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Phenotypic Characterization and Molecular Identification of Malaysian *Pseudomonas fuscovaginae* Isolated from Rice Plants

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ABSTRACT

Sheath Brown Rot (SBR) is one of the most important diseases that affect rice plant. The disease cause rots to the sheaths, panicles and leaves of rice plant. The disease also contaminating rice seeds through grain's discoloration thus leads in reduction of rice production. In this study, 50 bacteria isolates that fluoresced under UV illumination on King's B Selective (KBS) medium were obtained. Out of 50 isolates, 25 isolates were positive for both Hypersensitivity Reaction (HR) and pathogenicity tests. They also showed positive results for several biochemical reactions such as oxidase, arginine dihydrolase and trehalose utilization tests. Based on the preliminary tests, all isolates have been identified as *Pseudomonas fuscovaginae* (*P. fuscovaginae*). The 16S rDNA sequence analysis showed that all the isolates had 97-99% sequence similarities to *Pseudomonas fuscovaginae* in the GenBank database. This result was also supported by the constructed phylogenetic tree with the *Acidivorax avenae* outgroup. It was noted that all 25 isolates originated from the same node with the *P. fuscovaginae* group. The findings on characteristics of *P. fuscovaginae* in Peninsular Malaysia hopefully can be used in future studies especially on Malaysian *P. fuscovaginae* biology and their control.

Key words: Sheath brown rot, *Pseudomonas fuscovaginae*, malaysian isolates, characterization, pathogenicity, 16S rDNA

INTRODUCTION

Rice is a staple food in Malaysia as well as in the world. This crop has been considered as a strategic crop for economic development in Malaysia. However, several downfall factors have known to reduce rice production which are pests and diseases. Sheath brown rot caused by *P. fuscovaginae* is a devastating bacterial disease of rice that may become a threat to national food security due to its capability of causing rot to the sheath and grain of rice plant. The SBR disease that is caused by *P. fuscovaginae* was first discovered by Tanii *et al.* (1976) in Japan. Years after, this bacterial pathogen was later characterized and differentiated from other pseudomonads (Miyajima *et al.*, 1983). Rott *et al.* (1991) described a series of biochemical tests that would be useful in identification of the bacteria. In Malaysia, sheath brown rot had been well distributed in Peninsular Malaysia, where the first incidence was reported in Seberang Perak, Malaysia. The disease symptoms caused grain discoloration, necrosis of sheath and leaf flag and spikelet sterility (Cother *et al*., 2009). The discolored grain and spikelet sterility will directly affect the weight and quality of yield (Cottyn *et al*., 1996; Vidhyasekaran *et al*., 1984). To date, Razak *et al*. (2009) reported that the distribution of SBR in major granary in Peninsular Malaysia was almost over a half for most regions. Ever since the establishments of SBR in Malaysia, the characteristics of the causal agent of this country have not yet been described or documented in any report. The characteristic information is important for future studies, especially in determining control strategies of the pathogen. Therefore, our study aim to characterize *P. fuscovaginae* in Peninsular Malaysia by using morphology identification, biochemical characteristic, hypersensitivity and pathogenicity tests together with molecular identification.

MATERIALS AND METHODS

Sample collection: Leaf, sheath or grains showing SBR symptoms were collected from Tanjung Karang in Selangor, Kuala Muda in Kedah and Semarak and Gong Manak in Kelantan (Fig. 1). Five diseased plant samples were collected separately from each location and aseptically kept in bag given a reference number. Diseased plants were selected based on the procedures by Tilquin and Detry (1993) and Blair *et al*. (1970). All samples were taken to the Bacteriology laboratory at Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia for isolation and identification of the causal agent (Zeigler and Alvarez, 1989; Suslow *et al*., 1982; Schaad, 1980).

