African Journal of Biotechnology Vol. 10(59), pp. 12653-12656, 3 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1819
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

A pilot study on the isolation and biochemical characterization of *Pseudomonas* from chemical intensive rice ecosystem

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Accepted 19 May, 2011

In recent times, there has been a renewed interest in the search of plant growth promoting rhizobacteria (PGPR) for sustainable crop production. Rice is an economically important food crop, which is subjected to infection by a host of fungal, viral and bacterial pathogens. In this study, an attempt was made to isolate *Pseudomonas* spp., a potent PGPR in the rhizosphere. Through appropriate microbiological and biochemical methods, the study demonstrated the presence of fluorescent and non-fluorescent *Pseudomonads* in the rhizosphere of chemical intensive rice growing environments. Augmentation of such PGPR including, *Pseudomonads* in the rice ecosystems will ensure a healthy micro climate for rice.

Key words: *Pseudomonas*, rice, plant growth promoting rhizobacteria (PGPR).

INTRODUCTION

Rice is a staple food crop of economic importance, especially in Asia. Rice production is limited by diseases caused by fungi, bacteria and viruses, causing annual loss of 5% (Song and Goodman, 2001). Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that are found in the rhizosphere and rhizoplane which can improve plant growth. *Pseudomonas* spp. is one of the most promising groups of PGPR which can control plant pathogenic microbes in the soil (O'Sullivan and O'Gara, 1992). Rice is one of the major food crops grown in Malaysia, particularly in Kedah Darul Aman State. Exploitation of naturally occurring native *Pseudomonas* spp. can be a part of environmentally sustainable crop protection system.

Biological control using PGPR from the genus *Pseudomonas* is an effective substitute for chemical pesticides to suppress plant diseases (Compant et al., 2005). The biocontrol mechanism to suppress fungal pathogens by *Pseudomonas* spp. normally involves the production of antibiotics (Nagarajkumar et al., 2004). *Pseudomonas fluorescens* has a gene cluster that produces a suite of antibiotics, including compounds such as 2,4-diacetylphloroglucinol (2,4-DAPG), phenazine, pyrrolnitrin, pyoluteorin and biosurfactant antibiotics (Angayarkanni et al., 2005). The objective of this study was to isolate *Pseudomonas* spp. from rice rhizosphere and to further identify and characterize the isolates using standard microbiological and biochemical tests.

MATERIALS AND METHODS

Rhizobacteria were isolated from the rhizosphere of rice plants randomly selected from paddy fields in Sungai Petani, Kedah. The randomly selected rice plants were carefully pulled out from the soil without damaging the roots. The roots were shaken to dislodge any loosely adhering soil. Undamaged root pieces that were 2 to 3 cm long were used for the isolation of bacteria (Vidhyasekaran and

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Abbreviations: PGPR, Plant growth promoting rhizobacteria; **KMB**, King's medium B metals.

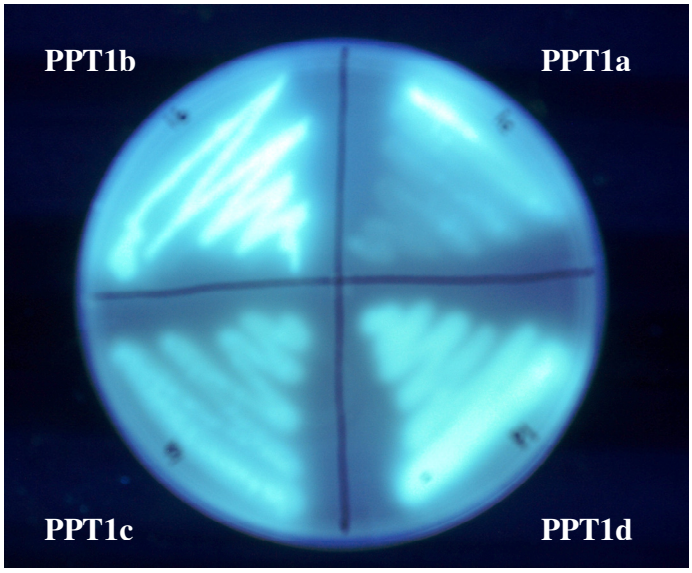

Figure 1. Isolates PPT1a, PPT1b, PPT1c and PPT1d showing fluorescence under UV (365 nm) light.

