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Potential Plant Growth-Promoting Activity of *Pseudomonas sp* Isolated from Paddy Soil in Malaysia as Biocontrol Agent

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Abstract

Pseudomonas fluorescens bacteria, a major constituent of Rhizobacteria, encourage the plant growth through their diverse mechanisms. In this investigation, 20 strains of *Pseudomonads* isolated from the rhizosphere soils of paddy areas in Malaysia and were screened for their plant growth promoting activity. All the 20 tested isolates of *Pseudomonads* were positive for the production of siderophores and HCN, while of the 20 antagonist bacteria strains, 15 strains (75%) showed positive for the production of plant growth-promoting hormone, IAA. Among the 20 isolates, 18 isolates (90%) produced phosphate solubilisation on NBRIP medium. All the twenty bacterial isolates (except DL21) inhibited the pathogen in the dual culture assay. Following API 20NE biochemical identification kit, of the 20 isolates, 15 strains were identified as *Pseudomonas fluorescens*, 3 isolates belong to the species of *P.luteola,* one isolates to the *P.aeruginosa* and a single isolate (TS14) showed a doubtful identification.

Keywords: Antagonism; Biochemical characterization; Hydrogen cyanide; IAA; PGPR; *Pseudomonas spp*

Introduction

Microorganisms has vital role in agriculture in order to promote the exchange of plant nutrients and reduce application of chemical fertilizers as much as possible. Plant Growth-Promoting Rhizobacteria (PGPR) is able to exert a positive effect leading plant growth. Beneficial plant– microbe interactions in the rhizosphere can influence plant vigor and soil fertility [1]. These beneficial effects of PGPR have direct or indirect performance on plants. Direct promotion of growth by PGPR including production of metabolites that enhances plant growth such as auxins [2], cytokinins, gibberellins and through the solubilization of phosphate minerals [3]. Indirect growth promotion occurs via the removal of pathogens by the production of secondary metabolites such as hydrogen cyanide and siderophores [4]. Plants are regularly concerned in interactions with a broad range of bacteria that colonize the rhizosphere (rhizobacteria), the phyllosphere (epiphytes), and the inside of plant tissues (endophytes) [5]. The application of plant growth-promoting rhizobacteria (PGPR) as crop inoculants for biofertilization, phytostimulation, and biocontrol would be an attractive alternative to decrease the use of chemical fertilizers which also effect environmental pollution [2]. *Pseudomonas sp*. is widespread bacteria in agricultural soils and has many traits that make them well-matched as PGPR. The most effective strains of *Pseudomonas* are gram negative, motile, rod shaped bacteria and have various phytobeneficial traits. Their plant growth promoting activities include production of HCN, siderophores, protease, antimicrobials, phosphate solubilizing enzymes [6]. In the present study, we investigated the production of HCN, siderophores, antimicrobials and phosphate solubilisation by 20 *Pseudomonas* isolated from rhizosphere soils of paddy areas cultivated in different localities Malaysia. The objectives of this study were (1) isolation and screening of *pseudomonas* isolates to develop them as biofertilizer and (2) to biochemically characterize *Pseudomonas fluorescens* strains.

Materials and Methods

Site description of soil sampling

Soil samples from the rhizosphere of rice plants was collected from irrigated paddy fields in different localities of Peninsular Malaysia at altitude of 900 to 1100 meters (Latitude: 04°0'N); Kedah, Perak, Kelantan, Selangor and Malacca.

Isolation and screening of *Pseudomonads fluorescens*

Bacteria isolated from the rhizosphere, root samples were shaken vigorously to remove loosely adhering soil and 4.5 ml of sterile physiological water was added to 0.5 g of rhizospheric soil and the mixture was shaken at 120 rpm for 2 min. Serial ten-fold dilutions were prepared from the extract and 0.1 ml of each dilution was seeded onto King B medium, supplemented with 100 µg ml^{-1} of cycloheximide to suppress fungi. To isolate and quantify *Pseudomonads fluorescens* UV light was used [7].