Isolation and morphological characteristics: Different plant parts (leaf, sheath, stem and grains) with disease symptoms were surface-sterilized in 5% (v/v) sodium hypochlorite (NaClO) and

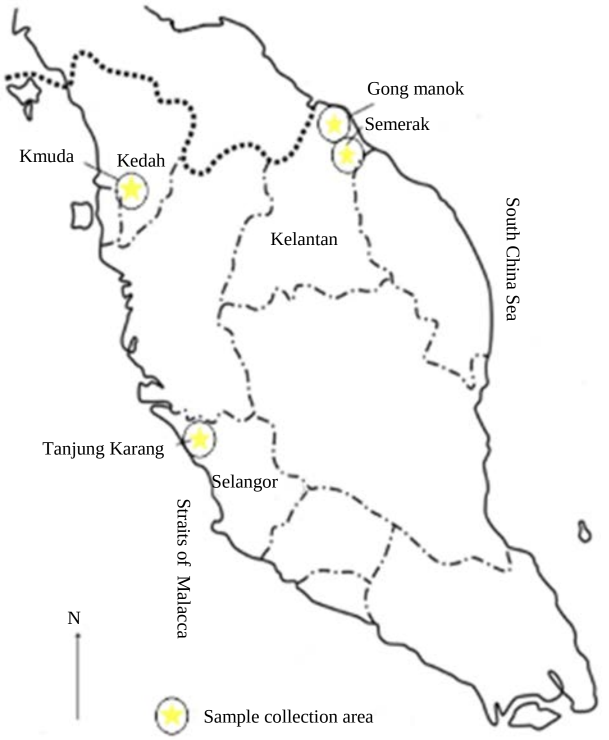

Fig. 1: Map of peninsular Malaysia showing the locations of rice granary where the *Pseudomonas fuscovaginae* isolates were obtained

rinsed twice with sterile distilled water. Then, the parts were cut into smaller pieces (approximately 3-5 mm) and soaked into sterile distilled water in autoclaved bottles. Each of the bottles was incubated for one hour at room temperature (26°C) for recovery of the bacterial cells. One hundred microliter bacterial suspension was streaked onto King's B Selective (KBS) agar plates (Rott *et al.*, 1989). The plates were incubated at 27°C. The plates were viewed after 24 h under the UV illumination where the fluorescent colonies will be re-isolated onto fresh KBS medium and agar slant to obtain pure culture and for storage purposes.

Hypersensitivity reaction and pathogenicity test: For Hypersensitivity Reaction (HR), 10^8 CFU mL^{-1} bacterial suspension was prepared from the overnight culture and 0.1 mL bacterial suspension was injected into *Nicotiana tabacum* cv. x*anthi* (five weeks old) leaf surface. The test plant was incubated on greenhouse's bench with 34/24°C (day/night temperature) condition. Sterile distilled water was used as a control. Any reaction on the leaf was observed for the next 24 h. Subsequently, 40-50 days old rice plant seedlings of MR219 variety were used in pathogenicity test. The seedling was injected with 0.1 mL 10^7 CFU mL^{-1} bacteria suspension (test samples) and sterile distilled water as negative control. Inoculated rice plants were placed on benches and incubated under greenhouse conditions at 34/24°C (day/night temperature). Lesion on the leaf sheath was observed after 24 h of inoculation. Suspected bacterial pathogens were re-isolated to fulfill Koch's Postulates.

Biochemical test: The isolates obtained were characterized by a series of biochemical tests that are useful in identifying *P. fuscovaginae* as suggested by Rott *et al.* (1991). The biochemical tests were oxidase, arginine dihydrolase, levan production, production of 2-ketogluconate and acid production from sucrose, sorbitol, inositol as well as trehalose. The biochemical characteristics (oxidase, arginine dihydrolase, production of 2-ketogluconate and levan) of the bacterial strains were determined by microbiological techniques as described by Fahy and Hayward (1983) and Lelliott and Stead (1987). Acid production from sucrose, inositol, sorbitol and trehalose (1%) was tested on the basal medium of Hugh and Leifson (1953).

Molecular identification: Total genomic DNA of the bacterial isolates was extracted using a commercial DNA extraction kit for bacteria as described in the protocol (Geneaid Extraction Kit DNA, Taiwan). Bacterial isolates were identified based on 16S rDNA gene sequences using universal primer pair of 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Turner *et al.*, 1999). PCR reaction mixture of 25 μL consist of 12.5 μL Dreamtaq Green Mastermix (Thermo Scientific), 0.5 μL forward primer (8F), 0.5 μL reverse primer (1492R), 3.0 μL DNA template and 8.5 μL nuclease free water. The cycle conditions were: an initial denaturation at 95°C for 3 min, 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 57°C) and extension (1 min for 72°C); followed by a final extension at 72°C for 10 min (Madiha *et al.*, 2012). The PCR products were running on 1% agarose gel stained with FloroSafe DNA Stain (First Base, mention country) to verify the PCR product. Then, the PCR products were purified using Gene Jet PCR purification kit (Thermo Scientific Fermentas, USA) according to the provided protocol. Sequencing was performed by a service provider (First Base Laboratories, Malaysia). The sequencing results were manually edited by using BioEdit software (v7.0.9). Edited sequences were subjected to Nucleotide Basic Alignment Search Tool (nBLAST) and compared with the sequences of *P. fuscovaginae* from the GenBank database (Altschul *et al.*, 1997; Benson *et al.*, 2008; Pearson and Lipman, 1988). Then, multiple sequences alignment were performed using a ClustalW software version 1.8 (Thompson *et al*., 1994). Subsequently, a phylogenetic tree was constructed with *Acidivorax avinae* outgroup from MEGA4 software using Neighbour Joining (NJ) method with 1,000 bootstrap replications (Tamura *et al*., 2007).