Rabindran, 1996). The King's medium B (KMB) was used to isolate *P. fluorescens* from the processed sample in the flask (King et al., 1954) as described by Vidhyasekaran and Rabindran (1996). The processed samples were serially diluted from 10^{-1} to 10^{-5} and 0.5ml of each dilution was aseptically spread onto Petri plates containing KMB. The plates were then incubated for 3 days at 30 ± 1°C. The growth of rhizobacterial colonies on KMB plates were observed and recorded continuously for 3 days. The selected isolates of rhizobacteria were subjected to further confirmatory biochemical tests.

Standard microbiological tests were conducted for rapid identification of *Pseudomonas* colonies on the KMB plates, which included colony morphology, Gram staining, motility test and fluorescent pigment test. Pure culture of *Pseudomonas* spp. was obtained following successive selection of fluorescing colonies on KMB under UV light at 365 nm (Rachid and Ahmed, 2005). The isolates were characterized to be identified as *P. fluorescens* by performing growth at 4 and 41°C and biochemical tests including oxidase, catalase, gelatin hydrolysis and nitrate reduction test (Reynolds, 2004). Motility of the isolates was determined using SIM (sulfide-indole-motility) medium. The ability of the isolates to grow at 4 and 41°C was determined by growing the isolates in Luria Bertani (LB) medium at respective temperatures. For oxidase test, bacterial inoculum was placed on a sterile filter paper and a drop of Kovac's reagent was added to the inoculum. Immediate colour change to purple gives positive scores (Reynolds, 2004). For the catalase test, the bacterial cultures on LB media were scraped with a toothpick and suspended in a drop of 3% H_2O_2 on a glass slide. Formation of bubbles indicates a positive reaction, while without any bubbles shows negative reaction (Smibert and Krieg, 1981). Gelatin hydrolysis test was performed by stabbing the inoculum of the isolates into the gelatin medium. Liquefied gelatin gives positive response, while solid gelatin shows negative response (Reynolds, 2004). Nitrate reduction test was conducted to determine the ability of the isolates to reduce nitrate to nitrite or further to free nitrogen gas. Nitrate broth with Durham tube was prepared in a screw-cap tube. The tubes were then inoculated with the isolates and incubated at 30 ± 1°C for 2 days. After incubation, the tubes were first checked for gas production. Then, nitrate reagent A and B were sequentially added to each tube. The appearance of red colour in the presence of nitrite gives a positive reaction. Negative reaction occurs when the solution turns pink-red after the addition of nitrate reagent C. Tube without colour change after the addition of reagent C, indicates that the isolate can reduce nitrate to nitrite and to nitrogen gas which also gives a positive response (Reynolds, 2004).

RESULTS AND DISCUSSION

All the isolates were large, circular, convex with an entire margin, and light to dark yellow in colour on KMB medium. The *Pseudomonas* isolates, PPT1a, PPT1b, PPT1c, PPT1d (Figure 1) and PPT2a, PPT2b, PPT3a and PPT3b (Figure 2) exhibited green fluorescence under UV (365 nm) light.

All the identified isolates, except for the control showed positive reaction on motility, oxidase, catalase and growth at 4°C (Table 1). However, negative responses were also identified for some *Pseudomonas* isolates such as for gelatin hydrolysis and nitrate reduction test as well as the ability of the bacteria to grow at 41°C.

Eight isolates of *Pseudomonas* species that nearly resemble *P. fluorescens* were identified from the total of 14 isolates. All the eight isolates were found to grow on the KMB with a typical *Pseudomonas* bacterial colony morphology. According to Todar (2004), more than half of the *Pseudomonas* bacteria produce pyocyanin which is a blue-green pigment, while the nonpathogenic saprophyte *P. fluorescens* produces fluorescent pigment that is soluble and greenish. In this study, all the eight identified gram-negative *Pseudomonas* isolates were found to be green fluorescent on KMB under ultraviolet light at 365 nm. All the isolates were motile, catalase and oxidase positive, confirming them to be *Pseudomonas* spp.