Quantitative estimation of IAA production

All the 20 isolates were screened for IAA production. In brief, test bacterial culture was inoculated in the nutrient broth containing L- tryphtophan (5 µg/mL), incubated at 28 ± 2°C for 5 days. Cultures were centrifuged at 3,000 rpm for 30 min and two mili liter of the supernatant was mixed with two drops of orthophosphoric acid and 4 mL of Salkowaskis reagent (50 mL, 35% perchloric acid; 1 mL 0.5 M $FeCl_3$). Appearance of red color indicates IAA production. OD(optimum density) was measured at 535 nm using spectrophotometer and shown as µg/mL [8].

Phosphate solubilization activity

All bacterial isolates were screened for inorganic phosphate solubilization. A loop full of fresh bacterial culture was streaked onto NBRIP medium containing inorganic phosphate and plates were incubated

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at 28 ± 2°C for 3 days. After 3 days, the colonies showing the clear halo zone around them indicated solubilization of mineral phosphate (2011). Phosphate solubilization activities were screened by measuring the clearing zone surrounding the developed bacterial colony via calculation of phosphate solubilization index [9]:

Phosphate Solubilization Index= A/B × 100

A= total diameter (colony+ halo zone)

B= diameter of colony

Assay for siderophore production

According to the methodology described by Gopalakrishnan [10], bacteria were streaked on the center of Chrome Azurol S (CAS) agar media and incubated at 28 ± 2°C for 48h. When the bacteria consume iron, present in the blue-colored CAS media, orange halos around the colonies indicate the incidence of siderophores.

Hydrogen cyanide production

The production of HCN was detected by spreading 1 ml of 24 h old broth culture of *Pseudomonas* on the Kings B medium and incubation of the plates with the Whatman filter paper flooded with the solution containing 0.5% picric acid in 2% sodium carbonate located in the upper lid of petri plate [11]. To avoid the escape of the gas, the plates were sealed with the parafilm. After 24-48 h, yellow to orange change in the color of the filter paper was observed.

In vitro antifungal activity

A 10 mm disk of a pure culture of *Prycularia oryzae* was placed at the centre of a Petri dish containing PDA (Potato Dextrose Agar). A loopful bacterial isolates were streaked on PDA 1.5 cm from the edge of each plate. Plate was cultured for 72h at 28°C and then the percent of radial growth inhibition (PIRG) were recorded by the following formula [12]:

PIRG= (R1-R2/ R1) ×100

R1 = Radial growth of *P.oryzae* in control plate

R2 = Radial growth of *P.oryzae* interacting with antagonistic bacteria

Data analysis

Data were subjected to analysis of variance using SPSS software (ver. 12) and means comparing were carried out by LSD method (α = 0.05).

Identification of the bacterial isolates by API 20NE kit

API 20NE (Biome´rieux, France) is another frequently used quick identification method based on the degradation of biochemical substrates. This method is suitable to identify Gram-negative rods at the species level by assessing the profile of 21 different biochemical reactions. The biochemical profile is specific for each species within this group of bacteria. These tests were performed according to the instructions of the manufacturer (Biome´rieux, France). The used tests include the oxidation of nitrate, indole production, anaerobic utilization of glucose, arginine and urea, production of β-glucosidase, protease and β-galactosidase, as well as the utilization of glucose, arabinose, mannose, mannitole, N-acetyl-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenyl-acetate. The API stripes were incubated for 48 h at 30°C under ambient air. The results were interpreted with the API Web™ software (version 7.0) [13].

Results and Discussion

All the fluorescents bacteria antagonists were gram negative, oxidase-positive, rod shaped and all produced yellowish green pigment on King B medium. Every isolate tested showed at least one of the growths promotion traits that were investigated (Table 1). All the 20 tested isolates of *Pseudomonads fluorescent* were positive for the production of siderophores in iron-deficient culture medium and HCN, while of the 20 antagonist bacteria strains, 15 strains (75%) showed positive for the production of plant growth-promoting hormone, IAA. Isolates TS3B6 and TS3A5 produced higher IAA (20.5 and 19.0 μgml^{-1}, respectively), whereas all the other isolates (except TS3A1, TS3C6, TS3C9 and TS3A2) produced IAA between 2.4 and 9.7 μgml^{-1} (Table 1). The isolates in this study presented several enviable features for PGPR, and multiple action mechanisms which suggest their potential for growth promotion. Production of IAA by PGPR generally affects the root system, increasing the size and number of adventitious roots and also the