RESULTS

Sample collection and morphological characteristics: From 80 diseased samples (Fig. 2) collected, isolates that fluorescent under UV illumination on KBS medium and producing yellowish green pigment were re-isolated and sub-cultured. There were 50 isolates that florescent under UV light on the medium and produced yellowish green pigment. The colonies which suspected to be *P. fuscovaginae* were consistently cream in colour, round in shape with raised margin, smooth elevation, diffused and produced yellowish green pigments under the colonies on KBS medium (Fig. 3). These isolates were then tested for hypersensitivity reaction test on *Nicotiana tabacum* cv. *xanthi* and pathogenicity test on *Oryza sativa* for further identification.

Hypersensitivity reaction and pathogenicity test: Result of Hypersensitivity Reaction (HR) test showed within 24 h, black lesion on the infected leaves can be observed, while no lesion was observed on control plant. Figure 4 showed the black lesion on tobacco leaves after inoculation. All experiments were repeated twice and with same results obtained. Out of 50 isolates, 25 isolates were positive for this test and were subjected to pathogenicity test. Meanwhile, for pathogenicity test on MR219 variety, all 25 isolates that previously positive in hypersensitivity reaction test

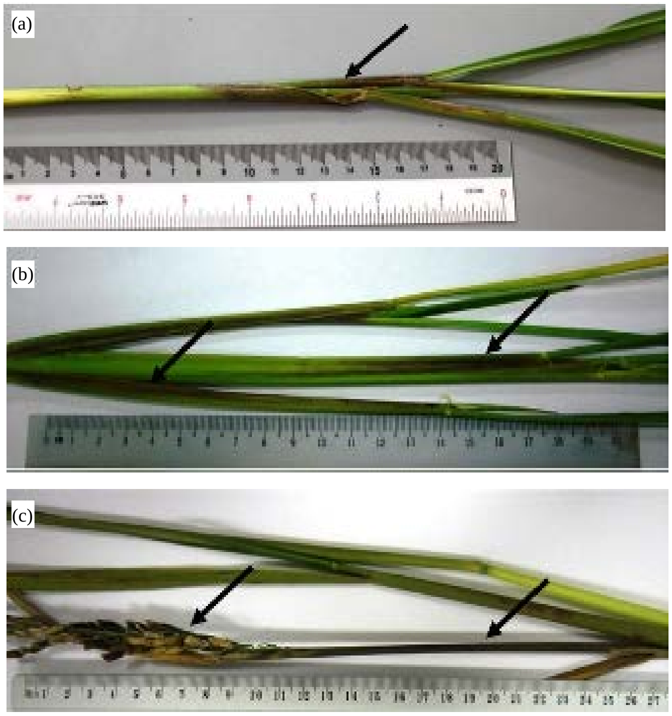

Fig. 2(a-c): Rice plants showing necrosis of sheath and grain discoloration symptoms; arrow pointed the symptoms, (a) Sample from Tanjung Karang, Selangor, (b) Sample from Kuala Muda, Kedah and (c) Sample from Semerak, Kelantan

