigment, etc., and was recorded based on Bergey's Handbook of Definitive Bacteria (Holt et al., 1994). Cell shape and gram response were also recorded using the standard procedure provided by Barthalomew & Mittewar (1950).

2.2. Biochemical and Physiological Characterization

2.2.1. Starch hydrolysis

Ten mL the overnight culture of the isolates was added to the center of a sterile starch agar plate and incubated at 30 ± 2°C for 24 - 48 h. After incubation, the plates were submerged in an iodine solution. A transparent area around the colony was considered a positive reaction for the test.

2.2.2. Hydrogen sulfide test

A culture stick was used to take the bacterial solution of overnight cultures of the isolates. Then, it was pierced deeply into sterilized hydrogen Sulfide Indole Motility agar and incubated for 48 h at 30 ± 2°C. Observation visualization of black colour along the line of inoculation indicated a positive reaction for the test.

2.2.3. Indole production

The cultured bacteria were inoculated overnight into sterilized SIM agar plates and incubated for 48 h at 30 ± 2°C. After incubation, 10 drops of Kovac's indole reagent was added to each tube. The observed red production was noted as positive for indole production.

2.2.4. Catalase test

This test was performed to study the presence of catalase enzyme in bacterial colonies. Fresh cultures of purified isolates were taken on a slide

and a drop of H_2O_2 (30%) was added. The appearance of gas bubbles indicated the presence of the catalase enzyme.

2.2.5. Oxidase test

The isolates were inoculated on a petri dish of sterile trypicase soy agar and incubated for 24 h at 30 ± 2°C. After incubation, 2 - 3 drops of N, N, N', N' - tetramethyl - p -phenylenediamine dihydrochloride (Wurster reagent) were added to the growth surface of each test organism. Isolations reported a color change to chestnut were as oxidase - positive.

2.2.6. Carbohydrate utilization

Purified bacteria were inoculated into peptone broth containing different carbohydrates (lactose, sucrose, dextrose and mannitol) to assess carbohydrate fermentation. The change in colour of peptone broth was observed for utilization of particular carbohydrate present in broth.

2.2.7. Methyl red test

Bacteria were inoculated into test tubes containing sterilized glucose phosphate broth and incubated at 28 ± 2°C for 48 h. After that, 5 drops of methyl red indicator were added to each tube and shaken gently. If the test tube was red, it was recorded as positive and yellow as a negative reaction. Methyl red test helped to distinguish the concentration of H^+ present in the medium after fermentation by microorganisms.

2.2.8. Voges Prausker's test

The test sample was inoculated into sterilized glucose phosphate broth and incubated at 37°C for 48 h. After incubation, 10 drops of Baritt reagent A were added and shaken gently, then adding 10 drops of Baritt reagent B. If the test tubes was pink, it was recorded as positive for the test.

2.2.9. Gelatin liquefaction

The cultured bacterial samples were inoculated overnight into sterilized nutrient gelatin deep test tubes and incubated for 24 h at 30 ± 2°C. Then, the tubes were kept in a refrigerator for 30 min at 4°C. The isolates showing liquefied gelatin were taken as positive and which resulted in solidification of gelatin on refrigeration were recorded as negative for the test.

2.2.10. String test

A sterile toothpick was used to collect the bacterial biomass grown on the medium plate and spread it on a drop of 3% KOH solution on a clean slide (the more biomass, the clearer was observed). The spinning ability of the bacteria was recorded.

2.2.11. Pigment pyoverdine test

The test colonies were inoculated on King's B agar and incubated at 25°C for at least 2 days. The plate was observed under UV light, *P. fluorescens* secretes a yellow green pigment pyoverdine that diffused in the medium along the inoculum and fluoresced under UV light.

2.2.12. Pigment pyocyanin test

Bacterial strains were inoculated on King's A medium, incubated at 30°C for 48 - 72 h. *Pseudomonas fluorescens* did not appear blue pyocyanin pigment on King's A medium. This test helped to distinguish between *P. fluorescens* and *P. aeruginosa* strains.

2.3. Investigation of some biological activities

2.3.1. IAA (β - Indol Acetic Acid) produce test

A color reaction with the denatured Salkowski reagent was used for determination. The IAA content was produced by *P. fluoresces*. Bacteria were shaken in TSB medium supplemented with 0.1 g/L tryptophan in the dark. After 4 days of shaking at 150 rpm, 30 ± 2°C, 1 mL of inoculum was collected and centrifuged to remove biomass, then 2 mL Salkowski modified reagent was added. Tthe mixture was incubated for 1 h in the dark, a positive reaction gave a light pink to red color.