Isolate	Phosphorous Solubilization (Psb Index)	Siderophore production	IAA production (1g ml^{-1})	HCN production	Antagonistic to P. oryzae (inhibition zone)
DL21	154 (EFGH)	+	**5.76 (F)**	+	5.76 (F)
DL17	308 (BC)	+	**4.23 (G)**	+	17 (BC)
TS3C8	341 (A)	+	**9.76 (C)**	+	52 (ABC)
TS3B5	146 (FGH)	+	**0 (J)**	+	65 (A)
TS3C	155 (EFGH)	+	**2.9 (HI)**	+	49 (AB)
TS3A5	178 (E)	+	**19.0 (B)**	+	26 (ABC)
TS3C4	138 (GH)	+	**8.06 (D)**	+	16 (BC)
TS3B9	141 (GH)	+	**6.73 (EF)**	+	13 (BC)
DL26	129 (H)	+	**3.16 (GHI)**	+	30 (ABC)
TS3B6	143 (GH)	+	**20.5 (A)**	+	33 (ABC)
TS3A1	0 (J)	+	**0 (J)**	+	0 (C)
TS3C6	289 (CD)	+	**0 (J)**	+	14 (BC)
TS25	152 (EFGH)	+	**9.76 (C)**	+	38 (ABC)
TS14	153 (EFGH)	+	**6.06 (F)**	+	16 (BC)
TS11	131 (GH)	+	**3.56 (GH)**		51 (AB)
TS3C9	269 (D)	+	**0 (J)**	+	27 (ABC)
DL22	176 (E)	+	**3.86 (GH)**	+	33 (ABC)
TS3A2	173 (EF)	+	**0 (J)**	+	19 (ABC)
TS3C1	316 (AB)	+	**7.60 (DE)**	+	10 (BC)
DL11	159 (EFG)	+	**2.40 (I)**	+	19 (ABC)

Table 1: The twenty potential isolates based on their plant growth promotion and biocontrol

Isolate	NO3	TRP	GLU	ADH	URE	ESC	GEL	PNG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	OX	ID
DL21	_	_	_	+	_	_	_	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*
DL17	_	_	_	+	_	_	+	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*
TS3C8	_	_	_	+	_	_	+	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*
TS3B5	+	_	+	+	_	+	_	+	+	+	+	+	+	+	+	_	_	+	+	+	_	*P.luteola*
TS3C	_	_	_	+	_	+	+	+	+	+	+	+	+	+	+	_	_	+	+	+	_	*P.luteola*
TS3A5	+	_	+	+	_	+	_	+	+	+	+	+	+	+	+	_	_	+	+	+	_	*P.luteola*
TS3C4	_	_	_	+	_	_	+	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*
TS3B9	_	_	_	+	_	_	+	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*
DL26	_	_	_	+	_	_	_	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*
TS3B6	_	_	_	+	_	_	+	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*
TS3A1	_	_	_	+	_	+	+	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.aeroginosa*
TS3C6	_	_	_	+	_	_	+	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*
TS25	_	_	_	+	_	_	_	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*
TS14	_	_	_	+	+	_	+	_	+	_	_	+	+	_	+	+	+	+	+	+	+	*?*
TS11	_	_	_	+	_	_	_	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*
TS3C9	_	_	_	+	_	_	+	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*
DL22	_	_	_	+	_	_	_	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*
TS3A2	_	_	_	+	_	_	_	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*
TS3C1	_	_	_	+	_	_	+	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*
DL11	_	_	_	+	_	_	_	_	+	_	+	+	+	_	+	_	_	+	_	+	+	*P.fluorescens*

Table 2: API 20NE examination results.