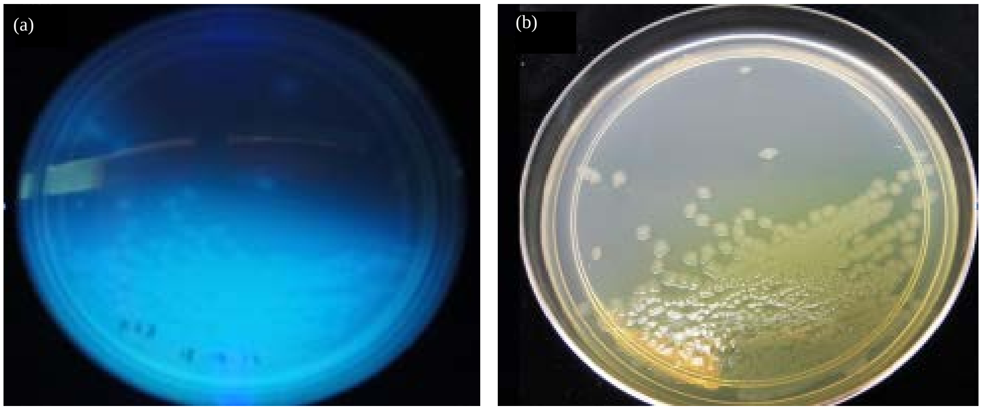

Fig. 3(a-b): Isolated pathogen on KBS agar, (a) Colonies fluorescent under or near UV light and (b) Colonies produce yellowish green crystal pigment on KBS agar

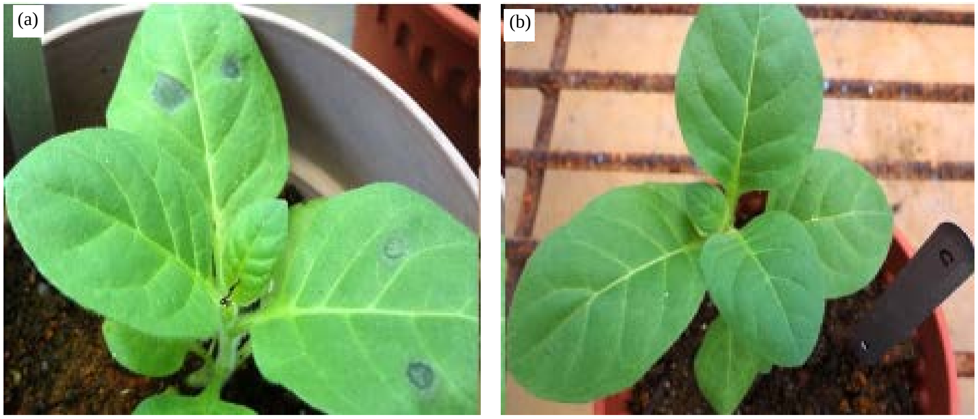

Fig. 4(a-b): Hypersensitivity reactions on *Nicotiana tabacum* cv. *Xanthi*, (a) Leaf injected with the isolate T1 and (b) Leaf injected with sterile distilled water (negative control)

produced identical symptoms of SBR around the inoculation area of infected plant within three days. The symptoms were lesions around the inoculation area. No symptom was produced on the control plant (Fig. 5). Isolates from the symptomatic plants were all isolated to fulfill the Koch's Postulates. These 25 positive bacterial isolates were then further characterized with biochemical test in order to compare their biochemical characteristics with the featured biochemical profile of *P. fuscovaginae*.

Biochemical test: In biochemical test, a total of 25 bacterial isolates which were positive in hypersensitivity and pathogenicity tests gave similar biochemical characteristic of *P. fuscovaginae*. These isolates were positive for kovac oxidase, arginine dihydrolase test and utilization of trehalose, negative for levan production, utilization of sorbitol, sucrose and inositol. Table 1 shows 25 isolates (from Selangor, Kedah and Kelantan) with positive biochemical patterns for *P. fuscovaginae* (X1) as described by Rott *et al.* (1991) and Jaunet *et al.* (1995). However, these tests

Table 1: Biochemical tests of bacterial isolates suspected to be *Pseudomonas fuscovaginae*, from Selangor, Kedah and Kelantan

		Isolates from Selangor									Isolates from Kedah								Isolates from Kelantan							
Characteristics	*X1	T1	T2	T3	T4	T7	T8	T10	T12	T13	K23	K2B	K21 Bi	K18 B	K2C	K21 Bii	K2A	K23B	S1 iii	S3 i	S3 ii	S3 iii	S7 Ni	GMS5i	GMS5iii	GM S3
Fluorescence under UV light	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Kovac oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Levan production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid production from:																										
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*X1: Reference characteristic of *P. fuscovaginae*, (+ positive reaction, - negative reaction)