2.3.2. Nitrogen fixtation test

Bacteria were grown in a nitrogen-free medium (MNFM) complemented by the addition of bromophenol blue as a pH indicator. Bacterial strains that changed the color of the medium after 2 days of culture were recorded (Gothwal et al., 2007; Latt et al., 2018).

2.3.3. Phosphate solubilization test

According to the method of Nautiyal (1999), the bacterial biomass was dissolved in $Ca_3(PO_4)_2$ phosphate buffer (0.5%) and diluted to a density of about 10^8 CFU/mL. About 0.002 mL of bacterial biomass was taken in physiological saline and inoculated at 1 point on Pikovskay agar, incubated at 30°C. The appearance of lysis rings around the colony was observed.

2.3.4. Dual culture test to evaluate the ability to inhibit *Phytophthora palmivora*

textitPhytophthora palmivora was isolated from infected durian fruit in Cam My district, Dong Nai province. It was isolated on V3 juice agar and then maintained at room temperature. For this experiment, a 7 days old V3 plate of *P. palmivora* was used.

Control plates comprised only PDA with *P. palmivora* and sterilized water. Three plates were prepared for each observation and incubated at room temperature (± 28°C) for 5 - 7 days. Inoculation of pathogen was done by placing a 0.5 mm diameter plug of *P. palmivora* facing down, 1.5 cm from the centre of the PDA petri dish. *P. fluorescens* strains were grown in nutrient broth for two days before. A filter paper was dipped in solution of *P. fluorescens* and thawed on sterile filter paper. It was then placed 3 cm away from the *P. palmivora* isolate. Growth of *P. palmivora* was taken by measuring the diameter of the mycelium growth. Data of the percentage growth inhibition (PGI) was calculated using the formula of Zivkovic et al. (2010).

$$PGI(\%) = \frac{R_1 - R_2}{R_1} \times 100$$

Where:

R1: Growth of pathogen alone without antagonist (control)

R2: Growth of pathogen along with the antagonist

2.3.5. Data prpcessing method

Data were collected, synthesized, calculated using Microsoft Excel, analyzed by 1-factor ANOVA and graded test using MINITAB 16.

3. Results and Discussion

3.1. Isolation of *Pseudomonas fluorescens*

Isolation results obtained 16 samples showing *Pseudomonas* sp. (16/56 bacteria isolated) from 70 samples of durian root soil collected in 10 durian growing provinces including Binh Duong, Binh Phuoc, Tay Ninh, Tien Giang, Vinh Long, Ben Tre, Dong Nai, Lam Dong, Dak Lak and Dak Nong. The 16 samples belonging to the genus *Pseudomonas* were identified based on the characteristics of gram negative, rod shaped, not fermenting sugars, clear colonies, regular or irregular borders, and mucilage.

However, resulting from the string test with 3% KOH, purifying and observing the ability to fluoresce under UV light of the bacterial strains showed that only 5 samples were able to fluoresce suspected *P. fluorescens*. Five samples were collected from Binh Duong, Dong Nai, Dong Thap, Tien giang, Lam Dong.

3.2. Cultural and morphological characterization

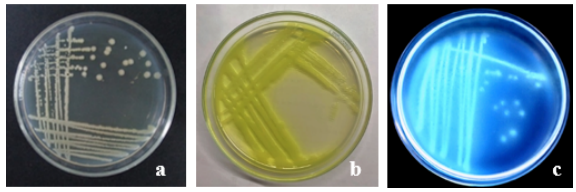

Figure 1. (a) *Pseudomonas fluorescens (P. F_{DN},* colonies on King's B agar medium, (b) *P. Fluorescens* secretes yellow-green pigment pyoverdine on King's B, (c) Colonies of *P. Fluorescens* was fluoresced under UV light on King's B.

Five *P. fluorescens* isolates, based on their colony morphology on King's B agar medium, cell morphology and gram reaction (Figure 1). All the isolates developed small to medium, smooth, glistening colonies, yellowish green colour with light green pigmentation. These isolates were gram negative, small, single isolated rods without sporulation when observed under microscope. This finding was similar to the description of the morphological characteristics of *P. fluorescens* P60 in the study of Soesanto et al. (2011).