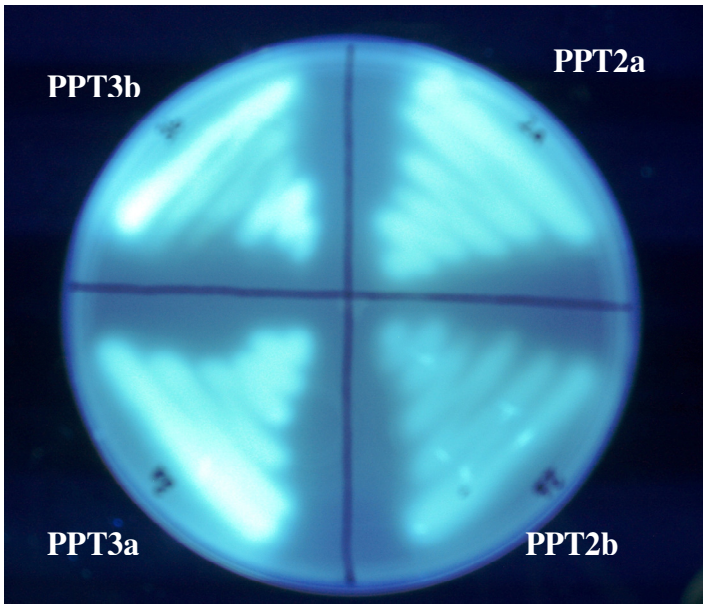

Figure 2. Isolates PPT2a, PPT2b, PPT3a and PPT3b showing fluorescence under UV (365 nm) light.

Table 1. Biochemical characterization of bacterial field isolates.

S/N	Bacterial field isolate	Motility	Growth at 4°C	Growth at 41°C	Oxidase test	Catalase test	Gelatin hydrolysis	Nitrate reduction
1	PKM1a	+	+	+	+	+	-	-
2	PKM1b	+	+	+	+	+	+	-
3	PKM2a	+	+	+	+	+	-	-
4	PKM2b	+	+	+	+	+	-	-
5	PKM3a	+	+	-	+	+	+	+
6	PKM3b	+	+	-	+	+	+	+
7	PPT1a	+	+	-	+	+	+	+
8	PPT1b	+	+	-	+	+	-	-
9	PPT1c	+	+	+	+	+	-	-
10	PPT1d	+	+	-	+	+	+	+
11	PPT2a	+	+	-	+	+	+	+
12	PPT2b	+	+	+	+	+	-	-
13	PPT3a	+	+	+	+	+	-	+
14	PPT3b	+	+	-	+	+	+	+

(Bergey's Manual of Determinative Bacteriology, 1974). Angayarkanni et al. (2005) reported that *P. fluorescens* can dissolve solid gelatin into a liquid form in room temperature with the presence of an enzyme known as PPT1a, PPT1d, PPT2a and PPT3b, were found to be positive for gelatin hydrolysis.

Some species such as *P. fluorescens* strains are capable of denitrification and able to grow anaerobically in nitrate media. Todar (2004) reported that incubation temperature around 30°C favours the growth of denitrifying biotypes of *P. fluorescens*, while temperatures above 37°C may be conducive for other *Pseudomonas* species. Based on the test results, isolate PPT1a, PPT1d, PPT2a, PPT3b, PKM3a, PKM3b and PPT3a showed nitrate reduction activity. Isolates PKM3a, PKM3b, PPT1a, PPT1d, PPT2a and PPT3b showed a positive response for oxidase, catalase, motility, gelatin liquefaction and growth at 4°C, but not at 41°C. The results of this study indicates that all the six identified *Pseudomonas* isolates have similar characteristics with that of *P. fluorescens*, and this confirms that these isolates may belong to the group of *P. fluorescens*. This

study is assumed to be important as the agriculturally beneficial antibiotic-producing *P. fluorescens* could be one of the potential candidates in the development of microbial pesticides to manage rice diseases, for sustained crop productivity.

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