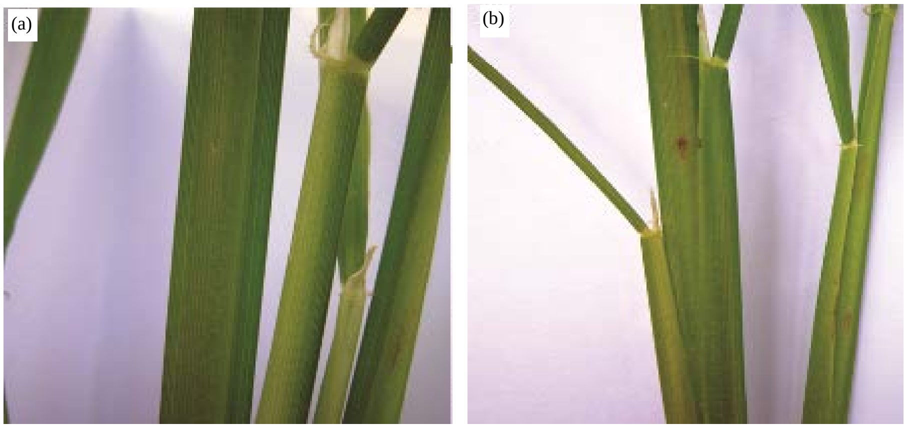

Fig. 5(a-b): Pathogenicity test on MR219 variety, (a) Sheath injected with sterile distilled water (negative control) and (b) Sheath injected with the isolate T1

Table 2: Local isolates and accession number

Region and isolates	Accession No.
Selangor	
T1	JX915743
T2	KC663615
T3	KP241916
T4	KP241917
T7	KP241918
T8	KC663616
T10	KP241919
T12	KP241920
T13	KP241921
Kedah	
K2A	KP241925
K2B	KP192026
K2C	KP241924
K18B	KP241922
K21Bi	KP241922
K21 Bii	KP192027
K23	KC663617
K23B	KP241926
Kelantan	
S1 iii	KP192028
S3 I	KP192029
S3 ii	KP241927
S3 iii	KP241928
S7N I	KP241929
GMS5i	KP241930
GMS5iii	KP241931
GMS3	KP192030

are inadequate to characterize and verify the causal agent of SBR. Therefore, molecular identification using 16S rDNA gene sequence was conducted for a definite verification of *P. fuscovaginae*.

Molecular identification: The Polymerase Chain Reaction (PCR) amplification on all isolates generated a 1500 Base Pairs (BP) amplicon each. Edited sequences of both forward and reverse primers were deposited in National Center Biotechnology Information (NCBI) for accessioning number of representative local isolates (Table 2). Following that, result of BLAST revealed that all

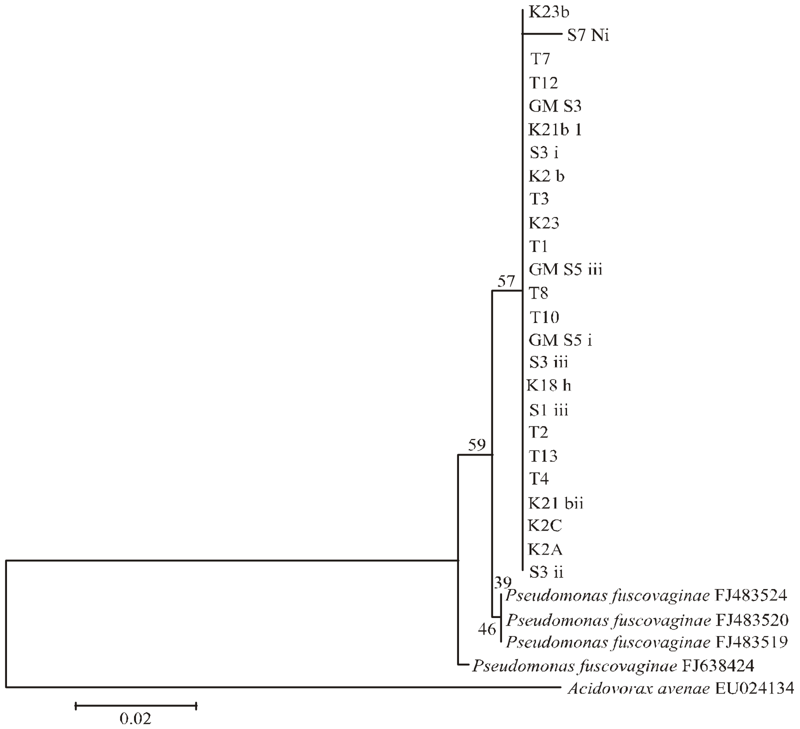

Fig. 6: Phylogenetic tree constructed using a neighbour-joining analysis on the 16S rDNA gene sequences; bootstrap values are shown at the branch points. The scale bar was 0.02 substitutions per nucleotide position. The accession numbers of *P. fuscovaginae* and *A. avenae* from NCBI database were indicated on tip-labeled