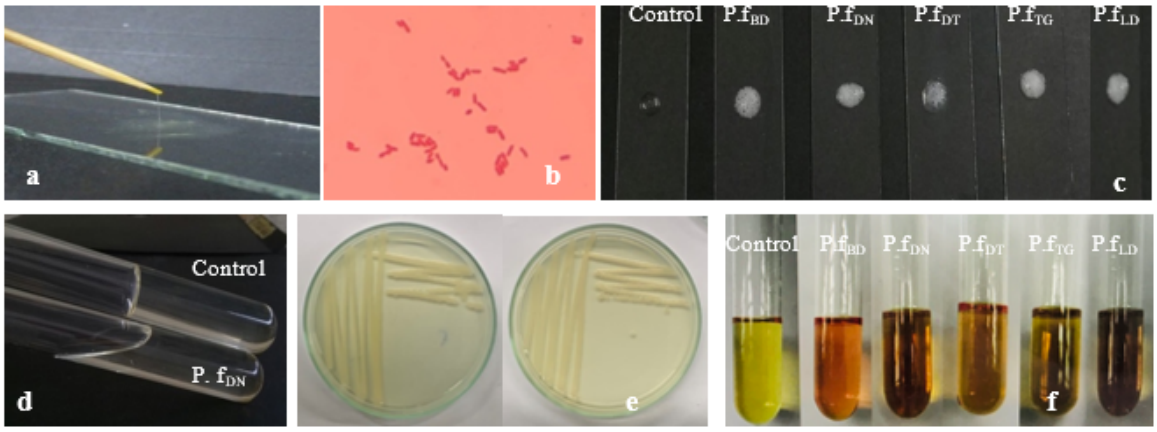

Figure 2. (a) String test spinning ability of *Pseudomonas* with KOH 3%, (b) Microscopic observation of *Pseudomonas*, (c) Catalase test of 5 *P. fluorescens* isolates, (d) Gelatine test, (e): Pyocyanin production test, (f) IAA production test with the modified Salkowski reagent.

Table 1. Biochemical and physiological characteristics of *P. fluorescens* isolates

Isolates	Oxidase test	Catalase test	Starch hydrolysis	Gelatine liquefaction	H_2S	Indole	MR	VP	Citrate utilization
P. f_{BD}	+	+	+	+	+	-	-	-	+
P. f_{TG}	+	+	+	+	+	-	-	-	+
P. f_{DT}	+	+	+	+	-	-	-	-	+
P. f_{DN}	+	+	+	+	+	-	-	-	+
P. f_{LD}	+	+	+	+	+	-	-	-	+

MR -Methyl red; VP – Voges Praskaur's test; + Positive result; - Negative result.

Table 2. Biological characteristics of *P. fluorescens* isolates

Isolates	Nitrogen fixtation test	Pyocyanin production	IAA production	Siderophore production	Phosphate solubilization
P. f_{BD}	+	-	+	+	+
P. f_{TG}	+	-	+	+	+
P. f_{DT}	+	-	+	+	+
P. f_{DN}	+	-	+	+	+
P. f_{LD}	+	-	+	+	+

+ Positive result; - Negative result.

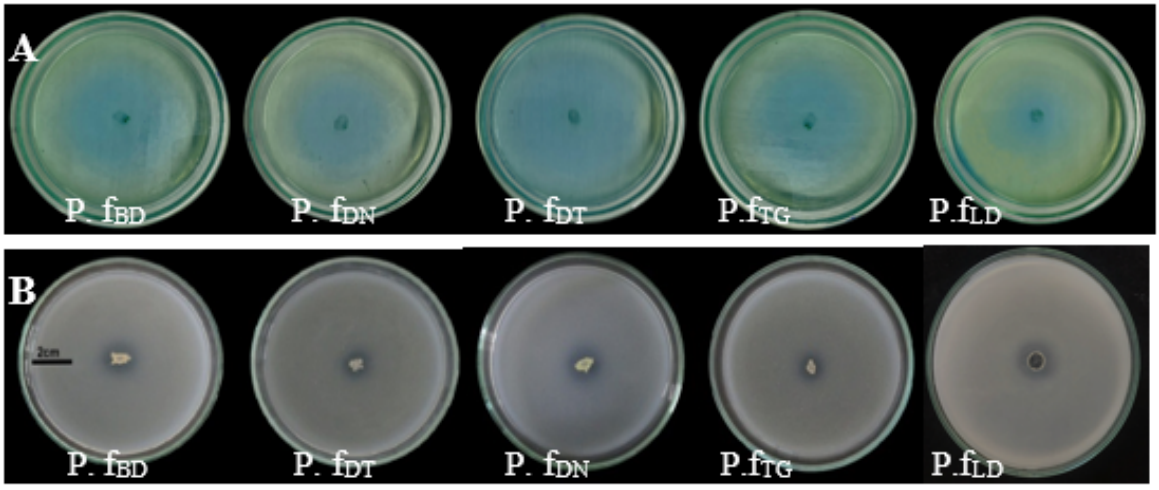

Figure 3. (A) Nitrogen fixtation test of *P. fluorescens* on a nitrogen-free medium after 2 days, (B) Phosphate solubilize test on Pikovskaya medium after 7 days.

