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 causes 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 (Miyagjima *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
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. xanthi (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' - AGAGTTTGATCCTGCTCAG-3') and 1492R (5' - GGTACCTTGTTACGACTT-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

| Characteristics | $X_1$ | T1  | T2  | T3  | T4  | T10 | T11 | K23 | K2B | K21 | K18 | B | K2C | B1 | K2A | K2B3 | B12 | T13 | K23B | K2B1 | Bi | K21 | Bi i | K2A | K2B3B | S1 | S3i | 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        |       |     |     |     |     |     |     |     |     |     |     |   |   |   | |++  |   |+    |+    |     |     |     |   |   |   |   |   |   |

$X_1$: Reference characteristic of *P. fuscovaginae*, (+ positive reaction, - negative reaction)
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 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*.
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, *Acidovorax 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               |
| K2                   | 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               |

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.
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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