25 isolates exhibited 97-99% maximum identity to *P. fuscovaginae* (Table 3). Moreover, on the Neighbour-Joining (NJ) analysis with 1,000 bootstrap replications, discovered that all 25 bacterial isolates were originated from the same node with the reference strain, *P. fuscovaginae* (Genbank accession nos. FJ483524, FJ483520 and FJ483519). Meanwhile, *Acidivorax avenae* was set as an outgroup. These 25 bacterial isolates however, were not clustered with *P. fuscovaginae* from Genbank database (Fig. 6).

DISCUSSION

Sample collection and morphological characteristic: In this study, *P. fuscovaginae*, the causal pathogen of SBR of rice in Malaysia has been successfully isolated, identified and characterized. In general, the symptoms of infected rice plants that we have collected were similar to SBR symptoms as described by Razak *et al.* (2009) and Rott *et al.* (1991) which lower parts of the infected leaf sheath appeared light or dark brown, the leaf sheaths became necrotic and dried, a number of flag leaves changed into light or dark brown as well as the infected panicles of the rice grains turned abnormal and discoloured . According to Cottyn *et al.* (1994), there are two main groups of phytopathogenic pseudomonads; the fluorescent group and the non-fluorescent group. Most fluorescent *Pseudomonas* spp., were reported to cause sheath rot and grain discoloration of rice plant in various parts of the world including *P. fuscovaginae* (Zeigler and Alvarez, 1987). Isolation and identification of the causal agent of SBR, *P. fuscovaginae* is always challenging due to the presence of other fluorescent and non-fluorescent pseudomonads, with most of them were

Table 3: Results of BLAST search based on 16S rRNA partial sequence gene of bacterial isolates from sheath brown rot

States and isolates	Identity (%)	Reference bacteria	Genebank accession No.
Selangor			
T1	98	*P. fuscovaginae*	FJ483519
T2	97	*P. fuscovaginae*	FJ483519
T3	98	*P. fuscovaginae*	FJ483519
T4	98	*P. fuscovaginae*	FJ483519
T7	98	*P. fuscovaginae*	FJ483519
T8	98	*P. fuscovaginae*	FJ483519
T10	98	*P. fuscovaginae*	FJ483519
T12	98	*P. fuscovaginae*	FJ483519
T13	98	*P. fuscovaginae*	FJ483519
Kedah			
K2B	98	*P. fuscovaginae*	FJ483524
K23	98	*P. fuscovaginae*	FJ483524
K21Bi	98	*P. fuscovaginae*	FJ483524
K18B	98	*P. fuscovaginae*	FJ483524
K2C	98	*P. fuscovaginae*	FJ483524
K21Bii	98	*P. fuscovaginae*	FJ483524
K2A	98	*P. fuscovaginae*	FJ483524
K23B	98	*P. fuscovaginae*	FJ483524
Kelantan			
S1 iii	98	*P. fuscovaginae*	FJ483524
S3 i	98	*P. fuscovaginae*	FJ483524
S3 ii	98	*P. fuscovaginae*	FJ483524
S3 iii	98	*P. fuscovaginae*	FJ483524
S7 N i	97	*P. fuscovaginae*	FJ483524
GMS5i	99	*P. fuscovaginae*	FJ483520
GMS5iii	99	*P. fuscovaginae*	FJ483520
GM S3	98	*P. fuscovaginae*	FJ483524

saprophytes commonly found in rice sheath rots and discolored grains. Therefore, combined approaches comprising isolation on selective medium, hypersensitivity reaction test, pathogenicity test, biochemical test and molecular identification were suggested in order to identify and characterize the causal agent (Miyajima *et al*., 1983; Rott *et al*., 1991; Jaunet *et al*., 1995).