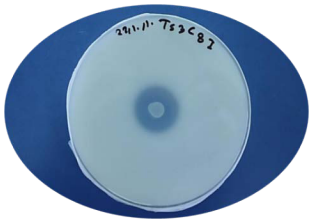

Figure 1: Phosphate solubilization halo produced by one of the most potential isolates.

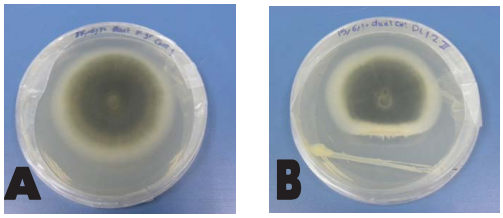

Figure 2: A: Control; B: Influence of one isolate on *Pyricularia oryzae* by dual culture assay

the root subdivision, enabling a bigger soil amount to be exploited by the roots, thus providing large amounts of nutrients accessible to the plant [14]. However, IAA production by PGPR can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability [15]. Among the 20 isolates, 18 isolates (90%) produced phosphate solubilisation on NBRIP medium. All the strains are identified as potential phosphate solubilizers based on their capacity to solubilize tricalcium phosphate [$Ca_3(PO_4)_2$] by the formation of clear halo zone on NBRIP medium (Figure 1). According to the PSB Index for each isolates, the maximum amount of soluble phosphates was released by TS3C8 (341) and the least by DL26 (129). A significant difference ($P<0.05$) was observed between all the isolates. Most of phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by the plants. The ability of bacteria to solubilize mineral phosphates has been shown of interest to agricultural microbiologists as it can enhance the availability of phosphorus and iron for plant growth. PGPR have been shown to solubilize precipitated phosphates and enhance phosphate availability to plant that represent a possible mechanism of plant growth promotion under field conditions [16]. Free-living P-solubilizing bacteria release phosphate from spare soluble inorganic and organic phosphate compounds in soil and so contribute to increase available phosphate for the plants [10]. A total of 20 rhizobacterial isolates were screened for siderophore production. All of these isolates grown on CAS agar and produced siderophores (Table 1). The color of the CAS agar was changed by rhizobacteria from the blue to orange. The variation in color changes in the CAS agar plate (orange, purple or purplish-red) recommend the production of siderophores of a differing nature by the variety of microorganisms isolated and the color intensity can be consequence of siderophore concentration. These siderophore producing microorganisms suppress some soil-borne fungal pathogens through direct role of siderophore-mediated iron competition in the biocontrol ability [17]. The microorganism investigated in this study was found to produce IAA and siderophore, which can chelate metal ions, related to the bound phosphorous and release phosphorous from complex [1]. Microbial production of HCN has been suggested as an important antifungal feature to control root fungi pathogen [18]. Cyanide acts as a general metabolic inhibitor to avoid predation or competition. The host plants are generally not harmfully affected by inoculation with HCN production bacteria and host-specific rhizobacteria can operate as biological control agents [19]. All the twenty bacterial isolates (except DL21) inhibited the pathogen in the dual culture assay, whereas isolates TS3B5, TS3C8 and TS11 showed the maximum percent inhibition of radial growth (PIRG) 65%, 52% and 51%, respectively (Figure 2). An inhibitory halo was observed suggesting the production of fungistatic metabolites secreted by the bacteria [20]. In order to identify 20 isolates, API 20NE was used. For identification of these twenty isolates we considered an isolate to be identified as *P. fluorescens* only in case of "good", "very good" or "excellent identification". Following this principle, of the 20 isolates, 15 strains were identified as *Pseudomonas fluorescens*, 3 isolates belong to the species of

the *P.luteola*, one isolates to the *P. aeruginosa* and a single isolate (TS14) showed a doubtful identification (Table 2). Thus, it is obvious from this investigation that the *Pseudomonads fluorescens* under these studies are able to produce plant growth promoting substances and antifungal substances. Therefore, they are potential candidates for the development of biofertilizer and bioinoculants for crop plants. The world over is changing from inorganic conventional farming towards organic ecofreindly farming methods. This not only requires the isolation of bioinoculants with high potential for use as biofertilizers but also several other factors right from appropriate application procedures to correct Marketing practices also being economically cheaper.

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