3.3. Biochemical and physiological characterization

After the study of cell morphology, the isolates of the *P. Fluorescens* (5 isolates) were tested for different biochemical such as IMVIC test, oxidase test, catalase test, carbohydrate fermentation, denitrification, H2S production, starch hydrolysis, gelatin liquefaction etc. (Table 1; Figure 2). All 5 isolates from *P. fluorescens* showed positive results from catalase test, oxidase test, starch hydrolysis, gelatine liquefaction, H2S and citrate utilization whereas which was negative for Voges Prausker's, methyl red and indole test. In this study, 5 *P. fluorescens* isolates had diffused pyoverdin pigment capable of fluorescence into King's B medium, that showing the potential of using pigments of these *P. fluorescens* to create iron biosensors (Meyer, 2000) for future research and applications. The biological characteristics studied in Table 2 showed that the 5 *P. fluorescens* isolates were capable of solubilizing phosphate, fixing N2, producing siderophore and IAA. Many studies has shown that strains of *Pseudomonas* spp. were used in biocontrol because they produced antibiotic with antifungal abilities in vitro (Raaijmakers et al., 2002). The results obtained suggest the potential to use native *P. fluorescens* samples of further studies.

3.4. Dual culture test to evaluate the ability to inhibit *Phytophthora palmivora*

Antagonistic potential of 5 *Pseudomonas fluorescens* isolates (P. F_{BD}, P. f_{TG}, P. f_{DT}, P. f_{DN}, P. f_{LD}), were tested against *P. palmivora* in dual culture under in vitro conditions (Figure 3). The growth of the fungus was lesser as compared to the control plate. Among them P. f_{DN} performed best which gave 51.85% inhibition of radial growth followed by P. f_{LD} (43.71%), P. f_{TG} (42.96%) to *P. palmivora*, whereas, P. f_{DT} strain was not inhibition of radial growth (0%), (Table 3). The results in the Table 3 showed that there was no significant difference among the strains in controlling the growth inhibition of *P. palmivora*. Gade & Lad (2019) recorded that *P. fluorescens* was able to maximally inhibit the growth of the mycelium *Phytophthora parasitica* by 34.35%. *Pseudomonas fluorescens* strain is collected in different geographical areas, it is possible to produce different secondary metabolites, so the difference in percentage inhibition of different mycelium for the pathogen will test (Meena et al., 2012; Singh et al., 2013). Research of Laha et al. (1992) showed that *P. fluorescens* resistance to *Rhizoctonia batticola* in agar plate might be due to the production of siderophores or possibly to volatile antifungal compounds.

Table 3. Efficacy of *Pseudomonas fluorescens* against *Phytophthora palmivora*

Isolates	Diameter mycelial growth (mm)	Percentage growth inhibition (%)
Control	90	N/A
P. f_{BD}	71.33	$40.30^{c} \pm 0.30$
P. f_{TG}	70.67	$42.96^{b} \pm 0.74$
P. f_{DT}	90	$0.00^{d} \pm 0.00$
P. f_{DN}	66.67	$51.85^{a} \pm 0.74$
P. f_{LD}	70.33	$43.70^{b} \pm 0.74$

Treatments with the same letters did not differ significantly ($P \leq 0.05$) according to the Duncan's multiple range test.

4. Conclusions

Seventy soil samples were collected from rhizospheric soil at durian farms in 10 provices in Vietnam. Five *Pseudomonas fluorescens* isolates were isolated and evaluated in detail for their morphological and biochemical characteristics. All the isolates of *Pseudomonas fluorescens* in this study showed small to medium, smooth, glistening colonies, yellowish green colour with light green pigment on King's B medium. These isolates were small, single, gram negative isolated rods without sporulation when observed under microscope. All of these were positive with catalase test, oxidase test, starch hydorolysis, gelatine liquefaction, H_2S, citrate utilization and negative with indole, Voges-proskauers, methyl red. They were also able to produced siderophore and diffused pyoverdin pigment of fluorescence into King's B medium. Study showed that, all 5 *P. fluorescens* isolates were capable of phosphate solubilizing activity, N_2 fixation, siderophore and IAA production, inhibition of *Phytophthora palmivora*. These product from *Pseudomonas fluorescens* isolates have multiple roles in exerting the growth of plant. Therefore, these isolates can be utilized for biofertilizer formulation under local agroclimatic conditions.

Among five *Pseudomonas fluorescens* isolates, P. f_{DN} strains has against best with *Phytophthora palmivora* which gave 51.85% inhibition of radial growth. Future study with these isolates using them in pot cultures and followed by field experiments will help in establishing their potential to be used as biofertilizers. The data obtained in the present study suggest that five *Pseudomonas flourescence* isolates, especially, P. f_{DN} would be ideal organisms for further study in pot culture and field experiments to exploit their PGPR potential for a good biofertilizers production.

Conflict of interest

The authors have no conflicts of interest to declare.

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