Some *Pseudomonas* species produced diffusible yellow-green pigments that are sometimes mistakenly perceived as fluorescent pigments. These features can be distinguished by examining the cultures on solid media with Ultraviolet (UV) illumination of short wavelength (254-360 nm), of which only the fluorescent pigments will fluoresce. In our study, KBS medium suggested by Rott *et al*. (1989) was applied to isolate *P. fuscovaginae*-SBR causing strains, concurrently to eliminate the growth of unwanted bacterial pathogen. Although a few previous reports have been suggested the importance of KB and Miyajima mediums (Cother *et al*., 2009, 2010) for *P. fuscovaginae* isolation and identification, some of the strains might weakly fluorescent on these mediums under UV illumination, distracting the isolation process. To overcome this, Rott *et al*. (1989) had discovered the KBS medium for a flexible isolation and identification of *P. fuscovaginae*.

Hypersensitivity and pathogenicity test: In hypersensitivity test, reactions elicited on *Nicotiana tabacum* cv. *xanthi* after 24 h of infiltration indicated all 25 fluorescent isolates had plant pathogen. A hypersensitivity reaction test on *Nicotiana tabacum* cv. *xanthi* was helpful as the first test to screen bacteria for pathogenicity (Klement and Goodman, 1967). If the injection used contains pathogenic bacteria, the injected tissue becomes necrotic within 24 h. Non-pathogenic bacteria caused only a faint chlorosis after a few days (Klement *et al*., 1964).

The pathogenicity results revealed that all 25 isolates which were previously positives in HR test were pathogenic to rice plant by causing SBR disease. Brown rot lesion on the inoculated sheath appeared was exactly similar in pathogenicity test reported by earlier findings

(Cother *et al*., 2009; Jaunet *et al*., 1995; Miyajima *et al*., 1983; Rott *et al*., 1991). Koch's postulates showed that all bacterial isolates were also pathogenic to the rice plant of MR219 variety.

Biochemical test: Rott *et al.* (1991) reported a series of biochemical test that are useful in identification of *P. fuscovaginae*. Jaunet *et al.* (1995) stated that these biochemical test are essential to differentiate *P. fuscovaginae* from other fluorescent pseudomonas especially *Pseudomonas syringae* pv. *syringae* which also bacterial pathogen of rice plant (Cortesi *et al*., 2008). These biochemical tests were kovac oxidase, arginine dihydrolase, acid production from trehalose, sucrose, sorbitol and inositol as well as levan production. In this experiment, all 25 isolates produced similar and consistent biochemical profile shown by *P. fuscovaginae* as described by Rott *et al.* (1991) and Jaunet *et al.* (1995). Based on the symptoms appearance in the field, morphological characteristics, HR test, pathogenicity test as well as biochemical test, all isolates have similar characteristic resembling *P. fuscovaginae* (Miyajima *et al*., 1983; Rott *et al.,* 1991; Jaunet *et al*., 1995).

Molecular identification: In this study, the analysis of phylogenetic tree showed all 25 isolates were originated from the same node with the reference strains; *P. fuscovaginae* (FJ483524, FJ483520, FJ483519, AB638424) from Genbank database and were neighbouring them on the outer branches. However, strains differences due to genetic polymorphism probably made all our isolates were in a separate cluster from these *P. fuscovaginae* reference strains. According to Onasanya *et al.* (2010), formation of outer branches derived from same node in phylogenetic tree was a proved of genetic polymorphism in *P. fuscovaginae* due to temporal, climate, elevation as well as geographical origin (Adorada *et al*., 2013). The BLAST analysis allowed comparison of local isolates 16S rRNA sequence to the *P. fuscovaginae* from Genbank Database. Thus, the result from the NJ dendogram obtained from this study showed that all 25 bacterial isolates were *P. fuscovaginae* with 97-99% similar to their reference *P. fuscovaginae* strains.

CONCLUSION

Our study revealed that a combination of techniques to identify and characterize the causal agent of SBR of rice is of necessary importance. The isolation and identification of *P. fuscovaginae* is often difficult, thus a combination phenotypic characterization is crucial for a robust identification of this pathogen. Indeed, ever since the establishment of SBR in Malaysia, this local causal pathogen has not yet been characterized and documented. Our results represent the first characterization of Malaysian *P. fuscovaginae* isolates based on phenotypic characteristics and molecular identification.

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